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Department of Experimental Biology

**Study of the biological significance of
hyaluronidases in the context of hyaluronan
metabolism and intercellular communication
in the gastrointestinal tract**

Doctoral thesis

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Abstract

Hyaluronan (HA) is a glycosaminoglycan widely distributed in vertebrate tissues and increasingly applied as a dietary supplement for its proposed health benefits. However, the mechanisms underlying its fate in the gastrointestinal tract and systemic bioavailability remain incompletely understood. This dissertation investigates the enzymatic degradation of HA, the fate of orally administered HA in the GIT, and the downstream metabolic and physiological effects associated with this process, with special attention to microbiota composition and the enzymatic potential of *Bacteroides* species.

The first part explores the evolutionary and structural diversity of HA-degrading enzymes across vertebrates, invertebrates, fungi, bacteria, and bacteriophages. Despite major sequence divergence across families, each family retains conserved catalytic motifs, with distinct mechanistic strategies reflecting adaptation to specific ecological environments. These insights provide a framework for understanding how HA degradation is achieved in the gut. The second part investigates the bioavailability of orally administered HA. *In vivo* experiments confirmed that intact HMW-HA is not absorbed across the intestinal barrier, demonstrating that its degradation depends on microbial activity. Supplementation combined with 16S rRNA sequencing revealed shifts in microbial composition, including increased abundance of *Bacteroides* species, consistent with their role as HA degraders. Metabolomic analyses further showed accumulation of HA-derived intermediates and fermentation products such as SCFAs, indicating that the physiological effects of oral HA are mediated by microbial breakdown products rather than systemic uptake of the intact polymer. The third part refines our understanding of HA degradation by addressing the limits of predicting microbial function from sequence identity alone.

Part of the abstract excluded for confidentiality (unpublished)

Abstrakt

Kyselina hyaluronová (HA) je glykosaminoglykan běžně přítomný v tkáních obratlovců a stále častěji využíváný jako doplněk stravy pro své předpokládané zdravotní přínosy. Mechanismy určující její osud v gastrointestinálním traktu (GIT) a systémovou biologickou dostupnost však dosud nejsou plně objasněny. Tato disertační práce se zaměřuje na enzymatickou degradaci HA, osud perorálně podané HA v GIT a následné metabolické i fyziologické účinky tohoto procesu, se zvláštním důrazem na roli mikrobioty a enzymatický potenciál rodu *Bacteroides*.

První část se věnuje evoluční a strukturní rozmanitosti enzymů štěpících HA u obratlovců, bezobratlých, hub, bakterií a bakteriofágů. Přestože mezi rodinami existuje výrazná sekvenční divergence, uvnitř jednotlivých rodin jsou zachovány konzervované katalytické motivy a rozdílné mechanistické strategie, které odrážejí adaptaci na specifická ekologická prostředí. Tyto poznatky poskytují rámec pro pochopení degradace HA ve střevě. Druhá část se zabývá biologickou dostupností perorálně podané HA. *In vivo* experimenty potvrdily, že HMW-HA není absorbována střevní bariérou a její degradace tedy závisí na mikrobiální aktivitě. Suplementace kombinovaná se sekvenováním 16S rRNA ukázala změny ve složení mikrobioty, včetně zvýšeného zastoupení rodu *Bacteroides*, což odpovídá jejich roli hlavních druhů podílejících se na degradaci HA. Metabolomické analýzy navíc prokázaly akumulaci meziproduktů a fermentačních produktů, jako jsou mastné kyseliny s krátkým řetězcem (short-chain fatty acids), což potvrzuje, že fyziologické účinky perorální HA jsou zprostředkovány produkty mikrobiální degradace, nikoli systémovým vstřebáváním intaktního polymeru. Třetí část zpřesňuje poznatky o degradaci HA tím, že ukazuje limity predikce mikrobiálních funkcí pouze na základě sekvenční identity.

Část abstraktu je vyloučena z důvodu utajení (nezveřejněná data).

DECLARATION

I hereby declare that I worked on this thesis on my own using the sources listed in the bibliography, under the supervision of Mgr. Kristýna Turková, Ph.D. I further acknowledge that I used OpenAI's ChatGPT as a supportive tool for text editing and language refinement, while all scientific ideas, interpretations, and conclusions are my own.

In Brno, 21.8.2025

Martin Šindelář

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List of publications included in the thesis

- I. **Martin Šindelář**; Jana Jílková; Lukáš Kubala; Vladimír Velebný; Kristýna Turková, ***Hyaluronidases and hyaluronate lyases: From humans to bacteriophages.*** Colloids and Surfaces B: Biointerfaces, 2021, doi: 10.1016/j.colsurfb.2021.112095. IF 5.999, Q1
I performed all in silico analyses, including multiple sequence alignments, phylogenetic analysis, and visualizations, and contributed substantially to the writing and structuring of the review. Total contribution estimated as 70%.
- II. Matěj Šimek; Kristýna Turková; Martin Schwarzer; Kristina Nešporová; Lukáš Kubala; Martina Hermannová; Tereza Foglová; Barbora Šafránková; **Martin Šindelář**; Dagmar Šrůtková et al. ***Molecular weight and gut microbiota determine the bioavailability of orally administered hyaluronic acid.*** Carbohydrate Polymers, 2023, doi: 10.1016/j.carbpol.2023.120880. IF 10.7, Q1
I performed post-processing of Illumina 16S rRNA sequencing data, including statistical evaluation of community composition and diversity, functional analysis, and visualization of results. Total contribution estimated as 10%.
- III. **Martin Šindelář**, Anna Kocurková, Matěj Šimek, Gabriela Ambrožová, Lukáš Kubala, Kristýna Turková. ***Proteomics identifies non-canonical enzymes involved in hyaluronan degradation in Bacteroides.*** Submitted to: mBio journal. IF 4.7, Q1
I performed all the bioinformatic analyses including protein comparative analysis, clustering, and visualization of proteomic data. I also contributed to the interpretation of results, as well as to the writing and revision of the manuscript. Total contribution estimated as 50%.
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List of other publications

- IV. Anna Marlene Schmid, Agnieszka Razim, Magdalena Wyszomolek, Daniela Kerekes, Melissa Haunstetter, Paul Kohl, Georgii Brazhnikov, Nora Geissler, Michael Thaler, Eliška Krčmářová, **Martin Šindelář**, Tamara Weinmayer, Jiří Hrdý, Katy Schmidt, Peter Nejsum, Bradley Whitehead, Johan Palmfeldt, Stefan Schild, Aleksandra Inić-Kanada, Ursula Wiedermann, Irma Schabussova. ***Extracellular vesicles of the probiotic bacteria E. coli O83 activate innate immunity and prevent allergy in mice.*** Cell Communication and Signaling, 2023, doi: 10.1186/s12964-023-01329-4. IF 8.2, Q1.
I performed proteomic data curation and post-processing, including protein comparison across datasets and volcano plot visualization. Total contribution estimated as <10%.
- V. Miriam Sandanusová; Kristýna Turková; Eva Pecháčková; Jan Kotouček; Pavel Roudnický; **Martin Šindelář**; Lukáš Kubala; Gabriela Ambrožová. ***Growth Phase Matters: Boosting immunity via Lacticasebacillus-derived membrane vesicles and their interactions with TLR2 pathways.*** Journal of Extracellular Biology, doi: 10.1002/jex2.169.
I performed proteomic data post-processing, including all visualizations and prediction of interaction network. Total contribution estimated as 10%.

1. Introduction

The mammalian gastrointestinal tract (GIT) harbors a complex microbial community, dominated by members of the phyla *Bacteroidota* and *Bacillota*, which exerts profound effects on host physiology, metabolism, immunity, and barrier integrity. Host–microbiota interactions occur through direct cell contact, microbial metabolites, and extracellular vesicles (EVs), with dietary polysaccharides playing a major modulatory role. While most complex polysaccharides bypass host digestion and are metabolized by specialized gut microbes into short-chain fatty acids (SCFAs) and other bioactive molecules, glycosaminoglycans (GAG) such as hyaluronic acid (HA) represent a relatively understudied but potentially significant component of this metabolic network.

HA is a high–molecular-weight (HMW) GAG broadly distributed in vertebrate tissues, where it supports extracellular matrix (ECM) structure, regulates tissue hydration, and mediates cell–cell and cell–matrix communication. Its turnover is catalyzed by hyaluronidases (HYALs) and HA lyases, enzymes found in eukaryotes, bacteria, and even bacteriophages. In recent years, HA has gained attention as a dietary supplement, promoted for benefits to skin health, joint function, and inflammation control. However, the bioavailability of orally administered HMW HA remains contentious. Due to its large size, direct absorption is limited, but microbial degradation in the GIT can produce smaller oligosaccharides that are more readily absorbed and may exert systemic effects.

The enzymatic capacity for HA degradation varies across bacterial taxa: Gram-negative bacteria like *Bacteroides* spp. utilize a compartmentalized polysaccharide utilization (PUL) locus-based system, whereas Gram-positive bacteria such as *Lactobacillus* and *Enterococcus* rely on extracellular enzymes and phosphotransferase-mediated uptake. This dissertation investigates the hypothesis that degradation of HMW HA in the GIT is primarily mediated by the gut microbiota through specialized polysaccharide lyases (PLs) and associated proteins, and that this process influences HA bioavailability and shapes microbial community dynamics.

1.1 Mammalian GIT and microbiota

The mammalian GIT harbors one of the most densely populated and metabolically active microbial ecosystems in nature, exerting profound effects on host physiology, nutrient metabolism, immune regulation, and epithelial barrier integrity (Maciel-Fiuza et al. 2023). This community collectively referred to as the gut microbiota is dominated by bacteria, but also contains archaea, fungi, protozoa, and viruses, including bacteriophages (Matijasic et al. 2020). The estimated microbial cell load ranges between 10^{13} and 10^{14} , approaching or exceeding the total number of host cells (Sender, Fuchs, and Milo 2016). Across mammalian hosts, bacterial communities are largely composed of members of the phyla *Bacteroidota* (formerly *Bacteroidetes*) and *Bacillota* (formerly *Firmicutes*), with smaller but consistent contributions from *Actinomycetota*, *Verrucomicrobiota*, and *Proteobacteria* (Mariat et al. 2009). Microbial composition is highly species-specific and is shaped by multiple factors, including host genetics, developmental stage, environmental exposure, and, most prominently, diet.

Although the general structural organization of the GIT is conserved among mammals, notable anatomical and physiological distinctions influence microbial biogeography and function (Fig. 1). In humans, the GIT is approximately 5–7 m in length, with a relatively small cecum and a large colon serving as the primary site of microbial fermentation. In contrast, the mouse, currently the most widely used experimental model, has a much shorter GIT (~50–55 cm) but a disproportionately enlarged cecum, which constitutes the major site of fermentation and microbe–host metabolic exchange (Hugenholtz and de Vos 2018). Gastric physiology also diverges, humans typically maintain a highly acidic stomach (pH ~1.5–3.0), whereas the murine gastric environment is less acidic (pH ~3–4), potentially permitting survival of a broader range of ingested microbes. Furthermore, gastrointestinal transit in mice is rapid (6–8 h) compared to humans (30–40 h), with implications for microbial load, biofilm development, and mucosal colonization patterns (Nguyen et al. 2015).

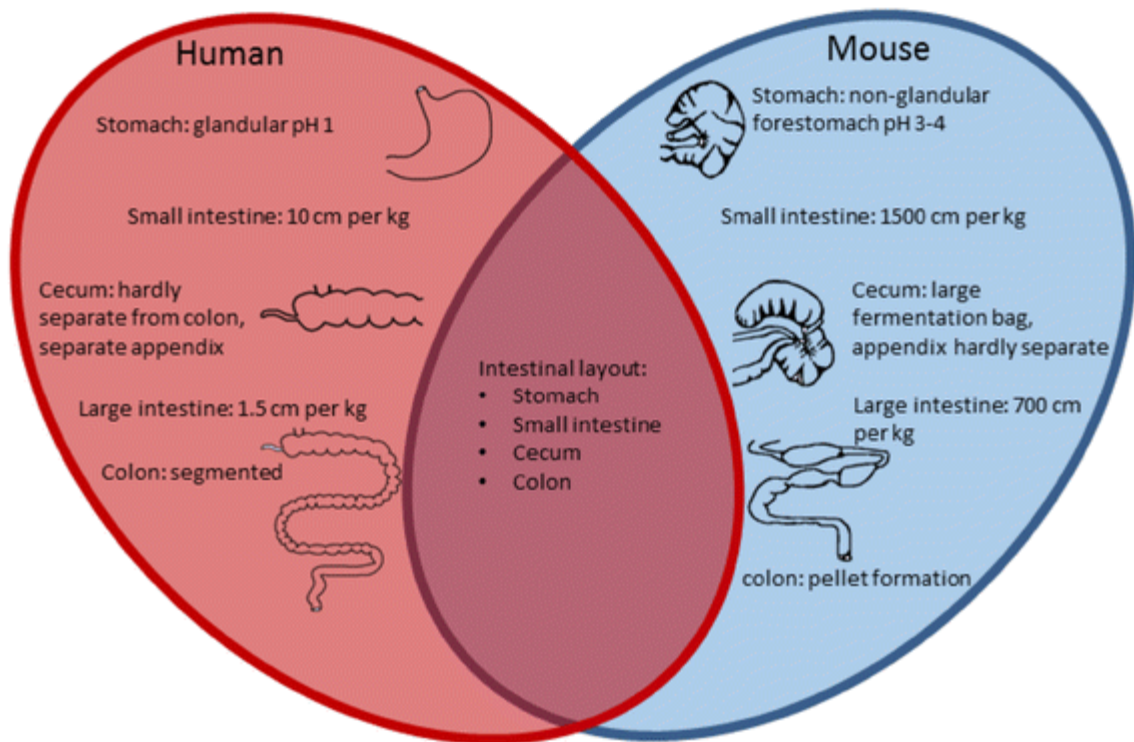


Fig.1: Structural and functional differences between human and murine GIT (Hugenholtz and de Vos 2018)

These differences notwithstanding, murine models remain central to microbiome research because they allow rigorous control of host genetics, diet, and housing, and provide unique experimental modalities including germ-free, gnotobiotic, and humanized systems that enable causal dissection of host–microbe–diet interactions (Nguyen et al. 2015). While limitations in taxonomic and functional equivalence to the human microbiome are well recognized, mice continue to be indispensable for mechanistic studies of dietary interventions, infection, and mucosal immunology.

The mammalian GIT is a spatially and functionally compartmentalized environment, with microbial communities shaped by gradients in pH, oxygen tension, nutrient availability, and host-derived secretions. In both humans and mice, the stomach and small intestine host relatively low microbial densities, dominated by facultative anaerobes such as *Lactobacillus*, *Enterococcus*, and *Streptococcus*. The colon, by contrast, supports the highest microbial densities exceeding 10^{11} cells per gram of luminal content and favors strict anaerobes, including *Bacteroides*, *Clostridium*, *Faecalibacterium*, and *Ruminococcus* (Tropini et al. 2017; Yang et al. 2025). These biogeographic patterns reflect the interplay of host-imposed selective pressures and microbial niche specialization, underscoring the finely tuned co-adaptation between mammals and their resident microbiota.

1.2 Microbiota–host interactions in the GIT

From birth, gut microbiota establishes intimate interactions with host epithelial and immune cells. In a state of health, gut microbiota supports host homeostasis through multiple mechanisms like competitive exclusion of pathogens, synthesis of metabolites that regulate immune and metabolic pathways, promotion of gut-associated lymphoid tissue (GALT) development, and maintenance of the epithelial barrier (Gutman and Litvak 2024). However, in disease contexts, the microbial community often undergoes a shift toward a dysbiotic state, characterized by reduced microbial diversity, increased representation of *Proteobacteria* and other pathobionts, and impaired metabolic output (Vester-Andersen et al. 2019). Dysbiosis encompasses not only compositional changes but also functional disruption of the microbial ecosystem, which may contribute to the onset or progression of various inflammatory, metabolic, and infectious diseases.

Overall microbiota-host interactions are multifactorial and occur via several mechanisms: direct microbial–host cell contact; microbial metabolism of dietary substrates and production of bioactive metabolites; and the release of EVs by both host and microbial cells. This mutual interaction can influence host physiology both locally within the gut and systemically throughout the body (Brown, Bashir, and Zeng 2025).

1.2.1 Direct interaction between microbial and host cells

Intimate cellular interactions between gut microbes and host epithelial cells are foundational to gastrointestinal homeostasis. Adhesin-mediated binding ensures microbial persistence and niche formation, while engagement of microbial pattern molecules with epithelial pathogen recognition receptors (PRRs) regulates immune signaling (Ardita et al. 2014). Epithelial defenses include barrier tight junctions, antimicrobial peptide secretion, and reactive oxygen species (ROS) production actively modulate these contacts. Additionally, organisms like segmented filamentous bacteria directly interface with M cells to drive GALT maturation (Yu et al. 2012). Together, these multifaceted interfaces maintain epithelial integrity, regulate immune responses, and shape microbial community structure through bidirectional communication mechanisms.

1.2.2 Interaction via products of microbial metabolism

The gut microbiota profoundly influences host physiology through a vast array of metabolites that act as signaling molecules. These microbial metabolites mediate host–

microbiota communication by engaging host receptors and pathways, effectively making the gut microbiota a second endocrine organ. Many of these compounds (e.g. SCFA), bile acids, tryptophan catabolites) are produced or modified exclusively by gut microbes.

In the last five years, research into host–microbiota interactions have elucidated numerous mechanistic pathways by which microbial metabolites influence human and animal physiology. SCFA emerge as multi-functional mediators that link diet to immune tolerance, gut barrier integrity, and metabolic regulation via GPCR signaling and epigenetic modifications (Losol et al. 2024). Bile acids, once only appreciated for digestion, are now understood as microbiota-modified ligands for FXR and TGR5 that shape metabolic and immune outcomes in both gut and liver (Guo and Chiang 2020). Tryptophan metabolites provide a chemical bridge between microbes and the host’s immune system and nervous system, chiefly through receptors like AhR that maintain mucosal homeostasis (Losol et al. 2024). Other microbial products from trimethylamine N-oxide to polyamines further expand this inter-kingdom dialogue, affecting cardiovascular risk, inflammatory pathways, and even longevity. Mechanistic insights into these interactions have greatly expanded, identifying key pathways by which microbial metabolites signal to the host and vice versa. Importantly, these findings have opened new avenues (e.g. metabolotherapy) for treating disease by targeting microbiome metabolites (Zhang et al. 2023).

1.2.2.1 Short-chain fatty acids

SCFAs (primarily acetate, propionate, and butyrate) are major fermentation products of dietary fibers by gut bacteria. They are produced in high concentrations in the colon with acetate > propionate > butyrate (Liu et al. 2024) and most (~95%) are rapidly absorbed by the host colonocytes. While acetate appears abundantly in peripheral circulation (hundreds of μM), butyrate is mostly consumed locally as the preferred fuel for colonocytes underscoring that SCFAs act both as locally acting molecules and as systemic messengers (Koh et al. 2016).

SCFAs signal through specific host G-protein-coupled receptors on cell surfaces. Key receptors include Free Fatty Acid Receptor 2 (FFAR2/GPR43), FFAR3 (GPR41), and GPR109A (HCAR2), which are expressed on intestinal epithelial cells and immune cells. Engagement of GPR41/43 by SCFAs on gut epithelial cells triggers downstream pathways that promote cytokine and chemokine production (Losol et al. 2024). In the gut lumen, SCFAs can thus bolster the first line of defense against pathogens by fortifying epithelial barrier function and innate immunity. Beyond the gut, SCFAs influence endocrine and metabolic responses. In

the colon, SCFAs stimulate L cells to secrete the incretin hormones GLP-1 and PYY, an effect mediated in part by GPR43 activation(Zeng et al. 2024). This links microbiota fermentation to host glucose metabolism and appetite regulation. Indeed, studies show that increased colonic SCFA levels (e.g. after dietary fiber) activate GPR43, raise GLP-1 secretion and improve glycemic control(Zeng et al. 2024). SCFAs can also modulate lipid metabolism. For instance, propionate absorbed into the portal circulation is taken up by the liver where it serves as a substrate for gluconeogenesis and inhibits HMG-CoA reductase, thereby reducing cholesterol synthesis(Liu et al. 2024). These metabolic actions illustrate how SCFAs act as systemic metabolic cues linking diet, microbiota activity, and host energy homeostasis.

1.2.2.2 Dietary oligosaccharides produced by microbial action

In GIT, dietary polysaccharides such as inulin, resistant starch, pectins, and complex plant cell-wall fibers are fermented by the resident gut microbiota into short-chain oligosaccharides e.g. fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), xylo-oligosaccharides (XOS), and monosaccharides. This depolymerization is accomplished by a rich repertoire of microbial enzymes, particularly glycoside hydrolases and polysaccharide lyases, which generate reducing oligosaccharides as intermediate products. Many of these fragments, including GOS, FOS, XOS, and others, may persist as nondigestible oligosaccharides that exert prebiotic effects by enriching beneficial microbes and supporting short-chain fatty acid production. Through these metabolites and signaling functions, these oligosaccharides influence gut barrier integrity, immune modulation, metabolic pathways (including bile acid metabolism), and overall host-microbial homeostasis(Klassen et al. 2021; Yuan et al. 2024).

Mannan oligosaccharides (MOS) produced by microbial breakdown of yeast or plant mannans were shown to modulate host lipid metabolism and cholesterol through altered bile acid excretion and gut immune modulation (e.g. decreased plasma cholesterol, reduced atherosclerosis) in mice(Cheong et al. 2023). While much of that effect is via microbiota changes and metabolites (bile acids), MOS themselves may interact with host physiology. Short chains from brown algae polysaccharide fermentation like alginate oligosaccharides (AOS) were for their ability to modulate gut microbiota and confer host benefits (immune regulation, barrier protection) (Song et al. 2025).

1.2.3 Interaction using EVs

Gut-resident microbes continuously secrete EVs. EVs are nanosized particles covered by membrane lipid bilayers released by all living cells, including bacteria, serving as vital

mediators of intercellular communication. In the GIT, both eukaryotic host cells and prokaryotic microbes produce EVs that play critical roles in maintaining homeostasis, modulating immune responses, and contributing to disease processes (Díaz-Garrido, Badia, and Baldomà 2021).

Bacterial extracellular vesicles (BEVs), particularly those derived from Gram-negative bacteria, are commonly referred to as outer membrane vesicles (OMVs), which originate from the budding of the bacterial outer membrane. These vesicles carry a diverse cargo, including proteins, lipopolysaccharides (LPS), DNA, RNA, and small molecules, allowing them to influence host cellular processes upon delivery (Xiao, Li, and Yang 2023). BEVs can traverse the intestinal mucus barrier and interact directly with epithelial and immune cells, thereby exerting both local and systemic effects.

One well-characterized mechanism of BEV-host interaction involves *Bacteroides fragilis*, whose OMVs contain polysaccharide A (PSA). PSA is known to interact with host dendritic cells via Toll-like receptor 2 (TLR2), promoting regulatory T cell (Treg) responses and maintaining intestinal immune homeostasis (Shen et al. 2012). This finding underscores the potential of BEVs to influence adaptive immune responses in a highly specific and beneficial manner. The potential of BEVs to support gut barrier function and modulate disease states has been highlighted in studies involving *Akkermansia muciniphila*. Wang et al. (2023) demonstrated that OMVs derived from this beneficial microbe can enhance epithelial barrier integrity, reduce gut inflammation, and influence host metabolism (Wang et al. 2023). These vesicles were also found to support colorectal cancer (CRC) immunotherapy, illustrating their potential as adjunct therapeutic agents. Similarly, Zheng et al. (2023) showed that *A. muciniphila*-derived OMVs protect against dextran sulfate sodium (DSS)-induced colitis in mice by reducing inflammation and enhancing barrier function, suggesting a broader role in preventing inflammatory bowel diseases (IBD) (Zheng et al. 2023).

Beyond direct host interactions, BEVs may contribute to shaping the gut microbiota itself. They can modulate microbial community structure by transferring genetic material or delivering antimicrobial compounds, creating a dynamic network of microbe-microbe and microbe-host communication (Díaz-Garrido, Badia, and Baldomà 2021).

These findings collectively highlight the multifaceted roles of BEVs in the gut ecosystem. Their ability to modulate immune responses, reinforce barrier function, and influence microbial composition makes them key players in gut physiology and potential targets for therapeutic development in diseases like IBD and CRC. Future research on their molecular cargo, targeting

mechanisms, and therapeutic applications may reveal new strategies for modulating host-microbe interactions.

1.3 Dietary polysaccharides

One of the most potent and modifiable regulators of gut microbiota, their metabolism and thus microbiota-host interaction, is diet. High-fiber, plant-based polysaccharide diets promote the growth of bacteria (e.g., *Bifidobacterium*, *Prevotella*) that ferment complex polysaccharides into beneficial SCFAs (Fu et al. 2022; Oliver et al. 2021). In contrast, high-fat or high-protein diets tend to enrich bile-tolerant and proteolytic species such as *Alistipes*, *Bilophila*, and certain *Clostridia*, often associated with pro-inflammatory profiles (Wang et al. 2021). Rapid changes in microbial community structure have been observed within 24–48 hours of dietary intervention, underscoring the plasticity of the gut ecosystem in response to nutritional inputs (David et al. 2014).

Dietary polysaccharides are the most abundant fermentable substrates available to the colonic microbiota and play a central role in regulating its composition and metabolic activity (Huang et al. 2022; Zhao et al. 2023). Complex polysaccharides escape digestion in the small intestine and are instead metabolized by specialized microbial taxa in the colon (or in caecum in mice), particularly *Bacteroides* and *Bifidobacterium* spp. but also key degraders from *Prevotella*, *Ruminococcus*, *Faecalibacterium*, and *Eubacterium*, as well as *Akkermansia* and certain *Clostridium* clusters IV and XIVa (Nishiyama et al. 2021). For example, *Bacteroides thetaiotaomicron* expresses an extensive array of glycoside hydrolases and polysaccharide lyases that allow it to degrade a broad range of dietary fibers (Zhang et al. 2022). Prebiotic oligosaccharides, including FOS, GOS, and XOS, selectively promote the growth of health-associated taxa such as *Bifidobacterium* and *Lactobacillus*. These bacteria contribute to intestinal homeostasis by competitively inhibiting pathogenic species, enhancing mucosal barrier function, and promoting regulatory immune responses (Gan, Wang, and Guo 2022).

Dietary polysaccharides include not only plant-derived fibers but also GAG such as chondroitin sulfate and HA. HA possesses potential to influence microbiota-host interactions, as its microbial degradation produces a range of bioactive compounds including shorter HA oligosaccharides and metabolites such as SCFAs. These molecules can modulate immune signaling pathways, stimulate the production of antimicrobial peptides, and enhance intestinal barrier integrity, thereby linking HA metabolism to both microbial ecology and host immune

homeostasis (Kim and de la Motte 2020; Zhang et al. 2024; Hill et al. 2012; Ray et al. 2023). In addition to its exogenous functions in host–microbiota interactions, endogenous HA is a vital component of the human body, where it contributes to many physiological processes.

1.3.1 HA and its functional role in GIT

HA, also commonly referred to as hyaluronan, is a well-characterized, high-molecular-weight glycosaminoglycan and a major structural component of the ECM. It is ubiquitously distributed across vertebrate tissues, including the skin, synovial fluid, vitreous humor, umbilical cord, and various connective tissues (Kim and de la Motte 2020). In addition to its endogenous presence in vertebrates, HA is also synthesized by certain microorganisms, most notably as a capsule-forming exopolysaccharide in *Streptococcus* spp. (Qiu et al. 2021).

Structurally, HA is a linear, non-sulfated polysaccharide composed of repeating disaccharide units of $\beta(1\rightarrow4)$ -linked D-glucuronic acid and $\beta(1\rightarrow3)$ -linked N-acetyl-D-glucosamine (Fig. 2). Its molecular weight can range from 10^5 to 10^7 Da, allowing the formation of long, unbranched polymer chains. Owing to its polyanionic nature, HA has a high affinity for water, enabling the formation of hydrated, viscoelastic ECM matrices that regulate tissue hydration, porosity, and mechanical resilience (Valachová, Hassan, and Soltés 2024).

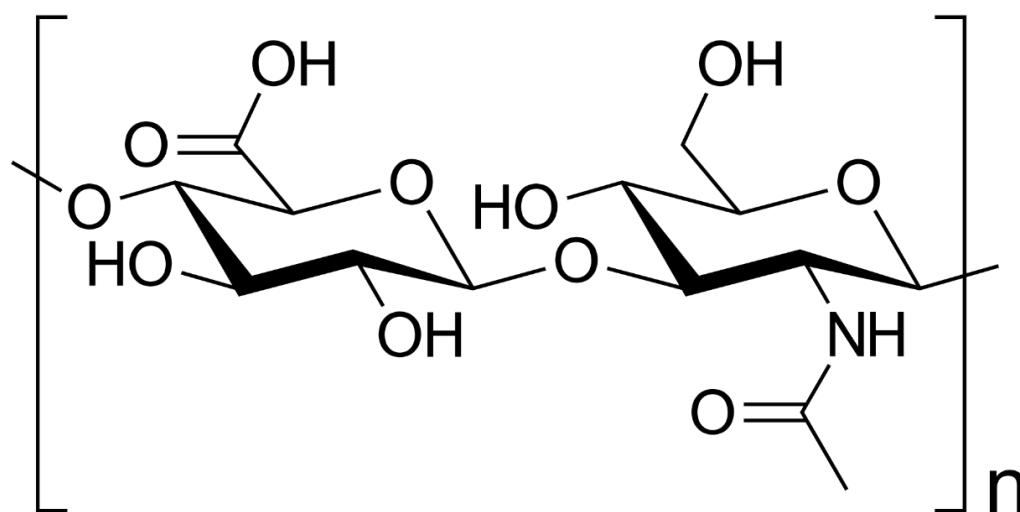


Fig. 2: Chemical structure of HA (Salih et al. 2024).

HA is synthesized at the inner surface of the plasma membrane by three isoforms of hyaluronan synthase (HAS1, HAS2, HAS3) and is secreted directly into the extracellular space. It is a highly dynamic molecule, with approximately one-third of total body HA undergoing daily turnover. Its half-life is tissue-dependent, ranging from several minutes in the bloodstream

to several days in cartilage(Cowman et al. 2015). Beyond its structural role, HA exerts key regulatory functions in tissue homeostasis, repair, and immune signaling. Through interactions with cell-surface receptors such as CD44, TLR2, and TLR4, HA modulates processes such as cell migration, cytokine production, and epithelial regeneration(Litwiniuk, Krejner, and Grzela 2016; de la Motte 2011).

In the GIT, HA plays an integral role in maintaining mucosal homeostasis and mediating responses to injury or inflammation. HMW-HA, which predominates in healthy tissues, contributes to gut barrier integrity by retaining water, stabilizing tight junctions, and forming a pericellular matrix that limits microbial translocation (Eckert et al. 2024; Gunasekaran et al. 2020). Additionally, HA enhances epithelial barrier function and supports the development of regulatory T-cell responses via CD44–STAT5 signaling, thereby participating in immune regulation and maintaining gut immune homeostasis (Bosi et al. 2022).

From an immunological perspective, HA exhibits size-dependent functional properties. HMW-HA is generally considered anti-inflammatory, contributing to tissue quiescence and epithelial protection. In contrast, during tissue injury or oxidative stress, HMW-HA is enzymatically cleaved into low-molecular-weight (LMW) fragments. These fragments were historically described as damage-associated molecular patterns (DAMPs) capable of engaging pattern recognition receptors such as TLR2 and TLR4, thereby activating immune cells and inducing pro-inflammatory cytokine production(Romo et al. 2022; Jiang, Liang, and Noble 2011; Collins et al. 2011). However, more recent evidence suggests that the immunomodulatory role of HA is highly context dependent. The biological activity of HA fragments is influenced not only by their size but also by cell type specificity, receptor expression, and the local microenvironment(Cowman et al. 2015). This complexity underscores the need to reassess simplistic models of HA as merely pro- or anti-inflammatory and instead view it as a finely tuned regulator of immune responses.

HA also plays a central role in mucosal repair processes. Following injury, HA levels increase locally and facilitate tissue regeneration by promoting fibroblast recruitment, epithelial proliferation, and angiogenesis. These reparative functions are mediated by HA's interactions with immune and stromal cells and are essential for restoring the integrity of the gut barrier(Fallacara et al. 2018).

Taken together, the structural and signaling functions of HA place it at the intersection of tissue architecture, immune modulation, and repair. Its diverse biological roles, ranging from

barrier stabilization to inflammation resolution and wound healing, make it a critical component in gastrointestinal physiology and host defense.

1.4 Degradation of HA in GIT

1.4.1 Host-cell degradation of HA

The cellular network responsible for HA degradation in the intestine involves the hyaluronidase (HYAL) enzymes HYAL1 and HYAL2, which are active in multiple intestinal cell types, including epithelial cells, fibroblasts, and immune cells, thereby contributing to tissue-level HA turnover. Additional non-lysosomal degradation pathways have been identified, notably those mediated by Cell Migration-Inducing Hyaluronidase (CEMIP/KIAA1199) and the cell-surface hyaluronidase Transmembrane Protein 2 (TMEM2), which participate in the breakdown of extracellular and surface-bound HA and influence processes such as cell migration and barrier regulation (Kim and de la Motte 2020). *In vivo* evidence from HYAL-deficient genetic models supports a role for host-driven HA catabolism in gut tissues (Fink and Triggs-Raine 2024). Mice lacking HYAL1 or HYAL2 show disrupted HA homeostasis, including the accumulation of HA fragments in connective tissues and altered lymphatic clearance, changes that likely involve the intestine and mesenteric lymph nodes (Bourguignon and Flamion 2016). In addition to enzymatic pathways, HA turnover in the gut may also occur via non-enzymatic mechanisms, including degradation induced by ROS, acidic pH, or mechanical shear stress, particularly under conditions of physiological stress or inflammation.

1.4.2 Microbial degradation of HA

Microbial degradation of HMW HA has been documented in both Gram-negative and Gram-positive bacteria, often alongside the breakdown of other GAG such as chondroitin sulfate. The underlying mechanisms differ substantially due to their distinct cell envelope architectures. Gram-negative bacteria, exemplified by *Bacteroides* spp., possess a tripartite structure comprising an outer membrane, periplasmic space, and inner membrane. This compartmentalization enables a stepwise process in which HA fragments are initially processed by specific outer membrane-associated enzymes and transporters, followed by further degradation within the periplasm (Ndeh et al. 2020; Overbeeke et al. 2022). In contrast, Gram-positive genera such as *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Lactobacillus* lack an outer membrane and instead feature a thick, peptidoglycan-rich cell wall. This architecture necessitates alternative strategies involving extracellular HA-degrading enzymes for polymer breakdown and subsequent uptake (Kawai et al. 2018). Such structural divergence shapes the

enzymatic repertoires across bacterial taxa, adding to the complexity of HA catabolism within the gut microbiome.

1.4.2.1 Degradation pathway for GAG in Gram-negative bacteria

The genus *Bacteroides* serves as a model for Gram-negative bacteria in GAG metabolism, including HA. In *Bacteroides*, the enzymatic machinery for polysaccharide degradation is organized within specialized genomic regions known as polysaccharide utilization loci (PULs), which enable the recognition, binding, and degradation of structurally diverse glycans (Ndeh et al. 2020). In *Bacteroides thetaiotaomicron*, the processing of multiple GAGs is coordinated by a single, versatile GAG-specific PUL that integrates several functional modules within one locus (Fig. 3a). This PUL encodes surface glycan-binding proteins for polymer recognition, SusC/D-like TonB-dependent transporters for importing partially degraded oligosaccharides into the periplasm, and both surface-associated and periplasmic polysaccharide lyases that cleave GAG backbones via β -elimination. It also contains glycoside hydrolases for converting unsaturated disaccharides into monosaccharides, sulfatases for removing sulfate groups from substrates such as chondroitin and dermatan sulfates, and a hybrid two-component regulatory system that detects substrate presence and activates gene expression accordingly. This modular organization supports sequential processing from extracellular cleavage to transport and periplasmic degradation allowing *B. thetaiotaomicron* to exploit HA, chondroitin sulfate, and dermatan sulfate by selectively deploying enzymes based on substrate structure and sulfation pattern (Fig. 3b) (Ndeh et al. 2020).

1.4.2.2 Degradation pathway for GAG in Gram-positive bacteria

In Gram-positive bacteria such as *Lactobacillus* and *Enterococcus*, GAG degradation follows a multi-step process distinct from Gram-negative systems due to the absence of an outer membrane. Cell-surface or extracellular polysaccharide lyases e.g. polysaccharide lyase 12 (PL12) family heparin lyase HepC, first depolymerize GAGs such as heparin into unsaturated disaccharides via β -elimination. These disaccharides are imported into the cytoplasm through a phosphotransferase system (PTS), where they undergo further processing. Cytoplasmic unsaturated glucuronyl hydrolase (UGL) cleaves them into unsaturated uronic acids and amino sugars. The unsaturated uronic acids spontaneously convert to 4-deoxy-L-threo-5-hexosulose uronic acid (DHU), which is metabolized to pyruvate and glyceraldehyde-3-phosphate via a sequential reaction catalyzed by ketol-isomerase KduI, NADH-dependent reductase KduD, kinase KdgK, and aldolase KdgA. This pathway, encoded within conserved GAG genetic clusters, enables Gram-positive gut bacteria to utilize host-derived GAGs as nutrient sources and may contribute to their intestinal colonization potential(Kawai et al. 2018)

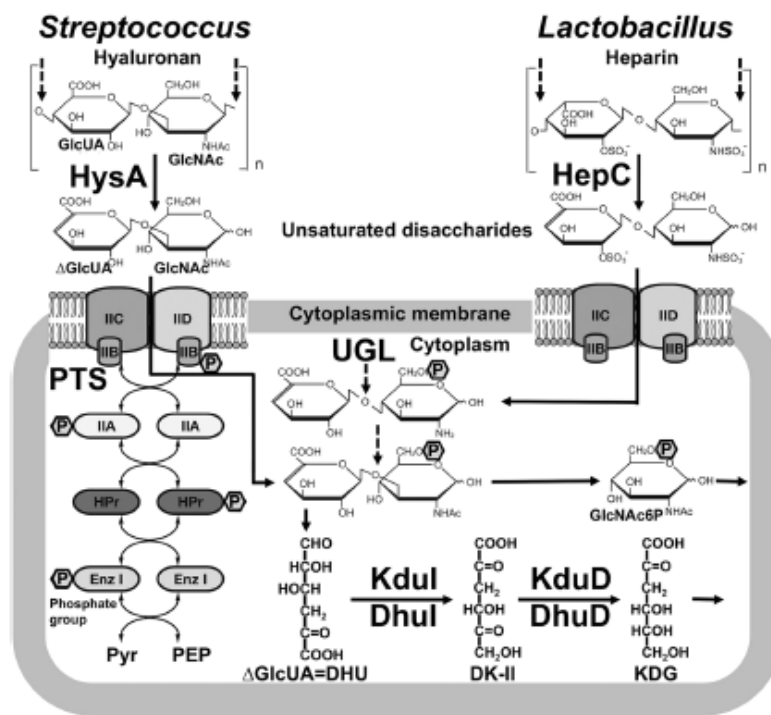


Fig.4: Proposed model for glycosaminoglycan degradation in the Gram-positive bacteria *Streptococcus* sp. and *Lactobacillus* sp.(Kawai et al. 2018).

1.5 Bioavailability of orally administered HA

The bioavailability of orally administered HA remains a subject of ongoing scientific debate, particularly regarding whether intact HA or its degraded oligosaccharide fragments can be absorbed across the GIT and distributed systemically. Numerous studies suggest that HMW HA is poorly absorbed in the upper GIT due to its large molecular size and hydrophilic nature. Although several *in vivo* studies have detected trace levels of HA or radiolabeled fragments in plasma following oral administration, these findings are inconsistent and generally suggest that systemic absorption is limited to LMW fragments or microbial degradation products like SCFAs. For example, Balogh et al. (2008) demonstrated that radiolabeled HMW HA could be detected in connective tissues, including joints, after oral administration indicating partial uptake from the gut, although absolute absorption levels were low (Balogh et al. 2008). Similarly, Kimura et al. (2016) reported that HA was not recovered in feces but was instead degraded by cecal microbiota into oligosaccharides, which were subsequently absorbed through the large intestine and distributed to peripheral tissues, including the skin (Kimura et al. 2016). Supporting this, Mannino et al. (2024) observed a dose-dependent increase in HA concentrations in both plasma and lymph after oral supplementation, with the highest uptake occurring for the smallest HA fragments suggesting that LMW HA is more efficiently absorbed via both portal and lymphatic pathways (Mannino et al. 2024).

Taking it together, these findings challenge the notion that intact HMW HA undergoes substantial systemic absorption. Instead, they support a more nuanced pharmacokinetic model in which microbial metabolism plays a central role, owing to the presence of diverse bacterial taxa in the GIT that are enzymatically equipped to degrade HA (Ndeh et al. 2020; Kawai et al. 2018). In parallel, host-derived enzymatic pathways may also contribute to HA turnover in the GIT. These endogenous mechanisms act independently of microbial metabolism and likely target HA that reaches the intestinal mucosa. However, under physiological conditions, the mammalian intestine is densely colonized, suggesting that both host- and microbiota-mediated degradation pathways are likely to act in concert. The relative contribution and efficiency of each pathway remain insufficiently defined, but it is plausible that they function in a context-dependent and complementary manner, shaping HA catabolism via a dynamic host–microbe interplay.

2. Hypothesis

HMW HA degradation in the GIT is primarily mediated by gut microbiota, particularly *Bacteroides* species, through specialized PUL which encode polysaccharide lyases and associated proteins essential for substrate recognition, uptake, processing and shaping microbial community dynamics.

3. Aims

- 1) Comprehensive characterization of distinct HYALs and HA lyases by employing phylogenetic analysis and multiple sequence alignment to elucidate their evolutionary relationships and functional diversity.
- 2) Evaluating the presence of HA lyases as microbiota-produced enzymes in GIT on bioavailability of orally administered HMW HA and impact on gut microbiota composition.
- 3) In-depth analysis of the proteomic profiles and genomic features of *Bacteroides* species, with a focus on their HMW HA degradation pathways.

4. Results

The results of my PhD thesis have been published in two scientific articles: one as first author (Article I – review article), and another as co-author (Article II). A third manuscript related to the thesis, also as first author (Article III) has been submitted to the journal mBio (Q1) in August 2025.

In addition, I contributed to two other publications (Article IV and Article V) that are not directly tied to the dissertation topic but are focused on intercellular communication in GIT via EVs. In both, I was responsible for implementing and analyzing the proteomic data.

4.1 Comprehensive characterization of distinct HYALs and HA lyases by employing phylogenetic analysis and multiple sequence alignment

HA turnover is tightly coupled to the expression and enzymatic activity of HYALs. Genetic and biochemical studies have demonstrated that HA's rapid catabolism, its half-life in tissues often measured in hours to days, is driven substantially by specific HYALs which cleave HMW HA into oligosaccharides (Zadnikova et al. 2022).

Where HYALs expression is elevated, HA turnover accelerates; conversely, reduced HYALs activity due to genetic deficiency or regulatory suppression leads to HA accumulation in tissues (Triggs-Raine and Natowicz 2015). Thus, HA turnover closely mirrors HYALs expression patterns and enzymatic function, with both being central to the dynamic regulation of the HA matrix.

Due to the limited data available or old information from review wrote 10 years ago about HYALs I decided at the beginning of my PhD research to prepare a review article focused on comparing different HYALs families using phylogenetic analysis and multiple sequence alignments. Summarizing this knowledge became both essential and in high demand within the scientific community. The work was conducted primarily through *in silico* approaches, with a focus on elucidating sequence diversity, structural characteristics, and enzymatic mechanisms involved in HA cleavage. My research investigated these enzymes across a broad spectrum of biological sources including vertebrates, invertebrates, bacteria, micromycetes and bacteriophages with particular emphasis on their structural and functional properties. Consequently, the review was published (Article I – Sindelar et al., 2021: *Hyaluronidases and*

Hyaluronate Lyases: From Humans to Bacteriophages). The main findings from this review are summarized in the following sections.

4.1.1 Phylogenetic and structural analysis of HYALs and HA lyases

HYALs and HA lyases are enzymes that vary widely in their catalytic mechanisms and have historically been classified into two distinct groups based on their biochemical properties (Meyer and Rapport 1952):

- a) Hydrolases (vertebrate and leech-type HYALs which cleave β -1,4 or β -1,3 glycosidic bonds in HA by hydrolysis to reach saturated HA oligosaccharides.
- b) Lyases (bacterial and phage HA lyases) which employ β -elimination to cleave HA, producing unsaturated HA oligosaccharides.

Building on Karl Meyer's traditional classification, we expanded the review with categorization of HA-degrading enzymes by including newly identified proteins, such as CEMIP and TMEM2, which exhibit HA-degrading activity but do not fit into the classical groups based on biochemical properties (Yamamoto et al. 2017; Yoshida and Okada 2019).

Using all 92 annotated sequences of HYALs and HA lyases from UniProt and Carbohydrate active enzyme databases (CAZy), a phylogenetic analysis was performed, revealing six major enzyme families (Fig.5): glycoside hydrolase (GH) 56 (Vertebrate and venom HYALs), GH79 (Leech-type HYALs), polysaccharide lyases (PL) 8 (Bacterial HA lyases), PL16 (Bacteriophage HA lyases), GH16 (Micromycetes HYALs), CEMIP/TMEM2 cluster (Structurally distinct eukaryotic HA-degrading proteins).

Following the phylogenetic analysis, a multiple sequence alignment was performed to assess sequence identity among the investigated enzyme families. The alignment revealed a generally low overall sequence identity; however, key residues associated with substrate binding and catalysis were consistently conserved. In the glycoside hydrolase family GH56, characteristic of vertebrate HYALs, catalysis proceeds via a two-carboxylic acid mechanism mediated by the conserved residues Asp129 and Glu131. These residues are essential for the hydrolytic cleavage of $\beta(1\rightarrow4)$ glycosidic bonds in HA, producing saturated HA oligosaccharides. In contrast, bacterial HA lyases from polysaccharide lyase family PL8 cleave HA through a β -elimination mechanism, generating unsaturated HA oligosaccharides with a double bond between C4 and C5 of the non-reducing terminal glucuronic acid residue. PL8 enzymes possess conserved residues Asn349, His399, and Tyr408, which form the substrate-binding cavity, stabilized by aromatic residues Trp291 and Phe343 that facilitate substrate recognition and cleavage.

Among these enzyme families, GH16 HYALs from micromycetes remain the least characterized, with their substrate specificity and catalytic mechanisms still not fully elucidated. To address this gap, an in-depth *in silico* analysis was conducted to investigate the evolutionary relationships and functional diversity of these poorly described enzymes. The GH16 family is subdivided into 27 subfamilies, with micromycete HYALs belonging to subfamily GH16_1. This subfamily consists predominantly of fungal enzymes capable of processing HA as well as other substrates, including laminarin and xyloglucan. Multiple sequence alignment of GH16_1 members (Fig. 6) revealed two highly conserved, negatively charged residues Glu113 (E113) and Glu118 (E118) within the GEIDIXE motif, a sequence pattern characteristic of GH16_1 and broadly conserved across the entire GH16 family. In this catalytic arrangement, E113 functions as the nucleophile, whereas E118 serves as the general acid/base catalyst. Experimental mutagenesis studies have demonstrated that substitutions at these positions significantly reduce enzymatic activity, confirming their essential roles, in contrast to GH56 HYALs, which employ a distinct catalytic mechanism (Cheng et al. 2014).

Structural modeling further indicates that residues Glu113, Asp115, and Glu118 from the GEIDIXE motif have side chains oriented toward the substrate-binding cavity, directly facilitating catalysis. Conversely, Ile114 and Ile116 are oriented away from the active site, contributing to structural integrity through hydrophobic interactions that reinforce the β -sandwich fold. In addition to the GEIDIXE motif, aromatic residues Trp97, Trp101, and His131 play critical roles in substrate binding and catalytic efficiency, collectively shaping the functional architecture of GH16_1 HYALs.

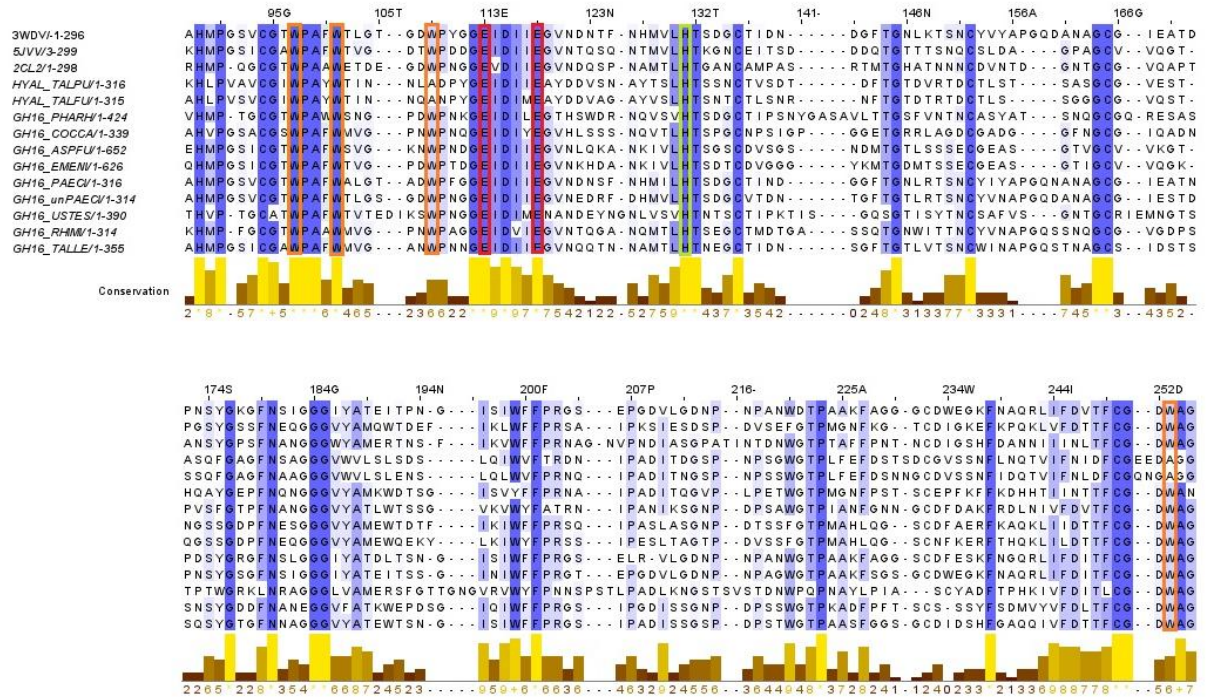


Fig. 6: Partial Multiple sequence alignment of the GH16_1 subfamily using 14 characterized enzyme sequences. The aa residues are numbered according to the secondary structure of 3WDV (*Paecilomyces thermophila* J18 β -1,3-1,4-glucanase). Blue represents conserved residues; green frame represents polar residuum responsible for the hydrogen bonding of ligand in the 3WDV structure (H131); orange frames represent aromatic residues that provide CH- π stacking (W97, W101, W108, W253); red frames represent catalytic residues (E113, E118). Alignment was constructed using the ClustalW algorithm and visualized in Jalview software(Thompson, Higgins, and Gibson 1994; Waterhouse et al. 2009). 3WDV (PDB ID); 5JVV (PDB ID); 2CL2 (PDB ID); GH16_PHARH (UniProt ID: O60019); GH16_COCCA (UniProt ID:O14421); GH16_ASPFU (UniProt ID:Q4X084); GH16_EMENI (UniProt ID:Q5BAP5); GH16_PAEQI (UniProt ID:G4WNE9); GH16_unPAEQI (UniProt ID:A0A386YJ14); GH16_USTES (UniProt ID:I7HBL1); GH16_RHMI (UniProt ID:H9BZ70); GH16_TALLE (UniProt ID:A0A168SIC2).

4.1.2 Summary

This chapter has outlined the remarkable enzymatic diversity of HA-degrading enzymes across vertebrates, invertebrates, fungi, bacteria, and bacteriophages. Comprehensive phylogenetic and structural analyses revealed that, despite substantial sequence divergence, key catalytic features remain conserved across families. Mechanistically, two major strategies dominate: hydrolases, primarily present in vertebrates and some eukaryotes, which hydrolyze $\beta(1\rightarrow4)$ linkages to yield saturated HA oligosaccharides; and lyases, prevalent in bacteria and bacteriophages, which employ β -elimination to produce unsaturated HA fragments. Both enzyme types are central to HA turnover in diverse biological settings, reflecting evolutionary adaptation to distinct ecological niches and physiological requirements.

These findings provide the conceptual framework for the following chapter, which examines the fate of orally administered HA within GIT, where both host-derived HYALs and microbial HA lyases may act independently or synergistically. *In vivo*, the metabolic processing of HA generates structurally distinct oligosaccharides with potential signaling roles, while also influencing microbial community composition, SCFA production, and intestinal homeostasis. Elucidating how the enzymatic mechanisms described here translate into GIT degradation pathways is essential for understanding microbiota-mediated effects of exogenous HA supplementation.

4.2 Evaluating the presence of HA lyases as microbiota-produced enzymes in GIT on bioavailability of orally administered HMW HA

Following the comprehensive analysis of enzymes involved in HA degradation, we collaborated with Contipro a.s. to investigate the fate of orally administered HA in the human body (Aim 2). HA has a long history of use as both a dietary supplement and a therapeutic agent most notably in dermatology (for skin hydration and as a dermal filler), osteoarthritis management (via viscosupplementation), and wound healing (through its tissue-regenerative and anti-inflammatory properties). Its beneficial effects on hydration, structural support, inflammation modulation, and tissue repair are well documented in the scientific literature (Migliore and Procopio 2015; Antoszewska, Sokolewicz, and Baranska-Rybak 2024; Simek et al. 2025).

Despite this, the literature presents inconsistent and sometimes contradictory information regarding the absorption, bioavailability, and mechanism of action of orally supplemented HA. This gap underscores the need to clarify whether HMW HA can traverse the gastrointestinal barrier intact, or whether it undergoes fermentation by gut microbiota, and how such processes may influence gastrointestinal microbial composition. To address these questions, we conducted a targeted study, the results of which were published in Article II (*Simek et al., 2023: Molecular weight and gut microbiota determine the bioavailability of orally administered hyaluronic acid*). The principal findings are summarized in the following sections.

4.2.1 The fate of perorally administered HMW HA

This study comprised two complementary parts: a pharmacokinetic analysis and a long-term supplementation experiment. In the first part, ¹³C-labeled HA combined with LC–MS was used to investigate the absorption and metabolic fate of orally administered HMW HA in germ-free and conventionally raised wild-type mice. The primary objective was to trace the degradation products of HMW HA along the gastrointestinal tract. As hypothesized, HMW HA underwent depolymerization during its passage through the GIT. In conventional mice, a variety of unsaturated HA oligosaccharides of differing lengths were detected in the small intestine, whereas the cecum and colon, regions with high microbial density and activity, contained predominantly unsaturated disaccharides. In contrast, germ-free mice exhibited no detectable unsaturated HA oligosaccharides, and only a minor reduction in HA molecular weight was observed, likely attributable to acid hydrolysis in the low-pH environment of the stomach.

These findings strongly indicate that microbial HA lyases are key contributors to the degradation, and thus the bioavailability, of orally administered HMW HA.

4.2.2 The impact of perorally administered HMW HA on microbial composition

Building on these findings, the next objective was to evaluate the effects of oral HA supplementation, alongside other dietary supplements, on gastrointestinal microbiota composition. Mice received daily supplementation for 28 days with one of the following HMW HA, pectin, *N*-acetylglucosamine, or water (control). At the end of the treatment period, animals were sacrificed, and cecal contents were collected for microbiota profiling via 16S rRNA gene sequencing. The experimental design is summarized in Fig. 9.

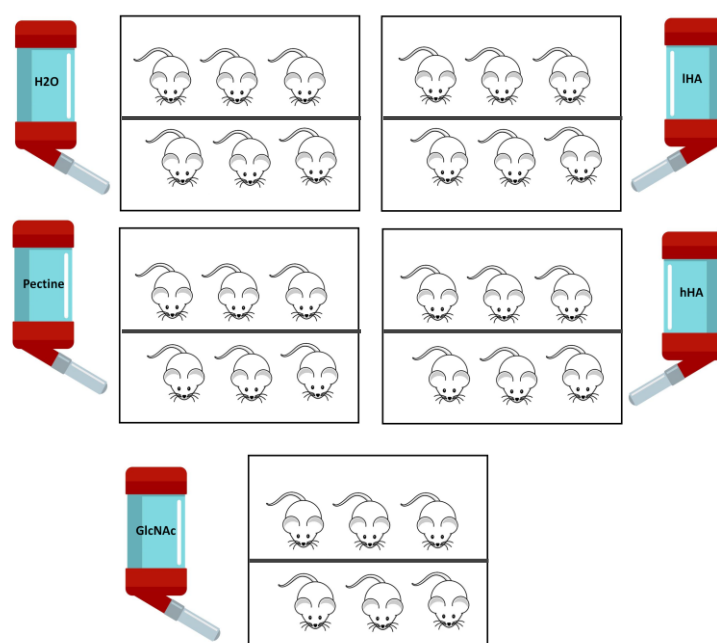


Fig.9: Experimental design of the 28-day oral supplementation study. Supplements included: H₂O – water (control), lHA – low dose of high-molecular weight hyaluronan (0.25 mg/mL), hHA – high dose of high-molecular weight hyaluronan (2.5 mg/mL), pectin (2.5 mg/mL), and GlcNAc – *N*-acetylglucosamine (2.5 mg/mL).

The V3–V4 region of the 16S rRNA gene was amplified and sequenced using the Illumina MiSeq platform (Eurofins Genomics, INVIEW Microbiome Profiling). My role in this stage was to process and analyze the raw sequencing data to determine relative taxonomic abundances and compare the effects of different supplements. Paired-end reads were processed using the open-source Mothur software package (v1.48.1), following the MiSeq Standard

Operating Procedure with modifications appropriate to the experimental design (Schloss et al. 2009); see methods in Article II for details.

Detecting statistically significant changes in microbiota composition in response to supplementation proved challenging. Principal Coordinate Analysis (PCoA) revealed no distinct clustering of samples (Fig. 10), and neither alpha nor beta diversity metrics showed statistically significant differences between treatment groups.

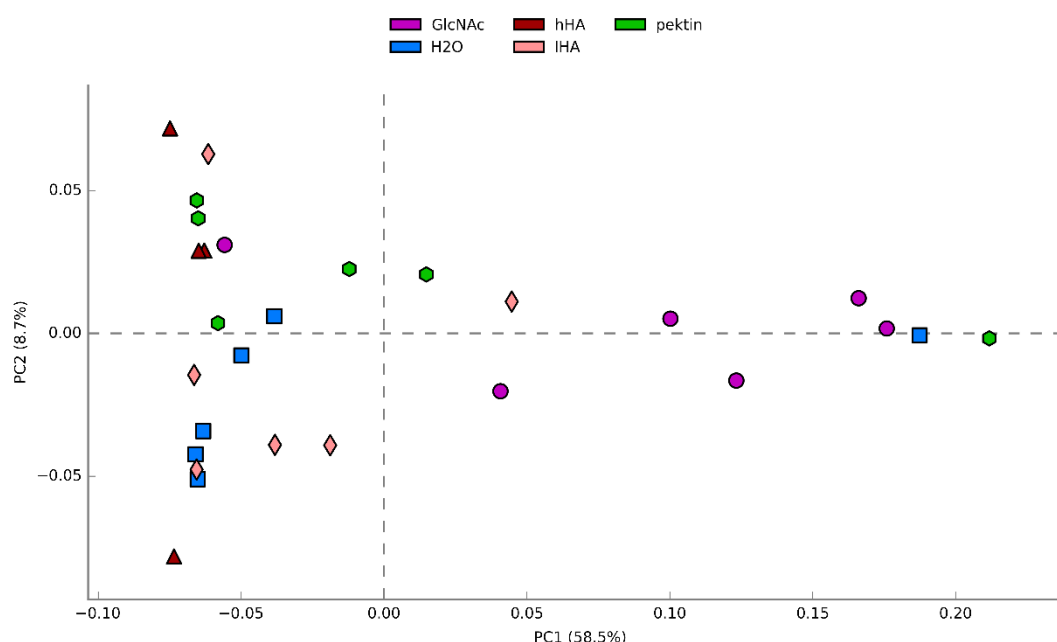


Fig. 10: Principal Coordinate Analysis (PCoA) plot based on OTU-level taxonomic profiles. Each point represents the microbial community of an individual mouse cecal sample after 28 days of supplementation. Samples are color-coded by treatment group: high dose of high-molecular-weight hyaluronan (hHA - red), low dose of high-molecular-weight hyaluronan (IHA - pink), pectin (green), GlcNAc (purple), and control (water; blue).

Subsequent genus-level analyses indicated that within-group variability exceeded the magnitude of any supplement-related effects, despite all animals being cohoused (Fig. 11A). Nevertheless, two genera with documented HA-degrading activity *Bacteroides* and *Enterococcus* displayed a noticeable increase in relative abundance in HA-supplemented groups (Fig. 11B). This trend, however, was not statistically significant due to the high variability among individuals.

Although not evident in higher-level taxonomic summaries, increases were also observed in certain operational taxonomic units (OTUs) belonging to the less abundant genera *Anaeroplasma*, *Clostridium* XIVb, and *Parabacteroides*, despite no database evidence for HA-

degrading capacity in these taxa. Similarly, the abundance of *Akkermansia muciniphila* increased in HA-treated groups although no HA-degrading activity for this species was detected in our study (Fig. 11B).

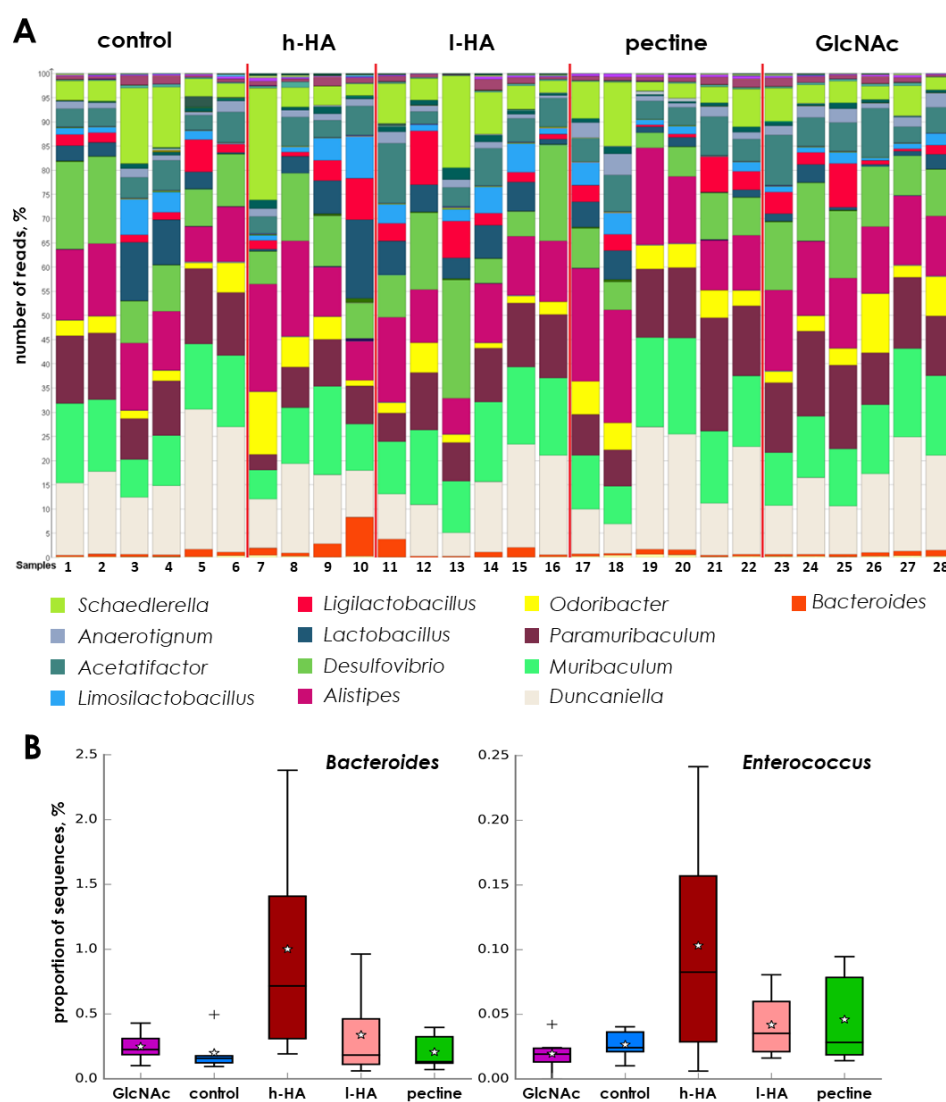


Fig. 11: Response of the gut microbiota to supplementation of HA, pectin and N-acetyl glucosamine. A) Structural comparison of gut microbiota at the genus level, B) Relative abundance of *Bacteroides* and *Enterococcus* in mice cecum after four-week supplementation with high doses of HMW HA (h-HA, 760 mg kg⁻¹ day⁻¹), low doses of HMW HA (l-HA, 90 mg kg⁻¹ day⁻¹), pectin (640 mg kg⁻¹ day⁻¹) and GlcNAc (650 mg kg⁻¹ day⁻¹) (Simek et al. 2023).

4.2.3 Metabolomic tracing and functional inference of gut microbial hyaluronan degradation

Metabolomic profiling (Article II; (Simek et al. 2023)) revealed that HA-derived unsaturated disaccharides were further metabolized via established bacterial pathways into phosphorylated intermediates, including GlcNAc-P and UDP-GlcNAc, indicating that HA can function both as an energy source and as a precursor for glycan biosynthesis. Significant ^{13}C incorporation was also detected in SCFAs and hydroxy acids, with labeling patterns consistent with propionate production via the *Bacteroides*-specific succinate pathway and butyrate synthesis through both the butyryl-CoA route and CO_2 fixation. These results demonstrate that *Bacteroides* spp. can completely catabolize HA to SCFAs and CO_2 . While intracellular intermediates such as UDP-GlcNAc were not detected in host plasma or liver, SCFAs known to enhance gut barrier integrity and exert immunomodulatory effects were efficiently absorbed, providing a plausible route for host benefit from microbial HA metabolism (Simek et al. 2023).

To complement these experimental findings, I performed predictive metagenomic analysis using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al. 2020). This computational framework infers microbial gene content and metabolic pathway abundances from 16S rRNA sequencing data by mapping OTUs to reference genomes and predicting functional gene presence based on phylogenetic relationships. As 16S rRNA sequencing offers only taxonomic resolution, PICRUSt2 provides an additional functional perspective, particularly valuable when metagenomic or metatranscriptomic datasets are not available (Douglas et al. 2020).

The aim of this analysis was to assess whether dietary supplementation particularly with HMW HA induced measurable shifts in predicted microbial metabolic capacities, such as GAG degradation, SCFA production, or synthesis of immunomodulatory compounds. Predicted pathway profiles were visualized via PCoA to evaluate functional clustering among treatment groups (Fig. 12). Minimal variation was observed between groups, with no distinct clustering, consistent patterns, or statistically significant differences attributable to supplementation. Although no substantial functional shifts were identified, these predictions complemented taxonomic data and reinforced the value of integrating functional inference tools into microbiome studies.

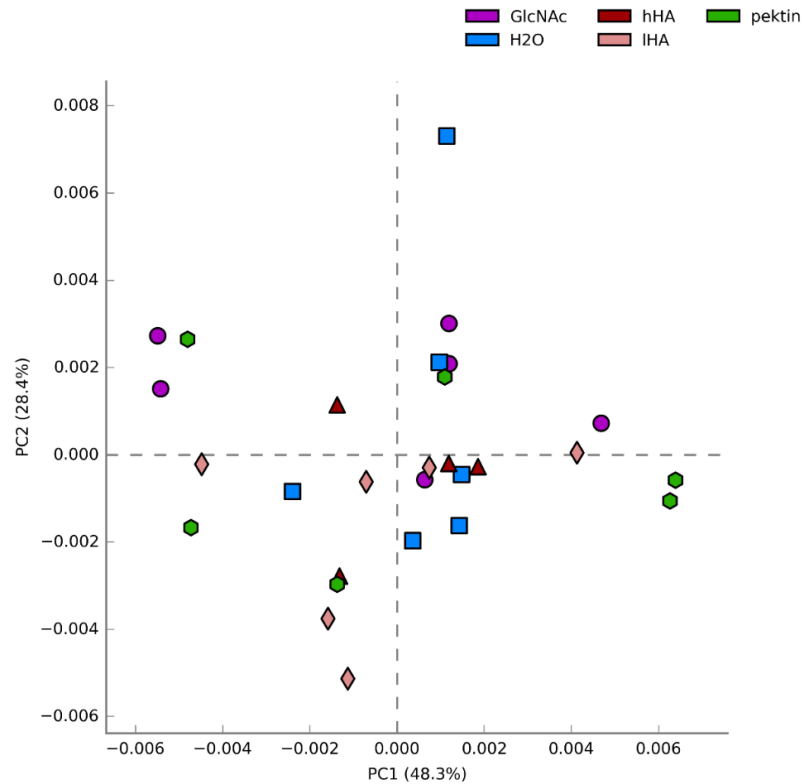


Fig. 12: Principal Coordinate Analysis plot based on predicted metabolic pathway abundances inferred using PICRUST2, visualized using STAMP software. Each point represents the functional profile of the gut microbiota from an individual mouse cecal sample after 28 days of supplementation. Samples are color-coded by treatment group: high dose of high-molecular-weight hyaluronan (hHA – red), low dose of high-molecular-weight hyaluronan (lHA – pink), pectin (green), GlcNAc (purple), and control (H₂O – blue).

4.2.4 Summary

This chapter examines the *in vivo* fate of orally administered HMW HA. Our findings indicate that the physiological effects of oral HMW HA are unlikely to result from its direct delivery to peripheral tissues such as joints or skin, as only unsaturated HA fragments were detected in wild-type mice. The absence of such fragments in germ-free animals confirms that gut microbial enzymes particularly HA lyases are essential for HA depolymerization during gastrointestinal transit.

The GIT hosts dense and metabolically versatile microbial communities capable of degrading complex polysaccharides, including HA, into unsaturated oligosaccharides. While HMW HA supplementation did not produce statistically significant shifts in overall microbiota composition, a modest increase in the relative abundance of *Bacteroides* and *Enterococcus* was

observed, consistent with HA degradation occurring predominantly in the distal gut (cecum and colon). However, the genus-level taxonomic resolution afforded by 16S rRNA V3–V4 sequencing limits attribution to specific HA-degrading species.

Metabolomic profiling demonstrated that HA-derived fragments were further metabolized by gut bacteria into phosphorylated intermediates, such as GlcNAc-P and UDP-GlcNAc, and into SCFAs via *Bacteroides*-specific and butyryl-CoA pathways. While intracellular intermediates were not detected in host plasma or liver, SCFAs were efficiently absorbed, representing a likely route for systemic effects of microbial HA metabolism. Collectively, the results support a model in which HMW HA is enzymatically depolymerized by gut microorganisms into unsaturated fragments, which are further fermented to SCFAs. Given the established roles of acetate, propionate, and butyrate in gut health and immune modulation, this microbial processing is likely to have systemic consequences. Notably, recent evidence suggests that SCFAs can directly inhibit colorectal cancer cell proliferation and modulate antitumor immunity, with context-dependent effects that may be either protective or, under certain conditions, pro-tumorigenic (Cheng, Li, and Yang 2024).

4.3 In-depth analysis of the proteomic profiles and genomic features of *Bacteroides* species, with a focus on their HMW HA degradation pathways

Our previous results indicate that the genus *Bacteroides* increases in relative abundance following *in vivo* supplementation with HMW HA (Article II) (Simek et al. 2023). In that study, gut microbiota composition was assessed using 16S rRNA gene sequencing targeting the V3–V4 hypervariable regions (~408 bp amplicon). While this approach enabled genus-level profiling, the combination of marker-gene sequencing and downstream bioinformatic processing limited taxonomic resolution to the genus level, precluding robust species-level identification. This represents a critical limitation, as many microbial functions including HA degradation are species-specific rather than conserved across an entire genus (Overbeeke et al. 2022).

Species-level resolution and functional potential are more reliably achieved through shotgun metagenomic sequencing; however, financial constraints rendered this approach impractical for the present work (Durazzi et al. 2021; Rashidi et al. 2024). Consequently, I

explored alternative strategies to more precisely resolve *Bacteroides* to the species level, with a particular focus on identifying taxa capable of HA degradation. Such species are likely to play a pivotal role in shaping gut ecosystem responses to long-term HA supplementation.

The results of this work are presented in Article III (Sindelar et al.: *Proteomics identifies non-canonical enzymes involved in hyaluronan degradation in Bacteroides*), which has been submitted to mBio journal as a first-author manuscript. Because the manuscript remained unpublished at the time of dissertation submission, the findings are described here in greater detail than for the first two aims.

4.3.1 Introduction

The mammalian GIT harbors a taxonomically diverse microbial community essential for host metabolic function, immune regulation, and barrier integrity (Maciel-Fiuza et al. 2023). Among its dominant members, *Bacteroides* spp. are particularly notable for their ability to degrade structurally complex polysaccharides, a capacity mediated by PULs, which encode proteins for glycan recognition, depolymerization, and uptake. The diversity of PULs underpins the ecological success of *Bacteroides* and enables adaptation to distinct glycan substrates (Rawat et al. 2022).

GAGs including chondroitin sulfate, heparan sulfate, and HA represent diet-associated polysaccharides of particular interest. Certain *Bacteroides* spp. utilize GAGs as nutrient sources, providing them with a selective advantage, and oral HA administration has been shown to enrich *Bacteroides* populations *in vivo* (Simek et al. 2023). Mechanistic insights into this process have been gained from studies of GAG-specific PULs, especially in *Bacteroides thetaiotaomicron*, which serves as a model for GAG metabolism (Ndeh et al. 2020). However, the ability to degrade GAGs varies markedly across species, ranging from non-degraders to strains capable of utilizing multiple substrates, including HA (Akazawa et al. 2023; Fang et al. 2024; Kawai et al. 2018; Overbeeke et al. 2022).

The enzymatic machinery underlying GAG degradation is best characterized for *B. thetaiotaomicron* and includes polysaccharide lyases 8 (PL8) and glycoside hydrolase 88 (GH88) family members, which act on GAG-derived substrates, and PL33 enzymes, suggested to exhibit higher specificity for HA (Pan et al. 2021; Yin et al. 2025). Yet, due to limited annotation of PL33 homologs, PL8 and GH88 enzymes remain the most practical targets for comparative analyses despite their limited substrate specificity (Overbeeke et al. 2022; Drula et al. 2022).

This chapter addresses the hypothesis that *Bacteroides* species can be functionally grouped according to their GAG-degrading potential, as inferred from the presence and conservation of PL8- and GH88-family enzymes and proteins encoded by GAG-specific PULs. To test this, I combined *in silico* comparative analyses with cultivation experiments on HA, followed by proteomic profiling of PUL-encoded proteins. This approach aims to identify functional signatures of HA utilization, uncover unrecognized degradative capacities, and improve predictions of metabolic potential in poorly characterized *Bacteroides* species.

4.3.2 Comparative clustering of PL8 and GH88 enzymes in *Bacteroides* reveals limited predictive power for HA-degrading phenotypes

Excluded for confidentiality (unpublished)

5. Discussion

The bioavailability of orally administered HA remains a matter of debate, particularly concerning whether intact HMW polymers can cross the GIT and enter systemic circulation. Current evidence for direct uptake of intact HMW HA is sparse and inconsistent, making it unlikely that the reported physiological benefits result from the incorporation of native polymers into peripheral tissues (Fin et al. 2024). The physicochemical properties of HMW HA, its large size and strong hydrophilicity, further argue against efficient trans-epithelial transport. While a few animal and human studies have detected trace amounts of radiolabeled HA or HA-like material following oral administration, these signals are sporadic and typically correspond to LMW fragments or downstream microbial metabolites. For instance, Balogh et al. (2008) reported radiolabeled HMW HA in connective tissues after supplementation, though overall absorption was minimal (Balogh et al. 2008). Similarly, Kimura et al. (2016) demonstrated that fecal HA was absent because cecal microbiota degraded it into oligosaccharides, which were subsequently absorbed in the large intestine and distributed to peripheral tissues, including skin (Kimura et al. 2016). More recently, Mannino et al. (2024) described dose-dependent increases in plasma and lymphatic HA following supplementation, with uptake favoring the smallest fragments, consistent with absorption through portal and lymphatic routes (Mannino et al. 2024).

Taken together, these findings suggest that the health effects of oral HMW HA supplementation such as improved skin hydration, enhanced joint lubrication, and reduced inflammation (Montero-Vilchez et al. 2025; Wang et al. 2021; Oe et al. 2016) are unlikely to depend on systemic distribution of intact HMW HA. Instead, emerging evidence points toward indirect, microbiota-mediated mechanisms. Microbial degradation of HMW HA generates oligosaccharides and SCFAs, metabolites known to modulate mucosal immunity, reinforce epithelial barrier integrity, and exert systemic anti-inflammatory effects (Bosi et al. 2022; Huang et al. 2022; Mao et al. 2021). This view reframes oral HMW HA not as a directly absorbed macromolecule, but rather as a substrate for microbial fermentation, with its physiological benefits arising from functional host–microbiome crosstalk.

Here, the diversity of HA-degrading enzymes (HYALs and HA lyases) provides critical context. In vertebrates, HYALs regulate ECM turnover and tissue homeostasis, while in bacterial pathogens they act as spreading factors that promote invasion and immune evasion. In gut microbes, HA lyases instead function as nutrient acquisition tools, breaking down dietary

HA into smaller fragments that can be fermented or shared through cross-feeding. As detailed in Article I of this dissertation (Sindelar et al. 2021), six major enzyme families were identified through phylogenetic and structural analyses: although they differ in sequence and catalytic strategy, they share conserved active-site features, reflecting evolutionary adaptation to distinct ecological niches. This enzymatic diversity underpins how both host and microbial systems process HA into bioactive products (saturated HA oligosaccharides produced by human HYALs and unsaturated HA oligosaccharides produced by bacterial HA lyases) that shape downstream physiological responses. Thus, interindividual variability in gut microbiota composition can play a role in therapeutic response (Venkataraman et al. 2016). Individuals with a higher abundance of HA lyase-producing bacteria, or with greater metabolic capacity for HA degradation, may experience more pronounced benefits, whereas others may show minimal improvement.

In this thesis, I hypothesized that HMW HA degradation in the GIT is driven by gut microbiota through specialized polysaccharide lyases and associated proteins for substrate recognition, uptake, and processing. Our *in vivo* study Article II (Simek et al. 2023) supported this hypothesis, showing that depolymerization of orally administered HMW HA is entirely microbiota dependent. In germ-free mice only minor acid hydrolysis occurred, with no microbial lyase activity or significant absorption. In contrast, conventional mice degraded over 97 % of HMW HA, producing mainly small fragments and the unsaturated HA disaccharide, along with SCFAs. These metabolites, rather than intact HA, were available to the host.

Following this, in our long-term HA supplementation experiment, only a limited number of bacterial taxa showed statistically significant changes between HA-treated and control groups. In the group feeding with high dose of HA, we observed a reduction in unclassified members of the family *Prevotellaceae* and in the genus *Blautia*. *Prevotellaceae* are typically associated with the degradation of dietary fibers and complex polysaccharides, and their abundance often reflects carbohydrate availability (Precup and Vodnar 2019). Their decline in the context of HA supplementation may therefore indicate a substrate shift away from plant-derived polysaccharides toward glycosaminoglycan metabolism. *Blautia*, generally regarded as a beneficial genus due to its ability to produce SCFAs and modulate host inflammation, also decreased in relative abundance (Kim, Park, and Kim 2014). While *Blautia* has been linked to protective effects in metabolic and inflammatory disorders, its role appears to be species- or strain-specific, and not uniformly beneficial across contexts.

Conversely, the genus *Anaeroplasm* increased under both hHA and lHA supplementation. This genus has been negatively associated with diet-induced obesity and metabolic dysbiosis, suggesting that its enrichment could represent a favorable outcome of HA supplementation (Zeng et al. 2015). Under lHA conditions, *Anaerotruncus* and *Mucispirillum* decreased in abundance. *Anaerotruncus* is notable for its ability to degrade mucin and produce butyrate, implying a dual role in both compromising mucosal integrity and supporting SCFA production. Its reduction could therefore reflect a balance between limiting potential mucin degradation and reducing beneficial butyrate output (Raimondi et al. 2021). Similarly, *Mucispirillum*, a mucosa-associated genus represented in rodents by *M. schaedleri*, is considered a pathobiont with context-dependent effects. It can exacerbate colitis in immunodeficient hosts but protect against enteric infections in immunocompetent ones. Its decline here may suggest a stabilization of host–microbiome interactions by reducing taxa with opportunistic behavior (Herp et al. 2021).

Of relevance to HA degradation, a modest increase in *Bacteroides* was detected in HA-supplemented groups, consistent with their established role as primary HA degraders. Although this enrichment did not reach statistical significance due to high inter-individual variability, the trend supports a selective advantage of *Bacteroides* under HA supplementation. This observation aligns with recent in vitro fermentation studies demonstrating that HA of varying molecular weights promotes *Bacteroides* proliferation while shaping distinct microbiota structures and metabolite outputs (Zhao et al. 2023). Importantly, the molecular weight of HA appears to influence both microbial enrichment and metabolic outcomes, with smaller HA fragments linked to different metabolite profiles compared to high-molecular-weight forms.

Our ¹³C-tracing experiments provide mechanistic confirmation of this process, showing that *Bacteroides* spp. cleave HA into ΔAN2, which is subsequently metabolized via established carbohydrate catabolic pathways into phosphorylated intermediates (GlcNAc-P, UDP-GlcNAc) and ultimately into SCFAs and CO₂. While intracellular intermediates such as UDP-GlcNAc were not detected in host tissues, SCFAs were efficiently absorbed and are known to reinforce barrier integrity and modulate immune responses. Collectively, these findings highlight that HA supplementation influences gut microbiota primarily through selective enrichment of HA-degrading taxa such as *Bacteroides* and through the downstream production of bioactive metabolites. The molecular weight of HA further modulates these interactions, determining both the taxa favored and the resulting metabolite landscape available to the host.

Differences in HA molecular weight likely influence microbial metabolism through both enzymatic accessibility and transport constraints. HMW HA requires extracellular depolymerization by surface or potential secreted HA lyases before uptake, which tends to favor species possessing complete and well-adapted HA-targeting PUL. In contrast, LMW fragments can more readily diffuse or be transported into bacterial cells, bypassing some extracellular steps and potentially favoring taxa with less specialized HA-degrading systems (Lapébie et al. 2019). This aligns with my comparative analysis, which showed that many *Bacteroides* species lack key surface-binding proteins (BT3329, BT3330) and the PL29 (BT3328), or possess only low-identity homologs (Article III, Sindelar et al. submitted). Such deficiencies likely constrain their ability to capture and initiate degradation of intact HMW HA, making them more reliant on smaller HA fragments generated by other community members. This variation in initial processing routes could alter metabolic flux and lead to distinct end-product profiles, as seen in the differential metabolite enrichment across HA sizes.

This scenario fits within a cross-feeding framework, supported by our observation of upregulated GAG-specific PUL proteins together with the accumulation of HA-derived oligosaccharides in the culture medium (Article III; Šindelář et al., submitted). In this model, primary degraders such as *B. thetaiotaomicron* initiate extracellular depolymerization of long-chain or structurally complex HA to obtain energy, generating shorter oligosaccharides and unsaturated disaccharides as by-products that subsequently become accessible to other community members in the gut (Overbeeke et al. 2022).

Part of the discussion excluded for confidentiality (unpublished)

In a similar way, vesicle-mediated mechanisms may also extend the accessibility of HA-derived products beyond the producing cell. BEVs and vesicle-containing enzymes and signals can disseminate to neighboring taxa as well as host tissues, providing an additional route for intercellular communication in the GIT (Sartorio et al. 2023). BEVs provide a compelling mechanistic bridge between microbial HA catabolism and host responses. BEVs are nanoscale bilayered particles released by Gram-negative commensals that can traverse mucus, reach the epithelium, and deliver complex cargo proteins, lipids, glycans, and nucleic acids capable of modulating immune and epithelial pathways locally and systemically. HA supplementation could reshape BEV production and cargo profiles by influencing the metabolism of HA-degrading bacteria (such as *Bacteroides*), potentially altering the vesicle-driven molecular signals delivered to host tissues (Sartorio et al. 2023).

Multiple lines of evidence support this model. First, BEVs produced by *B. thetaiotaomicron* were shown to activate NF- κ B in monocytes through a TLR4/TIRAP-dependent mechanism, as confirmed in BEV–monocyte co-culture experiments. *In silico* analysis further predicted that the same BEVs engage distinct components of the Toll-like receptor pathway in macrophages, dendritic cells, and monocytes, with the specific targets varying by cell type and health status (Gul et al. 2022). Such context-dependent targeting aligns with our observation that HA catabolism (and the resulting oligosaccharides/SCFAs) likely shapes epithelial and immune signaling rather than relying on systemic delivery of intact polymer. Second, the composition of *B. thetaiotaomicron* BEVs is highly adaptable. *In vivo*, their proteome changes in response to environmental and host-derived cues, suggesting that the available carbon source (e.g., HA versus nutrient-rich medium) can reshape vesicular cargo and alter the host pathways these vesicles interact with (Stentz et al. 2022). This plasticity is relevant to our proteomics results, which showed a broad induction of the GAG degradation pathway in *B. thetaiotaomicron* during HA growth; therefore, it is reasonable to hypothesize that *B. thetaiotaomicron* BEVs would undergo corresponding remodeling under these conditions. This represents a promising avenue for future research, particularly in investigating the proteomic cargo of BEVs under defined carbon sources such as HA and assessing their role in mediating bacteria–bacteria and bacteria–host communication. Although not directly connected to the central topic of this dissertation, I have gained experience in this area through the analysis of BEV proteomes from *Escherichia coli* O83 (Article IV) and *Lactocaseibacillus rhamnosus* CCM7091 (Article V). In these studies, I performed comprehensive downstream analyses of proteomic datasets, including prediction of functional families, subcellular localization profiling, and statistical evaluation of differentially abundant proteins, with visualization through volcano plots. Such methodological approaches could be adapted to examine *B. thetaiotaomicron* BEVs in future work, enabling a deeper understanding of their potential role in HA-dependent microbial interactions and host modulation.

6. Conclusion

This dissertation explores the degradation of HA in the GIT and its interplay with the gut microbiota, emphasizing how microbial communities shape the fate and functional impact of orally administered HA. By integrating comparative protein analysis, metabolomics, proteomics, and *in vivo* experimentation, it highlights the central role of commensal bacteria in linking dietary HA to host physiology.

The work builds on the broader enzymatic context introduced in Chapter 1, which underscored the remarkable diversity of HYALs and HA lyases across vertebrates, invertebrates, fungi, bacteria, and bacteriophages. Despite their structural and mechanistic differences, these enzymes converge on a common outcome which is fragmentation of HA into smaller oligosaccharides saturated (produced by HYALs) and unsaturated (produced by bacterial HA lyases). Understanding this diversity provides an essential foundation for studying how host- and microbiota-derived HYALs may act independently or synergistically in the GIT.

Taken together, the findings presented here support a shift in perspective: rather than intact HMW HA being absorbed into systemic circulation, its biological effects are more plausibly mediated through microbial processing. The resulting breakdown products, including oligosaccharides and fermentation-derived metabolites such as SCFAs, can influence local gut ecology, modulate immune function, and potentially affect systemic health through host–microbiome signaling.

Looking ahead, future research should expand on how interindividual differences in microbiota composition shape these outcomes and determine variability in clinical responses to oral HA. Emerging directions include examining the role of BEVs in HA metabolism, exploring species- and strain-level enzymatic diversity within microbial pathways for glycan degradation, and integrating host-focused analyses of immune and metabolic pathways. Such efforts will be critical for translating mechanistic insights into microbiota-informed strategies that harness the therapeutic potential of HA.

7. List of abbreviations

BTxxxx – Proteins encoded by genes from *Bacteroides thetaiotaomicron*, where xxxx indicates the gene position in the reference genome.

CRC – Colorectal cancer

ECM – Extracellular matrix

EV – Extracellular vesicle

FFAR2 – Free fatty acid receptor 2, also termed GPR43

FFAR3 – Free fatty acid receptor 3, also termed GPR41

GH – Glycoside hydrolase

GIT – Gastrointestinal tract

GPR41 – Free fatty acid receptor 3

GPR43 – Free fatty acid receptor 2

HA – Hyaluronan

HCAR2 – Hydroxycarboxylic acid receptor 2, also termed GPR109A

HMW – High-molecular-weight

HYAL_TALSN – Hyaluronidase from *Talaromyces stipitatus*

IBD – Inflammatory bowel disease

LMW – Low-molecular-weight

MOS – Mannan oligosaccharides

PDB – Protein Data Bank

PLxx – Polysaccharide lyase, where xx indicates the family number

PSA – Polysaccharide A

PUL – Polysaccharide utilization loci

ROS – Reactive oxygen species

SCFA – Short-chain fatty acid

TLR2/TLR4 – Toll-like receptors 2 and 4

TMEM2 – Cell-surface hyaluronidase transmembrane protein 2

8. References

- Akazawa, H., I. Fukuda, H. Kaneda, S. Yoda, M. Kimura, R. Nomoto, S. Ueda, Y. Shirai, and R. Osawa. 2023. 'Isolation and identification of hyaluronan-degrading bacteria from Japanese fecal microbiota', *Plos One*, 18.
- Antoszevska, M., E. M. Sokolewicz, and W. Baranska-Rybak. 2024. 'Wide Use of Hyaluronic Acid in the Process of Wound Healing-A Rapid Review', *Scientia Pharmaceutica*, 92.
- Ardita, C. S., J. W. Mercante, Y. M. Kwon, L. P. Luo, M. E. Crawford, D. N. Powell, R. M. Jones, and A. S. Neish. 2014. 'Epithelial Adhesion Mediated by Pilin SpaC Is Required for *Lactobacillus rhamnosus* GG-Induced Cellular Responses', *Applied and Environmental Microbiology*, 80: 5068-77.
- Balogh, L., A. Polyak, D. Mathe, R. Kjrally, J. Thuroczy, M. Terez, G. Janoki, Y. T. Ting, L. R. Bucci, and A. G. Schauss. 2008. 'Absorption, Uptake and Tissue Affinity of High-Molecular-Weight Hyaluronan after Oral Administration in Rats and Dogs', *Journal of Agricultural and Food Chemistry*, 56: 10582-93.
- Bateman, A., M. J. Martin, S. Orchard, M. Magrane, R. Agivetova, S. Ahmad, E. Alpi, E. H. Bowler-Barnett, R. Britto, B. Bursteinas, H. Bye-A-Jee, R. Coetzee, A. Cukura, A. Da Silva, P. Denny, T. Dogan, T. Ebenezer, J. Fan, L. G. Castro, P. Garmiri, G. Georghiou, L. Gonzales, E. Hatton-Ellis, A. Hussein, A. Ignatchenko, G. Insana, R. Ishtiaq, P. Jokinen, V. Joshi, D. Jyothi, A. Lock, R. Lopez, A. Luciani, J. Luo, Y. Lussi, A. MacDougall, F. Madeira, M. Mahmoudy, M. Menchi, A. Mishra, K. Moulang, A. Nightingale, C. S. Oliveira, S. Pundir, G. Y. Qi, S. Raj, D. Rice, M. R. Lopez, R. Saidi, J. Sampson, T. Sawford, E. Speretta, E. Turner, N. Tyagi, P. Vasudev, V. Volynkin, K. Warner, X. Watkins, R. Zaru, H. Zellner, A. Bridge, S. Poux, N. Redaschi, L. Aimo, G. Argoud-Puy, A. Auchincloss, K. Axelsen, P. Bansal, D. Baratin, M. C. Blatter, J. Bolleman, E. Boutet, L. Breuza, C. Casals-Casas, E. de Castro, K. C. Echioukh, E. Coudert, B. Cuhe, M. Doche, D. Dornevil, A. Estreicher, M. L. Famiglietti, M. Feuermann, E. Gasteiger, S. Gehant, V. Gerritsen, A. Gos, N. Gruaz-Gumowski, U. Hinz, C. Hulo, N. Hyka-Nouspikel, F. Jungo, G. Keller, A. Kerhornou, V. Lara, P. Le Mercier, D. Lieberherr, T. Lombardot, X. Martin, P. Masson, A. Morgat, T. B. Neto, S. Paesano, I. Pedruzzi, S. Pilbout, L. Pourcel, M. Pozzato, M. Pruess, C. Rivoire, C. Sigrist, K. Sonesson, A. Stutz, S. Sundaram, M. Tognolli, L. Verbregue, C. H. Wu, C. N. Arighi, L. Arminski, C. M. Chen, Y. X. Chen, J. S. Garavelli, H. Z. Huang, K. Laiho, P. McGarvey, D. A. Natale, K. Ross, C. R. Vinayaka, Q. H. Wang, Y. Q. Wang, L. S. Yeh, J. Zhang, and UniProt Consortium. 2021. 'UniProt: the universal protein knowledgebase in 2021', *Nucleic Acids Research*, 49: D480-D89.
- Bobkova, L., D. Smirnou, M. Kremer, J. Kulhanek, M. Hermannova, L. Franke, and V. Velebny. 2018. 'Discovery and Characteristic of Hyaluronidases from Filamentous Fungi', *Current Biotechnology*, 7: 8.
- Bosi, A., D. Banfi, M. Bistoletti, P. Moretto, E. Moro, F. Crema, F. Maggi, E. Karousou, M. Viola, A. Passi, D. Vigetti, C. Giaroni, and A. Baj. 2022. 'Hyaluronan: A Neuroimmune Modulator in the Microbiota-Gut Axis', *Cells*, 11.
- Bourguignon, V., and B. Flamion. 2016. 'Respective roles of hyaluronidases 1 and 2 in endogenous hyaluronan turnover', *Faseb Journal*, 30: 2108-14.
- Brown, J. A., H. Bashir, and M. Y. Zeng. 2025. 'Lifelong partners: Gut microbiota-immune cell interactions from infancy to old age', *Mucosal Immunology*, 18: 509-23.
- Cartmell, A., E. C. Lowe, A. Baslé, S. J. Firbank, D. A. Ndeh, H. Murray, N. Terrapon, V. Lombard, B. Henrissat, J. E. Turnbull, M. Czjzek, H. J. Gilbert, and D. N. Bolam. 2017. 'How members of the human gut microbiota overcome the sulfation problem posed by

- glycosaminoglycans', *Proceedings of the National Academy of Sciences of the United States of America*, 114: 7037-42.
- Cockburn, D., and B. Svensson. 2013. 'Surface binding sites in carbohydrate active enzymes: an emerging picture of structural and functional diversity', *Carbohydrate Chemistry: Chemical and Biological Approaches*, Vol 39, 39: 204-21.
- Cockburn, D., C. Wilkens, C. Ruzanski, S. Andersen, J. W. Nielsen, A. M. Smith, R. A. Field, M. Willemoës, M. Abou Hachem, and B. Svensson. 2014. 'Analysis of surface binding sites (SBSs) in carbohydrate active enzymes with focus on glycoside hydrolase families 13 and 77-a mini-review', *Biologia*, 69: 705-12.
- Collins, S. L., K. E. Black, Y. Chan-Li, Y. H. Ahn, P. A. Cole, J. D. Powell, and M. R. Horton. 2011. 'Hyaluronan Fragments Promote Inflammation by Down-Regulating the Anti-inflammatory A2a Receptor', *American Journal of Respiratory Cell and Molecular Biology*, 45: 675-83.
- Cowman, M. K., H. G. Lee, K. L. Schwertfeger, J. B. McCarthy, and E. A. Turley. 2015. 'The content and size of hyaluronan in biological fluids and tissues', *Frontiers in Immunology*, 6.
- Cuyvers, S., E. Dornez, J. A. Delcour, and C. M. Courtin. 2012. 'Occurrence and functional significance of secondary carbohydrate binding sites in glycoside hydrolases', *Critical Reviews in Biotechnology*, 32: 93-107.
- David, L. A., C. F. Maurice, R. N. Carmody, D. B. Gootenberg, J. E. Button, B. E. Wolfe, A. V. Ling, A. S. Devlin, Y. Varma, M. A. Fischbach, S. B. Biddinger, R. J. Dutton, and P. J. Turnbaugh. 2014. 'Diet rapidly and reproducibly alters the human gut microbiome', *Nature*, 505: 559-+.
- de la Motte, C. A. 2011. 'Hyaluronan in intestinal homeostasis and inflammation: implications for fibrosis', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 301: G945-G49.
- Díaz-Garrido, N., J. Badia, and L. Baldomà. 2021. 'Microbiota-derived extracellular vesicles in interkingdom communication in the gut', *Journal of Extracellular Vesicles*, 10.
- Douglas, G. M., V. J. Maffei, J. R. Zaneveld, S. N. Yurgel, J. R. Brown, C. M. Taylor, C. Huttenhower, and M. G. I. Langille. 2020. 'PICRUSt2 for prediction of metagenome functions', *Nature Biotechnology*, 38: 685-88.
- Drula, E., M. L. Garron, S. Dogan, V. Lombard, B. Henrissat, and N. Terrapon. 2022. 'The carbohydrate-active enzyme database: functions and literature', *Nucleic Acids Research*, 50: D571-D77.
- Durazzi, F., C. Sala, G. Castellani, G. Manfreda, D. Remondini, and A. De Cesare. 2021. 'Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota', *Scientific Reports*, 11.
- Eckert, J. V., K. S. Moshal, K. Burge, A. Wilson, and H. Chaaban. 2024. 'Endogenous Hyaluronan Promotes Intestinal Homeostasis and Protects against Murine Necrotizing Enterocolitis', *Cells*, 13.
- Fallacara, A., E. Baldini, S. Manfredini, and S. Vertuani. 2018. 'Hyaluronic Acid in the Third Millennium', *Polymers*, 10.
- Fang, Z. Y., M. F. Ma, Y. M. Wang, W. Dai, Q. S. Shang, and G. L. Yu. 2024. 'Degradation and fermentation of hyaluronic acid by *Bacteroides* spp. from the human gut microbiota', *Carbohydrate Polymers*, 334.
- Fin, M. T., C. Diedrich, C. S. Machado, L. da Silva, A. P. S. Tartari, I. C. Zittlau, S. H. Peczek, and R. M. Mainardes. 2024. 'Enhanced Oral Bioavailability and Biodistribution of Voriconazole through Zein-Pectin-Hyaluronic Acid Nanoparticles', *Acs Applied Materials & Interfaces*, 17: 513-23.

- Fink, S. P., and B. Triggs-Raine. 2024. 'Genetic Deficiencies of Hyaluronan Degradation', *Cells*, 13.
- Fu, J. X., Y. Zheng, Y. Gao, and W. H. Xu. 2022. 'Dietary Fiber Intake and Gut Microbiota in Human Health', *Microorganisms*, 10.
- Gan, L. P., J. R. Wang, and Y. M. Guo. 2022. 'Polysaccharides influence human health via microbiota-dependent and -independent pathways', *Frontiers in Nutrition*, 9.
- Gul, L., D. Modos, S. Fonseca, M. Madgwick, J. P. Thomas, P. Sudhakar, C. Booth, R. Stentz, S. R. Carding, and T. Korcsmaros. 2022. 'Extracellular vesicles produced by the human commensal gut bacterium *Bacteroides thetaiotaomicron* affect host immune pathways in a cell-type specific manner that are altered in inflammatory bowel disease', *Journal of Extracellular Vesicles*, 11.
- Gunasekaran, A., J. Eckert, K. Burge, W. Zheng, Z. X. Yu, S. Kessler, C. de la Motte, and H. Chaaban. 2020. 'Hyaluronan 35 kDa enhances epithelial barrier function and protects against the development of murine necrotizing enterocolitis', *Pediatric Research*, 87: 1177-84.
- Guo, G. C. L., and J. Y. L. Chiang. 2020. 'Is CYP2C70 the key to new mouse models to understand bile acids in humans?', *Journal of Lipid Research*, 61: 269-71.
- Gutman, E., and Y. Litvak. 2024. 'Gut dopamine kick: How gut microbes turn on host receptors to fight pathogens', *Cell Host & Microbe*, 32: 623-24.
- Herp, S., A. C. D. Raj, M. S. Silva, S. Woelfel, and B. Stecher. 2021. 'The human symbiont *Mucispirillum schaedleri*: causality in health and disease', *Medical Microbiology and Immunology*, 210: 173-79.
- Hill, D. R., S. P. Kessler, H. K. Rho, M. K. Cowman, and C. A. de la Motte. 2012. 'Specific-sized Hyaluronan Fragments Promote Expression of Human β -Defensin 2 in Intestinal Epithelium', *Journal of Biological Chemistry*, 287: 30610-24.
- Huang, G. X., L. Su, N. Zhang, R. X. Han, W. K. Leong, X. A. Li, X. C. Ren, and W. L. W. Hsiao. 2022. 'The prebiotic and anti-fatigue effects of hyaluronan', *Frontiers in Nutrition*, 9.
- Hugenholtz, F., and W. M. de Vos. 2018. 'Mouse models for human intestinal microbiota research: a critical evaluation', *Cellular and Molecular Life Sciences*, 75: 149-60.
- Cheng, W. Y., F. Li, and R. C. Yang. 2024. 'The Roles of Gut Microbiota Metabolites in the Occurrence and Development of Colorectal Cancer: Multiple Insights for Potential Clinical Applications', *Gastro Hep Advances*, 3: 855-70.
- Cheng, Y. S., C. H. Huang, C. C. Chen, T. Y. Huang, T. P. Ko, J. W. Huang, T. H. Wu, J. R. Liu, and R. T. Guo. 2014. 'Structural and mutagenetic analyses of a 1,3-1,4- β -glucanase from *Paecilomyces thermophila*', *Biochimica Et Biophysica Acta-Proteins and Proteomics*, 1844: 366-73.
- Cheong, K. L., S. T. Chen, B. Teng, S. Veeraperumal, S. Y. Zhong, and K. R. Tan. 2023. 'Oligosaccharides as Potential Regulators of Gut Microbiota and Intestinal Health in Post-COVID-19 Management', *Pharmaceuticals*, 16.
- Jiang, D. H., J. R. Liang, and P. W. Noble. 2011. 'Hyaluronan as an Immune Regulator in Human Diseases', *Physiological Reviews*, 91: 221-64.
- Kawai, K., R. Kamochi, S. Oiki, K. Murata, and W. Hashimoto. 2018. 'Probiotics in human gut microbiota can degrade host glycosaminoglycans', *Scientific Reports*, 8.
- Kim, C. H., J. Park, and M. Kim. 2014. 'Gut microbiota-derived short-chain Fatty acids, T cells, and inflammation', *Immune Netw*, 14: 277-88.
- Kim, D. E., D. Chivian, and D. Baker. 2004. 'Protein structure prediction and analysis using the Robetta server', *Nucleic Acids Research*, 32: W526-W31.
- Kim, Y., and C. A. de la Motte. 2020. 'The Role of Hyaluronan Treatment in Intestinal Innate Host Defense', *Frontiers in Immunology*, 11.

- Kimura, M., T. Maeshima, T. Kubota, H. Kurihara, Y. Masuda, and Y. Nomura. 2016. 'Absorption of Orally Administered Hyaluronan', *Journal of Medicinal Food*, 19: 1172-79.
- Klassen, L., X. H. Xing, J. P. Tingley, K. E. Low, M. L. King, G. Reintjes, and D. W. Abbott. 2021. 'Approaches to Investigate Selective Dietary Polysaccharide Utilization by Human Gut Microbiota at a Functional Level', *Frontiers in Microbiology*, 12.
- Koh, A., F. De Vadder, P. Kovatcheva-Datchary, and F. Bäckhed. 2016. 'From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites', *Cell*, 165: 1332-45.
- Lapébie, P., V. Lombard, E. Drula, N. Terrapon, and B. Henrissat. 2019. 'Bacteroidetes use thousands of enzyme combinations to break down glycans', *Nature Communications*, 10.
- Litwiniuk, M., A. Krejner, and T. Grzela. 2016. 'Hyaluronic Acid in Inflammation and Tissue Regeneration', *Wounds-a Compendium of Clinical Research and Practice*, 28: 78-88.
- Liu, H. L., A. L. Shiver, M. N. Price, H. K. Carlson, V. V. Trotter, Y. Chen, V. Escalante, J. Ray, K. E. Hern, C. J. Petzold, P. J. Turnbaugh, K. C. Huang, A. P. Arkin, and A. M. Deutschbauer. 2021. 'Functional genetics of human gut commensal *Bacteroides thetaiotaomicron* reveals metabolic requirements for growth across environments', *Cell Reports*, 34.
- Liu, M. Y., Y. B. Lu, G. Y. Xue, L. Han, H. B. Jia, Z. Wang, J. Zhang, P. Liu, C. J. Yang, and Y. J. Zhou. 2024. 'Role of short-chain fatty acids in host physiology', *Animal Models and Experimental Medicine*, 7: 641-52.
- Losol, P., M. Wolska, T. P. Wypych, L. Yao, L. O'Mahony, and M. Sokolowska. 2024. 'A cross talk between microbial metabolites and host immunity: Its relevance for allergic diseases', *Clinical and Translational Allergy*, 14.
- Maciel-Fiuza, M. F., G. C. Muller, D. M. S. Campos, P. D. S. Costa, J. Peruzzo, R. R. Bonamigo, T. Veit, and F. S. L. Vianna. 2023. 'Role of gut microbiota in infectious and inflammatory diseases', *Frontiers in Microbiology*, 14.
- Mannino, F., N. Irrera, G. Pallio, and A. Bitto. 2024. 'Steady state plasma and tissue distribution of low molecular weight hyaluronic acid after oral administration in mice', *Natural Product Research*, 38: 773-80.
- Mao, T. Y., C. W. Su, Q. R. Ji, C. Y. Chen, R. J. Wang, D. V. Kumar, J. G. Lan, L. F. Jiao, and H. N. Shi. 2021. 'Hyaluronan-induced alterations of the gut microbiome protects mice against *Citrobacter rodentium* infection and intestinal inflammation', *Gut Microbes*, 13.
- Mariat, D., O. Firmesse, F. Levenez, V. D. Guimaraes, H. Sokol, J. Doré, G. Corthier, and J. P. Furet. 2009. 'The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age', *Bmc Microbiology*, 9.
- Matijasic, M., T. Mestrovic, H. C. Paljetak, M. Peric, A. Baresic, and D. Verbanac. 2020. 'Gut Microbiota beyond Bacteria-Mycobiome, Virome, Archaeome, and Eukaryotic Parasites in IBD', *International Journal of Molecular Sciences*, 21.
- Meyer, K., and M. M. Rapport. 1952. 'Hyaluronidases', *Advances in Enzymology and Related Subjects of Biochemistry*, 13: 199-236.
- Migliore, A., and S. Procopio. 2015. 'Effectiveness and utility of hyaluronic acid in osteoarthritis', *Clinical Cases in Mineral and Bone Metabolism*, 12: 31-33.
- Montero-Vilchez, T., P. Gálvez-Martín, R. Sanabria-de la Torre, C. Cuenca-Barrales, A. Molina-Leyva, D. Martinez-Puig, J. Velasco-Alvarez, and S. Arias-Santiago. 2025. 'Oral Supplementation with a New Hyaluronic Acid Matrix Ingredient Improves Skin Brightness, Hydration, Smoothness, and Roughness: Results from a Randomized, Double-Blinded, Placebo-Controlled Study', *Dermatology and Therapy*, 15: 2099-116.

- Ndeh, D., A. Baslé, H. Strahl, E. A. Yates, U. L. McClurg, B. Henrissat, N. Terrapon, and A. Cartmell. 2020. 'Metabolism of multiple glycosaminoglycans by *Bacteroides thetaiotaomicron* is orchestrated by a versatile core genetic locus (vol 11, 646, 2020)', *Nature Communications*, 11.
- Nguyen, T. L. A., S. Vieira-Silva, A. Liston, and J. Raes. 2015. 'How informative is the mouse for human gut microbiota research?', *Disease Models & Mechanisms*, 8: 1-16.
- Nishiyama, K., T. Yokoi, M. Sugiyama, R. Osawa, T. Mukai, and N. Okada. 2021. 'Roles of the Cell Surface Architecture of *Bacteroides* and *Bifidobacterium* in the Gut Colonization', *Frontiers in Microbiology*, 12.
- Oe, M., T. Tashiro, H. Yoshida, H. Nishiyama, Y. Masuda, K. Maruyama, T. Koikeda, R. Maruya, and N. Fukui. 2016. 'Oral hyaluronan relieves knee pain: a review', *Nutrition Journal*, 15.
- Oliver, A., A. B. Chase, C. Weihe, S. B. Orchanian, S. F. Riedel, C. L. Hendrickson, M. Lay, J. M. Sewall, J. B. H. Martiny, and K. Whiteson. 2021. 'High-Fiber, Whole-Food Dietary Intervention Alters the Human Gut Microbiome but Not Fecal Short-Chain Fatty Acids', *Msystems*, 6.
- Overbeeke, A., B. Hausmann, G. Nikolov, F. C. Pereira, C. W. Herbold, and D. Berry. 2022. 'Nutrient niche specificity for glycosaminoglycans is reflected in polysaccharide utilization locus architecture of gut *Bacteroides* species', *Frontiers in Microbiology*, 13.
- Pan, L., X. Z. Ai, T. Y. Fu, L. Ren, Q. S. Shang, G. Y. Li, and G. L. Yu. 2021. 'In vitro fermentation of hyaluronan by human gut microbiota: Changes in microbiota community and potential degradation mechanism', *Carbohydrate Polymers*, 269.
- Precup, G., and D. C. Vodnar. 2019. 'Gut *Prevotella* as a possible biomarker of diet and its eubiotic versus dysbiotic roles: a comprehensive literature review', *British Journal of Nutrition*, 122: 131-40.
- Qiu, Y., Y. Ma, Y. Huang, S. Li, H. Xu, and E. Su. 2021. 'Current advances in the biosynthesis of hyaluronic acid with variable molecular weights', *Carbohydr Polym*, 269: 118320.
- Raghavan, V., E. C. Lowe, G. E. Townsend, D. N. Bolam, and E. A. Groisman. 2014. 'Tuning transcription of nutrient utilization genes to catabolic rate promotes growth in a gut bacterium', *Molecular Microbiology*, 93: 1010-25.
- Raimondi, S., E. Musmeci, F. Candeliere, A. Amaretti, and M. Rossi. 2021. 'Identification of mucin degraders of the human gut microbiota', *Scientific Reports*, 11.
- Rashidi, A., H. Gem, J. S. McLean, K. Kerns, D. R. Dean, N. Dey, and S. Minot. 2024. 'Multi-cohort shotgun metagenomic analysis of oral and gut microbiota overlap in healthy adults', *Scientific Data*, 11.
- Rawat, P. S., A. S. S. Hameed, X. F. Meng, and W. F. Liu. 2022. 'Utilization of glycosaminoglycans by the human gut microbiota: participating bacteria and their enzymatic machineries', *Gut Microbes*, 14.
- Ray, S., E. M. Y. Huang, G. A. West, M. Mrdjen, M. R. McMullen, C. de la Motte, and L. E. Nagy. 2023. '35kDa hyaluronan ameliorates ethanol driven loss of anti-microbial defense and intestinal barrier integrity in a TLR4-dependent manner', *Matrix Biology*, 115: 71-80.
- Romo, M., C. López-Vicario, N. Pérez-Romero, M. Casulleras, A. I. Martínez-Puchol, B. Sánchez, R. Flores-Costa, J. Alcaraz-Quiles, M. Duran-Güell, A. Ibarzábal, J. J. Espert, J. Clària, and E. Títos. 2022. 'Small fragments of hyaluronan are increased in individuals with obesity and contribute to low-grade inflammation through TLR-mediated activation of innate immune cells', *International Journal of Obesity*, 46: 1960-69.
- Salih, A. R. C., H. M. U. Farooqi, H. Amin, P. R. Karn, N. Meghani, and S. Nagendran. 2024. 'Hyaluronic acid: comprehensive review of a multifunctional biopolymer', *Future Journal of Pharmaceutical Sciences*, 10.

- Sartorio, M. G., E. J. Pardue, N. E. Scott, and M. F. Feldman. 2023. 'Human gut bacteria tailor extracellular vesicle cargo for the breakdown of diet- and host- derived glycans', *Proceedings of the National Academy of Sciences of the United States of America*, 120.
- Sender, R., S. Fuchs, and R. Milo. 2016. 'Revised Estimates for the Number of Human and Bacteria Cells in the Body', *Plos Biology*, 14.
- Shen, Y., M. L. G. Torchia, G. W. Lawson, C. L. Karp, J. D. Ashwell, and S. K. Mazmanian. 2012. 'Outer Membrane Vesicles of a Human Commensal Mediate Immune Regulation and Disease Protection', *Cell Host & Microbe*, 12: 509-20.
- Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. 'Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities', *Applied and Environmental Microbiology*, 75: 7537-41.
- Simek, M., D. Rubanová, K. Nesporova, S. Skoroplyas, K. Lehká, P. Raptová, V. Velebny, and L. Kubala. 2025. 'Pharmacokinetics of the systemic application of hyaluronic acid for joint arthritis treatment', *International Journal of Biological Macromolecules*, 307.
- Simek, M., K. Turková, M. Schwarzer, K. Nesporová, L. Kubala, M. Hermannová, T. Foglová, B. Safránková, M. Sindelár, D. Srutková, S. Chatzigeorgiou, T. Novotná, T. Hudcovic, and V. Velebny. 2023. 'Molecular weight and gut microbiota determine the bioavailability of orally administered hyaluronic acid', *Carbohydrate Polymers*, 313.
- Sindelar, M., J. Jilkova, L. Kubala, V. Velebny, and K. Turkova. 2021. 'Hyaluronidases and hyaluronate lyases: From humans to bacteriophages', *Colloids and Surfaces B-Biointerfaces*, 208.
- Song, M. L., L. Chen, C. Dong, M. H. Tang, Y. Wei, D. P. Lv, Q. C. Li, and Z. Chen. 2025. 'Alginate Oligosaccharide and Gut Microbiota: Exploring the Key to Health', *Nutrients*, 17.
- Stentz, R., E. Jones, R. Juodeikis, U. Wegmann, M. Guirro, A. J. Goldson, A. Brion, C. Booth, P. Sudhakar, I. R. Brown, T. Korcsmáros, and S. R. Carding. 2022. 'The Proteome of Extracellular Vesicles Produced by the Human Gut Bacteria *Bacteroides thetaiotaomicron* Is Influenced by Environmental and Host-Derived Factors', *Applied and Environmental Microbiology*, 88.
- Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. 'CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice', *Nucleic Acids Research*, 22: 4673-80.
- Triggs-Raine, B., and M. R. Natowicz. 2015. 'Biology of hyaluronan: Insights from genetic disorders of hyaluronan metabolism', *World J Biol Chem*, 6: 110-20.
- Tropini, C., K. A. Earle, K. C. Huang, and J. L. Sonnenburg. 2017. 'The Gut Microbiome: Connecting Spatial Organization to Function', *Cell Host & Microbe*, 21: 433-42.
- Trott, O., and A. J. Olson. 2010. 'Software News and Update AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization, and Multithreading', *Journal of Computational Chemistry*, 31: 455-61.
- Valachová, K., M. E. Hassan, and L. Soltés. 2024. 'Hyaluronan: Sources, Structure, Features and Applications', *Molecules*, 29.
- Venkataraman, A., J. R. Sieber, A. W. Schmidt, C. Waldron, K. R. Theis, and T. M. Schmidt. 2016. 'Variable responses of human microbiomes to dietary supplementation with resistant starch', *Microbiome*, 4.
- Vester-Andersen, M. K., H. C. Mirsepasi-Lauridsen, M. Prosberg, C. O. Mortensen, C. Träger, K. Skovsen, T. Thorkilgaard, C. Nojgaard, Vind, K. A. Krogfelt, N. Sorensen, F.

- Bendtsen, and A. M. Petersen. 2019. 'Increased abundance of proteobacteria in aggressive Crohn's disease seven years after diagnosis', *Scientific Reports*, 9.
- Wang, B. T., L. L. Wang, H. J. Wang, H. Y. Dai, X. Y. Lu, Y. K. Lee, Z. N. Gu, J. X. Zhao, H. Zhang, W. Chen, and G. Wang. 2021. 'Targeting the Gut Microbiota for Remediating Obesity and Related Metabolic Disorders', *Journal of Nutrition*, 151: 1703-16.
- Wang, X. Y., S. S. Lin, L. Wang, Z. P. Cao, M. M. Zhang, Y. F. Zhang, R. Liu, and J. Y. Liu. 2023. 'Versatility of bacterial outer membrane vesicles in regulating intestinal homeostasis', *Science Advances*, 9.
- Waterhouse, A. M., J. B. Procter, D. M. Martin, M. Clamp, and G. J. Barton. 2009. 'Jalview Version 2--a multiple sequence alignment editor and analysis workbench', *Bioinformatics*, 25: 1189-91.
- Xiao, M., G. D. Li, and H. F. Yang. 2023. 'Microbe-host interactions: structure and functions of Gram-negative bacterial membrane vesicles', *Frontiers in Microbiology*, 14.
- Yamamoto, H., Y. Tobisawa, T. Inubushi, F. Irie, C. Ohyama, and Y. Yamaguchi. 2017. 'A mammalian homolog of the zebrafish transmembrane protein 2 (TMEM2) is the long-sought-after cell-surface hyaluronidase', *Journal of Biological Chemistry*, 292: 7304-13.
- Yang, J., Y. Q. Xu, T. Miyakawa, L. J. Long, and M. Tanokura. 2020. 'Molecular Basis for Substrate Recognition and Catalysis by a Marine Bacterial Laminarinase', *Applied and Environmental Microbiology*, 86.
- Yang, K., G. Q. Li, Q. H. Li, W. Wang, X. Zhao, N. Shao, H. Qiu, J. Liu, L. Xu, and J. J. Zhao. 2025. 'Distribution of gut microbiota across intestinal segments and their impact on human physiological and pathological processes', *Cell and Bioscience*, 15.
- Yin, C. Y., H. Sadiq, W. H. Liao, H. N. Wang, Z. Fu, W. G. Yu, and F. Han. 2025. 'Identification and functional characterization of two novel hyaluronate lyases BxHly33 and BiHly33, from the human gut microbiome', *International Journal of Biological Macromolecules*, 313.
- Yoshida, H., and Y. Okada. 2019. 'Role of HYBID (Hyaluronan Binding Protein Involved in Hyaluronan Depolymerization), Alias KIAA1199/CEMIP, in Hyaluronan Degradation in Normal and Photoaged Skin', *International Journal of Molecular Sciences*, 20.
- Yu, L. C., J. T. Wang, S. C. Wei, and Y. H. Ni. 2012. 'Host-microbial interactions and regulation of intestinal epithelial barrier function: From physiology to pathology', *World J Gastrointest Pathophysiol*, 3: 27-43.
- Yuan, M. Y., Z. W. Zhang, T. Y. Liu, H. Feng, Y. H. Liu, and K. Chen. 2024. 'The Role of Nondigestible Oligosaccharides in Alleviating Human Chronic Diseases by Regulating the Gut Microbiota: A Review', *Foods*, 13.
- Zadnikova, P., R. Sinova, V. Pavlik, M. Simek, B. Safrankova, M. Hermannova, K. Nesporova, and V. Velebny. 2022. 'The Degradation of Hyaluronan in the Skin', *Biomolecules*, 12.
- Zeng, B., S. S. Han, P. Wang, B. Wen, W. S. Jian, W. Guo, Z. J. Yu, D. Du, X. C. Fu, F. L. Kong, M. Y. Yang, X. H. Si, J. C. Zhao, and Y. Li. 2015. 'The bacterial communities associated with fecal types and body weight of rex rabbits', *Scientific Reports*, 5.
- Zeng, Y., Y. F. Wu, Q. Zhang, and X. H. Xiao. 2024. 'Crosstalk between glucagon-like peptide 1 and gut microbiota in metabolic diseases', *Mbio*, 15.
- Zhang, C., B. T. Wang, L. L. Yu, R. H. Zhao, Q. S. Zhang, C. C. Zhang, S. M. Cui, J. X. Zhao, A. Narbad, W. Chen, F. W. Tian, and Q. X. Zhai. 2024. 'Hyaluronic acid modulates gut microbiota and metabolites relieving inflammation: A molecular weight-dependent study', *Science Bulletin*, 69: 2683-87.
- Zhang, D., J. Liu, H. Cheng, H. Wang, Y. Tan, W. Feng, and C. Peng. 2022. 'Interactions between polysaccharides and gut microbiota: A metabolomic and microbial review', *Food Research International*, 160.

- Zhang, Y., R. Chen, D. D. Zhang, S. Qi, and Y. Liu. 2023. 'Metabolite interactions between host and microbiota during health and disease: Which feeds the other?', *Biomedicine & Pharmacotherapy*, 160.
- Zhao, R. H., C. Zhang, L. L. Yu, C. C. Zhang, J. X. Zhao, A. Narbad, Q. X. Zhai, and F. W. Tian. 2023. 'Fermentation of Hyaluronan with Different Molecular Weights by Human Gut Microbiota: Differential Effects on Gut Microbiota Structure and Metabolic Function', *Polymers*, 15.
- Zheng, T., H. N. Hao, Q. Q. Liu, J. K. Li, Y. K. Yao, Y. S. Liu, T. Zhang, Z. Zhang, and H. X. Yi. 2023. 'Effect of Extracellular Vesicles Derived from *Akkermansia muciniphila* on Intestinal Barrier in Colitis Mice', *Nutrients*, 15.

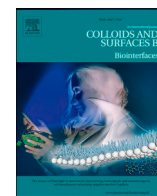
9. Appendices

Article I



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Colloids and Surfaces B: Biointerfaces

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Hyaluronidases and hyaluronate lyases: From humans to bacteriophages

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ABSTRACT

Hyaluronan is a non-sulfated negatively-charged linear polymer distributed in most parts of the human body, where it is located around cells in the extracellular matrix of connective tissues and plays an essential role in the organization of tissue architecture. Moreover, hyaluronan is involved in many biological processes and used in many clinical, cosmetic, pharmaceutical, and biotechnological applications worldwide. As interest in hyaluronan applications increases, so does interest in hyaluronidases and hyaluronate lyases, as these enzymes play a major part in hyaluronan degradation. Many hyaluronidases and hyaluronate lyases produced by eukaryotic cells, bacteria, and bacteriophages have so far been described and annotated, and their ability to cleave hyaluronan has been experimentally proven. These enzymes belong to several carbohydrate-active enzyme families, share very low sequence identity, and differ in their cleaving mechanisms and in their structural and functional properties. This review presents a summary of annotated and characterized hyaluronidases and hyaluronate lyases isolated from different sources belonging to distinct protein families, with a main focus on the binding and catalytic residues of the discussed enzymes in the context of their biochemical properties. In addition, the application potential of individual groups of hyaluronidases and hyaluronate lyases is evaluated.

1. Introduction

Hyaluronan (HA) is a large molecule with a molecular weight often exceeding 10³kDa, composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine connected by a β-1,3 glycosidic bond, with the disaccharide blocks joined together by a β-1,4 glycosidic bond [1,2]. HA is a non-sulfated negatively-charged linear polymer belonging to glycosaminoglycans (GAGs) and is distributed in most parts of the human body, where it is located around cells in the extracellular matrix of connective tissues and plays an essential role in the organization of tissue architecture [2–4]. Moreover, HA is involved in many biological processes such as wound healing, cell and organ development, inflammation, and cell proliferation, differentiation and migration [4–6]. The number of reviews published in this year alone clearly demonstrate the growing interest in HA applications in medicine,

cosmetology, drug delivery, and biotechnology, and in research around the world [7–13]. For this reason, hyaluronidases (Hyal) and hyaluronate lyases (HA lyases) have gained attention and importance as key and specific tools for HA depolymerization [1,14–16]. These previously neglected enzymes are thus becoming a subject of interest in many fields connected with HA applications [2,17].

Hyal activity was first observed in extracts of mammalian testis at the beginning of the twentieth century [18]. Karl Meyer classified Hyals into three classes of enzymes on the basis of their biochemical properties and generated end products [19]. These are 1) Eukaryotic hydrolase-type Hyals (vertebrate and venom Hyals), which randomly cleave β-1,4 glycosidic bonds, not only of HA but also chondroitin or chondroitin sulfate to yield saturated even-numbered oligosaccharides with N-acetyl-D-glucosamine at the reducing terminal [19,20]; 2) Leech hydrolase-type Hyals (endo-β-D-glucuronidase;

Abbreviations: Hyal, Hyaluronidase; Hyals, Hyaluronidases; HA lyase, Hyaluronate lyase; HA, Hyaluronan; GAGs, Glycosaminoglycans; GH, Glycoside hydrolase family; PL, Polysaccharide lyase family; CEMIP, Cell migration-inducing and hyaluronan-binding protein; TMEM2, Transmembrane protein 2; aa, amino acid; Lhyals, Leech hydrolase-type Hyals; CBM, Carbohydrate-binding module family; MSA, Multiple sequence alignment.

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hyaluronate-3-glycanohydrolases), which cleave β -1,3 glycosidic linkages of HA and generate saturated even-numbered oligosaccharides with D-glucuronic acid at the reducing end, but which are inert towards chondroitin and chondroitin 4- and 6-sulfate [19,21–23] and 3) Prokaryotic eliminase-type HA lyases, which cleave β -1,4 glycosidic bonds in HA, chondroitin, and chondroitin sulfates, using the β -elimination process, and yield even-numbered unsaturated oligosaccharides with double bonds between C4 and C5 on the uronic acid residue [19,20,24,25]. Recently, a couple of new proteins (CEMIP and TMEM2) that can bind and depolymerize HA in vertebrates and that are not included in Karl Meyer's three-group-system classification have been identified [26–28]. Interestingly, Hyal and/or HA lyase activity has even been detected in bacteriophages, yeasts, and micromycetes [29–32].

Wide ranges of HA applications require knowledge of effective enzymatic degradation; thus, demands for the characterization and discovery of new HA-degrading enzymes are increasing. This review presents a summary of annotated and characterized hyaluronidases and hyaluronate lyases isolated from different sources belonging to distinct protein families, with a main focus on the binding and catalytic residues of the discussed enzymes in the context of their biochemical properties. In addition, the application potential of individual groups of hyaluronidases and hyaluronate lyases is evaluated.

2. Evaluation of the relations between annotated hyaluronidases and hyaluronate lyases

HA-degrading enzymes are a group of enzymes detected in higher organisms like humans but also in bacteriophages that are invisible to the naked eye. Even though the reasons for the production of Hyals or HA lyases by various organisms are different, the ability of enzymes to cleave HA is common. To analyze the relations of Hyals and HA lyases from different organisms on the basis of amino acid sequence similarities, their characterized and annotated sequences (92) were downloaded from the UniProt and CAZy databases (Table S1) [33,34]. Due to a lack of phylogenetic relations between Hyals and HA lyases belonging to different protein families, a simple distance-based clustering

algorithm using the neighbor-joining method was used for phylogram construction. As shown in Fig. 1, the constructed phylogram creates 6 clusters, corresponding with protein family affiliations in the CAZy and UniProt databases, and includes 87 annotated Hyals and HA lyases sequences. The remaining 5 sequences – HYLY_STEMA (polysaccharide lyase family 5; PL5), HYLY_II_BACEL (PL30), HYLY_I_BACEL (PL33), HYAL_CLOPE (glycoside hydrolase family 84; GH84) and HYLY_STMKO (PL16) – are not grouped into any of these clusters and are found separately with low similarity to the sequences in the phylogram. Regarding the 6 clusters in the phylogram, the biggest cluster, which includes 58 sequences, is composed of vertebrate and venom Hyals belonging to the GH56 family. The well-known family of Hyals comprises major human Hyals, including HYAL1, HYAL2, and HYALP (sperm Hyal) as well as Hyals from animal venoms. However, the most recently studied HA-degrading vertebrate proteins (CEMIP and TMEM2) form a separate cluster in the phylogram with low similarity to Hyals from the GH56 family. Another separate cluster is created by Leech hydrolase-type Hyals (Lhyals), which are characterised by their ability to cleave β -1,3-glycosidic bonds and classified as GH79 family members. The next cluster is created by bacterial HA lyases belonging to the PL8 cluster. HA lyases depolymerize HA by the elimination mechanism to create unsaturated HA fragments and are often referred to as spreading factors for pathogenic bacterial invasion. Interestingly, the CAZy database describes seven of the bacterial HA lyases belonging to PL8 (HYLY_BACA50, HYLY_STRAG, HYLY_STRAG3, HYLY_STRCV, HYLY_STRIT, HYLY_STRPN, and HYLY_STRSU) also as members of the carbohydrate-binding module 70 family (CBM70) [34]. However, these seven annotated bacterial HA lyases are not grouped together despite being within PL8 cluster, so there is probably no closer relation between them (Fig. 1). The penultimate cluster is created by bacteriophage HA lyases of *Streptococcus* spp. belonging to PL16. Finally, the least explored HA-degrading enzymes, belonging to GH16 and originating from micromycetes, create an independent cluster with low proximity to other clusters. As shown in the phylogram (Fig. 1), annotated Hyals and HA lyases form individual groups mainly according to the enzyme families. The individual clusters representing enzyme families will be

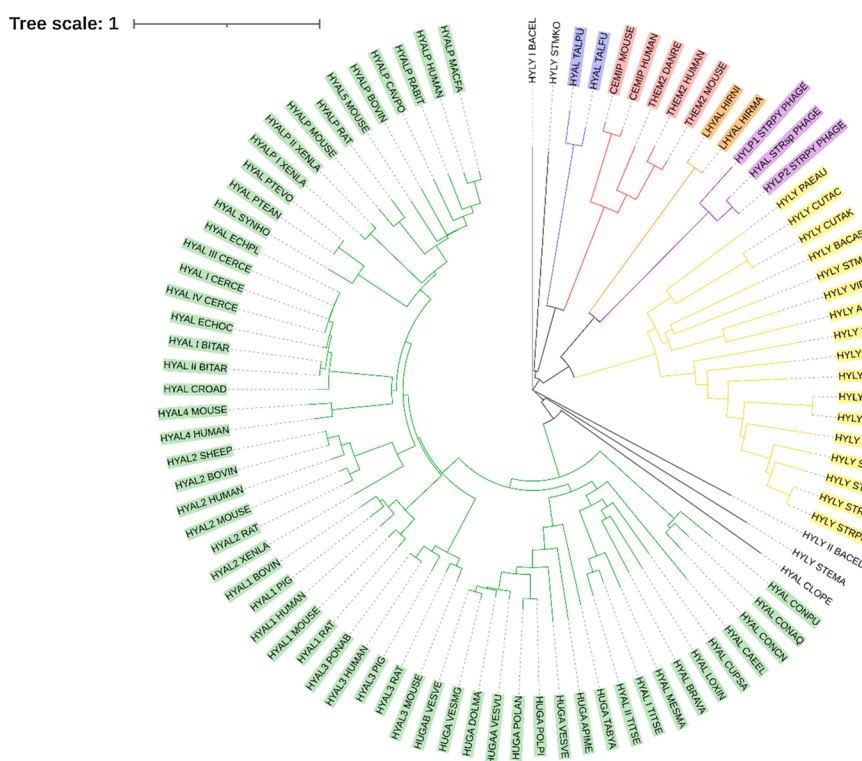


Fig. 1. A Phylogram of 92 annotated sequences of Hyals and HA lyases. The phylogram was constructed using the neighbor-joining method in MEGA-X software and visualized by the iTOL tool [206,207]. The length of branches represents the number of substitutions per site. Colours represent different clusters. Blue – Micromycete Hyals; glycoside hydrolase family 16 (GH16). Red – transmembrane Hyals and cell migration-inducing and hyaluronan-binding proteins (CEMIP family). Orange – Leech Hyals; glycoside hydrolase family 79 (GH79). Purple – Bacteriophage HA lyases; polysaccharide lyase family 16 (PL16). Yellow – Bacterial HA lyases; polysaccharide lyase family 8 (PL8). Green – vertebrate and venom Hyals; glycoside hydrolase family 56 (GH56). Carbohydrate-binding module family 70 (CBM70) enzymes are indicated by an asterisk. Uncoloured – Bacterial HA lyase belonging to PL5 (HYLY_STEMA); to PL30 (HYLY_II_BACEL); to PL33 (HYLY_I_BACEL); to GH84 (HYAL_CLOPE); and to PL16 (HYLY_STMKO).

discussed further in the following sections.

3. Vertebrate and venom hyaluronidases

Vertebrate and venom Hyals are the most explored and described Hyals in databases, as evidenced by the number of annotated hyaluronidases [33,34]. According to the CAZy database, vertebrate and venom Hyals are members of GH56 and possess the ability to randomly hydrolyze β -1,4 linkages between N-acetyl-D-glucosamine and D-glucuronate residues in HA [20,34]. In Fig. 1, a whole dataset of vertebrate and venom Hyals, with 58 members, is divided by secondary analysis into 11 subclusters, as depicted in Fig. S1. Herein, vertebrate Hyals such as HYAL1, HYAL2, HYAL3, HYAL4, sperm Hyal (HYALP) together with HYAL5; *Caenorhabditis elegans* Hyal; and animal venom Hyals such as conus venom Hyals, spider/scorpion venom Hyals, insect venom Hyals, fish venom Hyals, and snake venom Hyals create individual subclusters at different similarity levels (Fig. S1).

3.1. Vertebrate hyaluronidases

In vertebrates, HYAL1 and HYAL2 are predominantly considered as the major Hyals in somatic tissues, acting synergistically to degrade HA polymer at the cellular level in the human body. The current model, which is still under exploration, suggests that the internalization of HA through the coupled action of CD44 and HYAL2 on the cell surface allows the degradation of HA into approximately 20 kDa fragments. These fragments are partially transported into lysosomes, where HYAL1 continues its degradation into oligosaccharides followed by degradation by β -D-glucuronidase and β -N-acetyl-D-hexosaminidase into monosaccharides [20,35]. HA fragments can be exocytosed into the extracellular space [36], where they possess biological activity dependent on cell-type and the size of the fragment [1,2,36]. However, on the basis of knockout mice experiments, it was suggested that the described model of HA degradation is not the only means of the specific cleavage of HA in the human body. The accumulated levels of HA in mouse body (HYAL2^{-/-}) were significant but the amount of HA was not as high as expected. Thus, additional mechanisms of extracellular HA degradation independent of HYAL2 must exist [37,38]. One of the possibilities seems to be newly described proteins belonging to the CEMIP family i.e. CEMIP and TMEM2, which are able to bind and degrade HA and which will be discussed in the next section [28,39,40]. Unlike HYAL1 and HYAL2, HYAL3 does not belong to the main somatic Hyals. HYAL3 is reported to be present in bone marrow, in brain of adults, and in the testis [41]. However, the overexpression of HYAL3 indicates that this enzyme lacks Hyal activity, although HYAL1 activity was increased in HYAL3 overexpressing cells, suggesting a cooperative role for HYAL1 and HYAL3 in HA metabolism [42]. Moreover, HYAL4 is not able to cleave HA and possesses chondroitinase activity only [20,43,44]. HYAL1, HYAL2, HYAL3 and HYAL4 are acidic Hyals which are active at pH 3.0–4.0. By contrast, the human sperm Hyal (HYALP), also called PH20 (sperm adhesion molecule 1, SPAM 1), is a neutral Hyal which is active at pH 5.0–8.0 [45]. HYALP is one of the most active mammalian Hyals and plays an important role in sperm adhesion and penetration through the cumulus matrix [46]. In the vertebrate and venom phylogram, HYALPs create a subcluster together with mouse HYAL5 (Fig. S1). The gene for HYAL5 has been described in the mouse genome as a candidate enzyme involved in sperm penetration through the cumulus, exclusively expressed in the testis together with mouse HYALP [47]. However, the role of HYAL5 in mouse fertilization was questioned [48]. The missing *HYAL5* gene in the genomes of great apes and humans suggests the evolutionary progress to discard HYAL5 [43]. Finally HYAL6 (or HYALP1) is translated in most mammals with the exception of humans, where it is only transcribed, and is known as a pseudogene [43]. In humans, three genes (*HYAL1*, *HYAL2*, *HYAL3*) are located on chromosome 3p21.3 and have a near-identical homology [49]. The other two genes (*HYAL4* and *HYALP*) and one expressed pseudogene *HYAL6*

(*HYALP1*) are located on chromosome 7q31.3. The extensive homology between the different *Hyal* genes suggests gene duplication events at some point in evolution [43,50].

Regarding research applications, commercially available bovine testicular Hyal serves as a model enzyme for the examination of HA cleavage kinetics [51], for inhibitory studies [52], and for the evaluation of hydrolytic and transglycosylation reactions [53]. Bovine testicular Hyal is well studied in this field and has also recently become an extremely important tool in glycotechnology, where its transglycosylation reaction can be used together with hydrolysis to reconstruct various GAG chains [54–57]. Interestingly, the optimal pH for hydrolysis by bovine testicular Hyal was found to be about 5.0, whereas that for the transglycosylation reaction was about 7.0 [58]. There are many therapeutic uses of vertebrate Hyals belonging to GH56. The most commonly used Hyals for these purposes are mainly bovine testicular Hyal, ovine testicular Hyal, and, lately also, recombinant HYALP. Many commercial Hyal products are made from animal testicular preparations. For example, Vitrase (ISTA Pharmaceuticals, Inc., Irvine, CA, USA) uses an ovine testicular-derived Hyal, Amphadase (Amphastar Pharmaceuticals, Inc., CA, USA) uses a bovine testicular-derived Hyal, and Hylenex (Halozyme Therapeutics, Inc., San Diego, CA, USA) is produced by genetically engineered Chinese Hamster Ovary cells containing a DNA plasmid encoding for a soluble fragment of human HYALP [59]. Since Hyals can catalyze the degradation of HA in the extracellular matrix, allowing increased tissue permeability, their usage with local anesthesia showed increased diffusion of the injected anesthetic. The use of Hyals as an adjunct to local anesthetics has been described for, among others, ophthalmology, nail surgery, traumatology, dentistry, and dermatology [60–62]. Recently, Hyals were suggested as a treatment for managing extravasation caused by the administration of chemotherapy [63]. In addition to the use of animal-derived Hyals for local anesthetics, the use of recombinant human HYALP (so called rHuPH20) has recently become important for the subcutaneous administration of insulin [64], morphine [65,66], antibiotics [67], therapeutic monoclonal antibodies and immunoglobulins [68,69], and other medicaments [60,69,70]. From the biochemical point of view, the glycosylation of human Hyals plays an important role in the function and activity of Hyals. Results showed that an N-glycosylation-defective mutant of HYAL1 decreased its secretion and enzymatic activity, probably because of a change in conformation [71,72]. Similar results with an emphasis also on the importance of disulfide bonds were obtained using HYALP in its native or also recombinant form (rHuPH20), identifying *Pichia pastoris* as an expression system due to its ability to perform many post-translational modifications such as proteolytic processing, folding, disulfide bond formation, and glycosylation [73,74]. Alongside their already mentioned applications, vertebrate Hyals have a number of roles in the field of plastic surgery. The popularity of HA fillers in recent years has led to an increase in the usage of Hyals in the treatment of filler-related complications [60,75]. Several studies systematically assessed the degradability of different HA-fillers by Hyals [76–79] and suggested also possible uses of Hyals in neuroplasty [80].

3.2. Venom hyaluronidases

Venom Hyals are widely distributed in a variety of terrestrial and/or aquatic animals such as snakes [81,82], spiders [83], scorpions [84–86], bees [87,88], hornets [89], wasps [90], stonefish [91], lionfish [92] and conus [93]. Snake and spider bites, as well as scorpion sting envenoming is a significant cause of mortality and morbidity in some parts of the world [94]. Neurological complications vary according to the offending animal, and are often directly related to the toxic effects of the venom on the central nervous system, neuromuscular transmission, the cardiovascular system, and the coagulation cascade [95]. Generally, the non-toxic properties of animal venom Hyals have inspired little interest among researchers [82]. However, recently, Hyals in animal venom were found to have an important role as a spreading factor. Comparison

of the biodistribution of *Tityus serrulatus* venom in the absence or presence of anti-Hyal serum showed that Hyal plays a key role not only in spreading the venom from the inoculation point to the bloodstream, but also in venom biodistribution from the bloodstream to target organs, demonstrating that Hyal is indeed an important spreading factor and that its inhibition can be used as a novel first-aid strategy in envenoming [82,86,89,92,93,96].

3.3. Analysis of conserved residues within the vertebrate and venom hyaluronidase cluster

As mentioned before, vertebrate and venom Hyals belong to the GH56 family and share the same mechanisms of HA depolarization designed to produce fragments of HA of different size, i.e. by the hydrolytic cleavage of β -1,4 linkages in the substrate. The importance of particular amino acid residues with substrate binding properties and specific functional groups for catalytic activity was analyzed. The analysis of conserved residues was performed on the basis of the structures of Human HYAL1 (PDB ID: 2PE4) [97] and Bee venom Hyal (PDB ID: 1FCV) [88]. As MSA reveals, a highly conserved area is around two amino acids (aa), D129 and E131 (Human HYAL1 aa residue sequence numbers), where E131 is a catalytic residue (Fig. S2). Both aa residues are conserved throughout the whole vertebrate and venom cluster and are responsible for the two-carboxylic-acids mechanism, with the retention of HA substrate conformation, as reported before by other authors [20,97–100]. It is noteworthy that in the GH56 family (as well as in some other GH families), only one catalytic residue is sufficient for substrate cleavage. This is possible because the acetamido group of the substrate replaces the role of nucleophilic amino acid [101]. The analysis of point mutants of E131 highlights its essential function for catalysis [98]. The mutation of D129 reduces enzymatic activity to 5% and 3% of the wild type activity of HYAL1 and HYALP, respectively [98, 102]. Other residues were identified as the residue important for structural integrity (R265), as residues that position the carbonyl nucleophile for catalysis and/or release the fragments of HA (Y202; W321; Y247), as residues that are predicted to position the substrate (Y202; S245) and as a residue that modulates the ionization of the carboxyl groups (E268) [20,98,102]. Of the residues mentioned, Y202, E268 and W321 are conserved through all sequences and exhibit 100 % occurrence in the vertebrate and venom cluster. A point mutation at E268 causes a complete loss of activity [102]. According to their positions, Y202 and S245 were predicted to position the substrate acetamido group so that it can attack the anomeric carbon [98]. S245 stabilizes the Y202 side chain with a hydrogen bond and Y202 interacts with the acetamido group of HA. Unlike Y202, residue S245 is not conserved through the whole dataset and possesses only 79 % occurrence. Most of the changes are to asparagine, which could substitute hydrogen bonding. On the other hand, there is alanine at this site in HYAL4_MOUSE and HUGA_TABYA, preventing hydrogen interactions that can decrease catalytic efficiency.

Another important residue, this involved in the stabilization of the catalytic nucleophile, is Y247, which has 95 % occurrence and a point mutation that also eliminates the activity of the enzyme [98]. Interestingly, there are only three changes to this residue in the vertebrate and venom cluster, i.e. in HYAL_II_TITSE (Y247H), in HYAL4_MOUSE (Y247S), and in HYAL4_HUMAN (Y247G) (Fig. S2). Remarkably, according to the Hyal structure of bee venom (1FCV), a hydroxy group from Y247 interacts with the acetamido group from HA to form a hydrogen bond. Speculating about the functional consequences of Y247H mutation, it can be suggested that the change from tyrosine to histidine has a minor impact and that histidine could substitute hydrogen bonding, so that the Hyal activity of HYAL_II_TITSE should not be influenced. Next, regarding the Y247S mutation in HYAL4_MOUSE, the side chain of serine is also capable of hydrogen bonding, but is much smaller, and the acetamide of HA could be out of reach. Finally, the Y247G mutation in HYAL4_HUMAN disables any interactions. These

changes in HYAL4 sequences could be one of the reasons for their missing HA activity and for their specificity to chondroitin sulfate only.

Regarding the venom Hyals, they create separate subclusters according to the group of producing organisms in the vertebrate and venom phylogram (Fig. S1). Hyals isolated from several animal venoms are active at pH 5.0–8.0 [96]. Snake venom Hyals share the greatest sequence identity – specifically, more than 88.4 %. They are highly specific to HA as a substrate and show no significant activity towards chondroitin sulfate [103,104]. The specific subcluster containing insect venom Hyals (HUGA_Hyals) possesses one main difference compared to the rest of the Hyals in the vertebrate and venom cluster – specifically, the C-terminal end domain is missing [88,99]. The deletion of the C-terminal end domain negatively influences the activity and secretion of HYAL1 and HYALP [98,102]. It is also speculated that this domain in HYAL1 is an area of as yet undefined interaction [97].

4. CEMIP and TMEM2 hyaluronidases

Although HYAL1 and HYAL2 have been considered the main enzymes for HA catabolism in the human body, evidence about other proteins binding and depolymerizing HA has increased in the last decade [26,27]. The first of these, a protein encoded by the transcript KIAA1199, also called Cell migration-inducing protein (CEMIP) or hyaluronan-binding protein (HYBID), was described as a factor involved in HA degradation [26,105,106]. CEMIP possesses HA binding and degradation activity with the ability to catabolize HA as endo- β -N-acetylglucosaminidase via the clathrin-coated pit pathway to achieve intermediate fragments of 10–100 kDa [26,107]. However, it is not yet clear whether CEMIP itself catalytically cleaves HA [40]. The expression of CEMIP was highly tissue dependent, with levels of mRNA mainly detected in the brain, spleen, synovium, and skin dermis [27,40]. Surprisingly, no expression of CEMIP was detected in the lung, liver, colon, and/or kidney [27]. The main indispensable role of CEMIP in HA catabolism was demonstrated in skin dermis, where CEMIP knockdown in skin fibroblasts completely suppressed HA degradation [40], and in the brain, where CEMIP-deficient mice accumulated high-molecular-weight HA [108,109]. In addition, a close relationship between CEMIP-mediated HA metabolism and inflammation in the skin was suggested [110]. Shortly after identification of the CEMIP gene, point mutations were reported to cause hearing loss due to the folding change in protein structure [105]. Later on, CEMIP expression was associated with poor prognosis in various cancers and showed a tumor-promoting effect in breast [111], ovarian [112], pancreatic [113], gastric [114] and liver cancers [115]. In addition, CEMIP derived from tumor cells contributes to the growth of glioblastoma [116]. Generally, studies have proposed that CEMIP could be a promising diagnostic marker in patients with different types of tumors [117,118]. As gene-silencing experiments showed a decrease in CEMIP expression, this finding might be an index for the knockdown or silencing of CEMIP when necessary, suggesting that CEMIP suppression could be helpful in adjuvant therapy to prevent chemo-drug resistance and in the quest for novel chemo-agents or targeted gene treatments [116,117,119–122].

The second member of the CEMIP family is Transmembrane protein 2 (TMEM2), also called Cell migration-inducing hyaluronidase 2, which is a transmembrane protein with cell-surface Hyal activity [27]. According to *in vitro* studies, TMEM2 cleaves high-molecular HA into fragments of 5 kDa in the presence of calcium ions. The enzyme's optimal pH is around 6–7, and unlike CEMIP, TMEM2 does not require the participation of live cells for its Hyal activity. However, the precise mechanism of degradation has not yet been identified, though a lytic cleaving mechanism similar to that for bacterial HA lyase has been proposed [27,28]. It has been suggested that TMEM2 plays a key role in HA degradation in the human body, in addition to HYAL1 and HYAL2, which are considered the main somatic Hyals [28]. In comparison to CEMIP, the expression level of TMEM2 mRNA is much higher in the majority of tissues, except in brain, spleen, and synovium. The highest

levels of TMEM2 transcript have been identified in the stomach, large intestine, kidney, and bladder [27]. TMEM2 was identified as a potent modulator of the endoplasmic reticulum stress resistance. The breakdown of the HA by TMEM2 within the extracellular matrix altered endoplasmic reticulum stress resistance dependent upon the cell-surface receptor CD44 and the MAPK cell-signaling components [123]. TMEM2 could antagonize the effects of casein kinase 2 subunit alpha 3 on cell proliferation and hepatitis B virus infection via activation of the JAK-STAT pathway, showing its possible functionality in hepatitis B virus inhibition [124,125]. Also, the dysregulation of TMEM2 using microRNAs suppressed cell invasion and migration through the JAK-STAT pathway in breast cancer [126]. TMEM2 overexpression is associated with poor prognosis in pancreatic ductal adenocarcinoma patients [127]. It was shown that a variety of tumor cells are able to eliminate substrate-bound HA in a tightly-localized pattern corresponding to the distribution of focal adhesions and stress fibers. This focal adhesion-targeted HA degradation is mediated by TMEM2, which itself is localized at the site of focal adhesions, while TMEM2 depletion showed an inhibitory effect on the ability of tumor cells to attach and migrate in an HA-rich environment. These findings point to a critical role for TMEM2-mediated HA degradation in the adhesion and migration of cells on HA-rich extracellular matrix substrates, and provide a novel insight into the early phase of focal adhesion formation [128].

The aa sequences of CEMIP and TMEM2 create one cluster and share low homology with other Hyal sequences (Fig. 1). The CEMIP protein has an N-terminal signal sequence, one G8 domain, two GG domains, and four PbH1 domains, where the G8 and PbH1 domains probably play a role in extracellular ligand binding [26]. TMEM2 has an N-terminal cytoplasmic domain, a transmembrane domain, and a C-terminal extracellular domain. The C-terminal extracellular domain consists of one G8, one GG, and three PbH1 domains [27]. There is a 48 % sequence identity between CEMIP and the extracellular domain of TMEM2 in humans [27]. Only two reviewed CEMIP sequences (CEMIP_HUMAN, CEMIP_MOUSE) are available in the UniProt database [33]. They are highly conserved and share 91 % of sequence identity [107]. The comparison of available sequences of TMEM2 from mouse, human, and zebrafish also reveals conserved areas possessing 86.8 % (TMEM2_MOUSE/TMEM2_HUMAN), 58.1 % (TMEM2_MOUSE/TMEM2_DANRE), and 57.5 % (TMEM2_HUMAN/TMEM2_DANRE) identity. R265, D273, D275 and D286, and the presence of calcium ions are important for the Hyal activity of TMEM2_HUMAN, but the precise catalytic mechanism remains unknown [27]. Three out of four aa residues are also conserved in sequences of CEMIP, including R187 (R265 according to the aa numbering in sequence TMEM2), D195 (D273), and D208 (D286). The residue D275 from TMEM2 is missing in CEMIP. The R187 residue located in the GG domain of CEMIP seems to be important for Hyal activity because the mutation of this residue caused a reduction in catalytic activity [26,107]. It can be hypothesized that all these 3 residues (R187, D195, and D208, according to CEMIP aa numbering) could be important for the HA depolymerization activity of CEMIP. However, a dataset of analyzed CEMIP sequences containing only two sequences is too small for this assumption to be analyzed in more detail.

5. Leech hyaluronidases

Hyal activity in leeches was observed in the middle of the twentieth century [21]; however, the first leech Hyal-encoding gene was identified and characterized relatively recently [22]. Only two leech-type Hyals are annotated in databases [33,34]. One of them was detected in *Hirudo nipponia* (LHYAL_HIRNI) [22] and the second was isolated from *Hirudinaria manillensis* (LHYAL_HIRMA) [129]. Both Lhyals share 97 % identity; however, they possess a low sequence identity with other annotated Hyals in the dataset and are thus grouped in a separate cluster in the phylogram (Fig. 1). Unlike other Hyals and HA lyases, Lhyals cleave β -1,3-glycosidic bonds of HA hydrolytically in a non-processive endolytic mode, yielding oligosaccharides with D-glucuronic acid at the

reducing end [22,130]. LHYAL_HIRNI was cloned and successfully expressed in *Pichia pastoris* to obtain a high-level of the production of recombinant Hyal, which is substrate-specific to HA and inert towards chondroitin sulfate, chitin, and heparin [22,131,132]. From biochemical properties, an optimal temperature of 45 °C, a sensitivity to metal ions (Mn^{2+} , Cu^{2+} , Fe^{3+}), and an optimal pH of 6.5 were observed [22]. However, improved recombinant LHYAL_HIRNI with the modification of the N-terminus revealed a lower optimum pH (pH 5.0), a higher optimum temperature (50 °C), and a broader range of thermal stability (20–60 °C) [132]. In addition, recombinant LHYAL_HIRNI has no transglycosidase activity and thus is more suitable for medical applications compared to bovine testicular Hyal [22]. Generally, since the bioactivity of HA depends on its molecular weight, Lhyals attract intense attention due to their great potential for the enzymatic production of HA fragments of the desired length. The production of different fragment sizes is regulated by the incubation conditions and the amount of enzyme [22,130,133] or by using temperature-sensitive plasmid [134]. Other studies also showed the potential of Lhyals expressed in engineered organisms for the production of HA fragments on a large-scale [131,135,136]. Therefore Lhyals play an important role in the large-scale production of defined HA oligosaccharides with a narrow molecular mass distribution. In addition LHYAL_HIRNI was also used for disruption of the HA-capsule of *Corynebacterium glutamicum* recombinant strain overexpressing HA synthase type I in order to promote HA production [137].

Interestingly, LHyals share approximately 35 % sequence identity with five mammalian heparanases [22] (Fig. S3). In LHYAL_HIRNI, two residues, E176 and E290, are reported to be responsible for the catalytic cleavage of HA. This corresponds with the fact that E225 and E343 are identified as proton donors and the nucleophile residues responsible for the catalytic cleavage of HA by human heparanase [33,138] (Fig. S3). Additionally, the presence of two out of three binding sites for heparan sulfate in the sequence of LHYAL_HIRNI has been identified (T64 and H242). The third binding residue in heparanases is arginine, which is substituted by N249 in LHYAL_HIRNI and LHYAL_HIRMA (Fig. S3). The presence of catalytic residues and two out of three binding residues would suggest Lhyals to possess putative heparanase activity. However, the ability of both Lhyals to cleave heparin or heparan sulfate has not been observed [22,33]. One of the reasons could be the missing N-terminal region in Lhyals, which is important for heparanase enzymatic activity. Another could be the non-formation of heterodimer in Lhyals between two subunits, which is reported as essential for heparanase enzymatic activity [139]. On the other hand, the mutation of several residues throughout the LHYAL_HIRNI sequences has been shown to modulate Hyal activity and may extend substrate specificity to heparin [140].

6. Bacterial hyaluronate lyases

Prokaryotic type Hyals, called HA lyases, cleave β -1,4 glycosidic bonds in substrates using the β -elimination process to yield oligosaccharides with an unsaturated bond between C4 and C5 on the uronic acid residue [24,100]. However, HA lyase from *Streptomyces hyalurolyticus* could cleave both β -1,4 and β -1,3 glycosidic linkages, producing even and odd-numbered oligosaccharides [141]. Although *Streptomyces hyalurolyticus* was originally reported to cleave β -1,4 bonds and generates even-numbered oligosaccharides as the major products [142,143]. However, the minor amounts oligosaccharides having an odd number of units were also described [144]. HA lyases of certain bacterial species depolymerize HA of the animal extracellular matrix and thus enable the colonization and/or infection of the host – they serve as so-called spreading factors [145–149]. In addition, the created unsaturated oligosaccharides are mostly used by bacterial strains as a source of energy for their metabolism [146,150] or they can interact with host cells and cause a biological effect that is highly dependent on the length of the HA fragment [151,152]. Overall bacteria-derived HA lyases are in the

research spotlight because they can be produced by a simple fermentation process, are of high purity, and result in little environmental pollution, as compared with animal-derived Hyals are produced in low amounts, and are subject to the risk of virus infection [153,154]. Some HA lyases are frequently used by researchers as HA specific enzymes, such as the HA lyase from *Streptomyces hyalurolyticus*, which recognize HA from other GAGs [142,155,156]. In cosmetic or pharmaceutical applications, HA samples often contain a small amount of dermatan sulfate, which inhibits mammalian testicular Hyal. Instead of mammalian testicular Hyal, bacterial HA lyases from *Streptomyces hyalurolyticus* or *Streptococcus agalactiae* are used [157]. In chromatographic and mass spectrometric analysis, HA lyases produced by *Streptococcus pneumoniae* or *Streptococcus agalactiae* are frequently used for the preparation of HA-containing samples [158–161]. From the biochemical point of view, bacterial HA lyases are stable in different ranges of temperature and pH, these ranges highly dependent on the producing strain. Most HA lyases exhibit optimal temperatures in the range 37 °C–50 °C and optimal pH in the range 6.0–8.0 [153,162–164]. However, there are some exceptions; for example, marine *Vibrio* spp. produce HA lyases with an optimal temperature of 30 °C [149,165], while *Thermasporomyces composti* DSM22891 recombinant HA lyase has an optimal temperature of 70 °C [154].

HA lyases of gram-positive bacteria are produced extracellularly and can be secreted or anchored to the cell wall by means of a peptidoglycan-anchoring using a transpeptidation mechanism which requires a C-terminal sorting signal with a conserved LPxTG motif, e.g. *Streptococcus* spp. [33, 145,166,167]. HA lyases produced by gram-negative bacteria can also be extracellular enzymes released outside the bacterial cell and/or can be present on the surface of the outer membrane [25,149,166]. However, they are usually periplasmic enzymes, which degrade the fragments of HA into unsaturated oligosaccharides in the periplasmic area [25,145,147]. Generally, extracellularly and/or surface-connected HA lyases serve as a first step in the depolymerization of long chains of HA. Then, in the case of the gram-negative bacteria *Bacteroides thetaiotaomicron*, for example, the HA fragments are transported via outer membrane proteins into the periplasmic area, where the cleaving of HA fragments continues with the formation of unsaturated oligosaccharides [25]. Alternatively, in the case of the gram-positive bacteria *Streptococcus* spp. [167] and the gram-negative bacterium *Vibrio alginolyticus* LWW-9 [149], the extracellular HA lyases cleave HA into unsaturated disaccharides outside of the bacterial cell and the created products are transported into the cytoplasm by a phosphotransferase system, or, in the gram-negative bacterium *Streptobacillus moniliformis* [166,168], they are transported by a periplasmic solute-binding protein-dependent ATP-binding cassette transporter.

Using an initial random endolytic cleavage mechanism and a subsequent exolytic processive degradation mechanism, most HA lyases degrade substrates completely into unsaturated disaccharide units – for example, HA lyases produced by *Streptococcus* spp. or *Vibrio alginolyticus* LWW-9 [146,149,169–171]. In contrast, a few HA lyases degrade substrates by means of a non-processive endolytic degradation mechanism into a mixture of unsaturated oligosaccharides – e.g. HA lyases from *Streptomyces hyalurolyticus* or *Bacteroides thetaiotaomicron* [141,142,147, 172]. Meanwhile, *Cutibacterium acne* produces two variants of HA lyases encoded on specific genomic islands that differ in their ability to degrade HA and chondroitin sulfate, as well as to produce distinct HA fragments [148,173]. Regarding substrate specificity, bacterial HA lyases (EC 4.2.2.1.) cleave HA; however, with the exception of HA-specific lyase from *Streptomyces hyalurolyticus*, they can also act on closely-related substrates such as chondroitin or chondroitin sulfate [142,155], though most HA lyases act mainly on non-sulfated substrates like HA because the sulfation causes limited substrate accessibility [174]. Chondroitin and chondroitin sulfate are usually sulfated at C4 and C6. A free carboxylate group on the C4 glucuronate subunit of HA is essential for HA lyase activity because of the crucial interaction with the N349 residue [174,175]. However, the degradation of GAG by bacteria is a

complex process. For instance, the genome of the human gut bacteria *Bacteroides thetaiotaomicron* contains a polysaccharide utilization locus for the degradation of GAG, where cooperation between endo-acting sulfatases, putative chondroitinases, and HA lyase enables chondroitin sulfate, dermatan sulfate, and HA to be cleaved [25,147]. Surprisingly, the endo-acting O4 sulfatase is able to remove O4 sulfation and thus allow HA lyase to cleave sulfated substrates, where O4 would be inhibitory [25]. Compared to HA lyases (EC 4.2.2.1), the chondroitin-sulfate-ABC lyases (EC 4.2.2.20) degrade GAG of the chondroitin sulfate and dermatan sulfate type, but the enzyme can also act on HA at a much lower rate [33]. Even though the chondroitin-sulfate-ABC lyases (EC 4.2.2.20) are also able to cleave HA, for the purpose of this review only HA lyases, which are the enzymes belonging to EC 4.2.2.1, were added to the dataset.

Although many publications report many different bacteria depolymerizing HA, only a few HA lyases are annotated and characterized in databases (Tab.S1) [33,34]. Seventeen bacterial HA lyases belonging to PL8 create one cluster in the phylogram (Fig. 1). The remaining 5 sequences of bacterial HA lyases – HYLY_STEMA (PL5), HYLY_II_BACEL (PL30), HYLY_I_BACEL (PL33), HYAL_CLOPE (GH84) and HYLY_STMCO (PL16) – are not grouped into the cluster when a simple distance-based clustering algorithm and the neighbor-joining method are used. Hence, the bacterial HA lyases of different PL families share a low sequence identity. The best known and characterized are HA lyases produced by *Streptococcus* spp. (PL8), in which the mechanism of HA cleavage has been described by various authors [170,171,175]. The structures of *Streptococcus pneumoniae* and *Streptococcus agalactiae* HA lyases have also been revealed and are available in the PDB database [169, 176–178]. Meanwhile, the MSA of 17 bacterial HA lyases belonging to PL8 reveals conserved aa residues (Fig. S4). According to site-directed mutation studies on *Streptococcus pneumoniae* HA lyase, a group of 5 residues was considered to affect directly the catalytic process, with 3 of them, N349, H399, and Y408, involved in the catalytic process and the remaining two, R243 and N580, involved in substrate binding. Besides catalytic residues, there are three hydrophobic residues, W291, W292, and F343, creating a hydrophobic area in the cavity and interacting with HA via CH- π interactions [169,175]. All these aa residues are highly conserved through all examined sequences of bacterial HA lyases, except for R243 (82 % identity) and F343 (53 % identity) (Fig. S4). The C-terminal sorting signal with a conserved LPxTG motif is responsible for the transpeptidation mechanism and thus enables the anchoring of enzyme onto peptidoglycan, which was found in 6 sequences HYLY_STRIT, HYLY_STRPN, HYLY_STRR6, HYLY_STRPY, HYLY_STRCV, HYLY_STRSU.

Bacterial HA lyase HYLY_STEMA is produced by *Stenotrophomonas maltophilia* and belongs to the PL5 family [34]. HYLY_STEMA is annotated as a polysaccharide lyase with broad substrate specificity, which catalyzes the degradation of alginate, poly-beta-D-mannuronate, poly-beta-D-glucuronate, and also HA. The degradation of substrates is pH dependent, where the optimal pH for cleaving HA is pH 5 [179]. Two HA lyases produced by *Bacteroides cellulosilyticus* WH2 (HYLY_II_BACEL and HYLY_I_BACEL) and that belong to PL30 and PL33, respectively, were recently annotated as enzymes degrading HA [180,181]. HYAL_CLOPE (GH84) was originally annotated as hyaluronidase [182]. However, the authors were not able to observe the HA hydrolytic activity of this enzyme, which, on the other hand, readily cleaved GlcNAc of an artificial substrate. On the basis of sequence similarity, many GH84 family enzymes were labeled as hyaluronidases. Later studies throw doubt on the originally suggested activity [183] and show that HYAL_CLOPE is an exo-N-acetyl-D-glucosaminidase [184]. The last bacterial HA lyase belonging to PL16 is produced by the bacterial strain *Streptomyces koganeiensis* (HYLY_STMCO) and will be discussed in the next section.

7. Bacteriophage hyaluronate lyases

Bacteriophage HA lyases are a part of the phage particle and are not

released themselves into the extracellular milieu since they lack a signal peptide [185–187]. Their main function is to penetrate through the HA capsule of *Streptococcus pyogenes* and *Streptococcus equi* strains and to reach the cell surface of the bacteria to cause lysogenization [32, 187–189], even though they can also contribute to the penetration of streptococcal cells into the host [188] since the antibody response to bacteriophage HA lyase during streptococcal infection has been reported [190]. Bacteriophage HA lyases could also have an indirect role in streptococcal disease, where they transform the nonvirulent strain to a virulent strain, e.g. allowing dissemination of the phage-encoded erythrogenic toxin [191]. Bacteriophage sequences of HA lyases are present in the genome of *Streptococcus pyogenes* and *Streptococcus equi* as prophages [32,188,189]. As a therapeutic approach, the bacteriophage HA lyase HYLPI_STRPY_PHAGE was successfully used to suppress HA-mediated tumor growth and progression [192].

Compared to bacterial HA lyases, where aa sequences vary from 802aa to 1066aa, bacteriophage HA lyases are shorter (337–371aa) [33]. Unlike bacterial HA lyases, bacteriophage HA lyases were found to specifically cleave HA only [32,174,187,193], even though the bacteriophage HA lyase SEQ2045 possesses weak activity toward chondroitin-6-sulfate and dermatan sulfate [188]. Generally, bacteriophage HA lyases have an optimum pH of between 6.0 and 6.5 and digest HA into unsaturated tetra-, hexa-, octa- and decasaccharides using a random internal cut mechanism of action with a greatly extended substrate-binding region [32,187,193]. Three annotated sequences of bacteriophage HA lyases (HYAL_STRsp_PHAGE, HYLPI_STRPY_PHAGE, HYLPI2_STRPY_PHAGE) belonging to the PL16 family are currently published in the UniProt and CAZy databases [33,34,187,194]. Interestingly, bacteriophage HA lyases create a separate cluster showing very low sequence homology to bacterial HA lyases (Fig. 1) despite the fact that they share the same mechanisms of substrate degradation, i.e. the β -elimination of β -1,4 glycosidic bonds in HA. Bacteriophage HA lyases have an extended substrate-binding region compared to bacterial HA lyases [187]. Surprisingly, one HA lyase isolated from the bacterial strain *Streptomyces koganeiensis* (HYLY_STMKO) belongs to the same family as bacteriophage HA lyases i.e. PL16 [15,195]. However, the presented algorithm did not cluster bacterial HYLY_STMKO and bacteriophage HA lyases in closer proximity to each other in the phylogram in Fig. 1. The structure of HYLY_STMKO includes a triple-stranded β -helix, similarly to bacteriophage HA lyase [187,194,195]. HYLY_STMKO, however, is shorter and lacks the α -helix and the capping domains observed in bacteriophage HA lyases [187,194]. This difference causes lower cover (65 %) and increases the sequence distances; hence, the distance-based algorithm doesn't cluster them into one cluster. The residues D137 and Y149 located at the N-terminal end of the central β -helix are predicted to be involved in catalysis [187,196]. However, the interaction of Y149 with S129 makes tyrosine inaccessible for polysaccharide interaction and thus Y149 is probably not involved in substrate binding [194]. On the other hand, tyrosine Y264 indicates its suitability for catalytic interaction with respect to the loss of catalytic activity when Y264 is mutated [194].

8. Hyaluronidases produced by micromycetes

In recent literature, least information is available on Hyals produced by micromycetes. Only a few of these enzymes have been characterized and their genes annotated [29,34]. Culture broth supernatant of several filamentous fungi was screened for HA degradation, revealing Hyal activity by *Talaromyces stipitatus* DSM2425 (the highest activity at pH 3), *Schizophyllum commune* 127 (at pH 3–4), and *Fistulina hepatica* DSM4987 (at pH 5–6) [30]. *Fistulina hepatica* DSM4987 HA lyase was reported as the first to be isolated from higher fungi, with an optimal temperature of around 20 °C degrading HA upto unsaturated tetrasaccharides [30]. *T. stipitatus* DSM2425 Hyal possesses maximum activity at acidic pH 3 and hydrolyzes HA at an optimal temperature of 43 °C [30]. Another three strains of micromycetes, *T. purpureogenus*

IAM13753, *T. purpureogenus* IAM13754, and *T. funiculosus* IAM13752, cleave HA at acidic pH 4. In addition Hyal isolated from *T. purpureogenus* IAM13753 reveals its highest activity at 43 °C [29].

Up to now only two hyals produced by micromycetes are annotated in the databases, i.e. Hyal produced by *T. funiculosus* IAM13752 (HYAL_TALFU) and Hyal isolated from *T. purpureogenus* IAM13753 (HYAL_TALPU) [33,34]. Both Hyals produced by micromycetes belong to the GH16 family, according to the CAZy database, and create a separate cluster in the Hyal phylogram (Fig. 1). Interestingly, clusters of vertebrate and venom Hyals (the GH56 family in CAZy) and micromycete Hyals (the GH16 family in CAZy) share very low sequence similarity – around 15 %. However, Hyals belonging to both clusters share similarities with respect to the hydrolytic cleavage of the β -1,4 glycosidic linkage in HA and the production of saturated even-numbered HA oligosaccharide with N-acetyl-D-glucosamine on the reducing end [29,30]. Besides the very low sequence similarity, there are other differences between the Hyals from the vertebrate and venom cluster and the micromycete cluster. The major end products arising through non-processive endolytic processes are tetra- and hexasaccharides in the case of Hyals belonging to the vertebrate and venom cluster, and some of them also possess chondroitinase activity [20,100]. On the other hand, micromycete Hyals possess no chondroitinase activity and the final oligomer after the degradation of HA is a saturated disaccharide [29, 30]. These findings and the sequence similarity of micromycete Hyals (HYAL_TALFU, HYAL_TALPU) to enzymes in the GH16 family in the CAZy database support the idea of creating a new group of Hyals [29].

Generally, the members of the GH16 family are retaining enzymes that utilize a glycosyl-enzyme intermediate in the hydrolytic reaction [197]. Many of them are able to cleave β -1,3 and/or β -1,4 glycosidic bonds in polysaccharides [198,199]. The whole GH16 family is divided into 27 subfamilies, in which micromycete Hyals (HYAL_TALPU and HYAL_TALFU) belong to the subfamily GH16_1. GH16_1 is a subfamily containing almost exclusively fungal enzymes together with a few enzymes of a pathogenic Nematoda [200]. Currently, 14 enzymes are characterized within the GH16_1 subfamily, including three with already published structures in PDB: *Paecilomyces thermophila* J18 exo- β -1,3-glucosyltransferase/elongating β -transglucosylase (5JVV) [201], *Paecilomyces thermophila* J18 β -1,3-1,4-glucanase (3WDV) [198], and *Phanerochaete chrysosporium* K-3 β -1,3-1,6 glucan hydrolase (2CL2) [199]. However, there is no structure available for micromycete Hyals, although *Paecilomyces thermophila* glucanase (3WDV) shares almost 40 % sequence identity with micromycete Hyals, suggesting the possibility of homology modeling to obtain a structure model. Despite some sequence similarity, experimental findings showed that HYAL_TALFU lacks β -glucanase activity. With respect to the high homology of HYAL_TALPU and HYAL_TALFU, it was concluded that HYAL_TALPU does not possess β -glucanase activity either [29]. β -glucanase activity is also absent in *T. stipitatus* DSM2425 Hyal, which is not able to cleave β -1,3-1,6 glucan-schizophyllan, providing further evidence that such activity is absent in micromycete Hyals [30]. As far as we know, there are no further experimental data about the β -glucanase activity of micromycete Hyals.

To analyse primary sequences of 14 characterized enzymes belonging to the GH16_1 subfamily, an MSA was prepared (Fig. 2).

MSA analysis of the GH16_1 subfamily revealed that two negatively charged residues located in the active site (E113 and E118) are part of the highly conserved motif GEIDIXE, which is common not only for the subfamily GH16_1 but also for the whole GH16 family, where E113 acts as a nucleophile and E118 as a proton donor. The substitution of E113 and E118 has a negative effect on enzyme cleavage activity [198]. Thus, both acid/base and nucleophiles are required for enzymatic activity, unlike in GH56 hyaluronidases. E113, D115, and E118 have side chains oriented towards the binding cavity, while I114 and I116 are oriented in the opposite direction and their hydrophobic interactions help stabilize the β -sandwich fold. The motif is responsible for cleavage and binding functions, along with W97, W101 and H131 (Fig. S5). These three aa

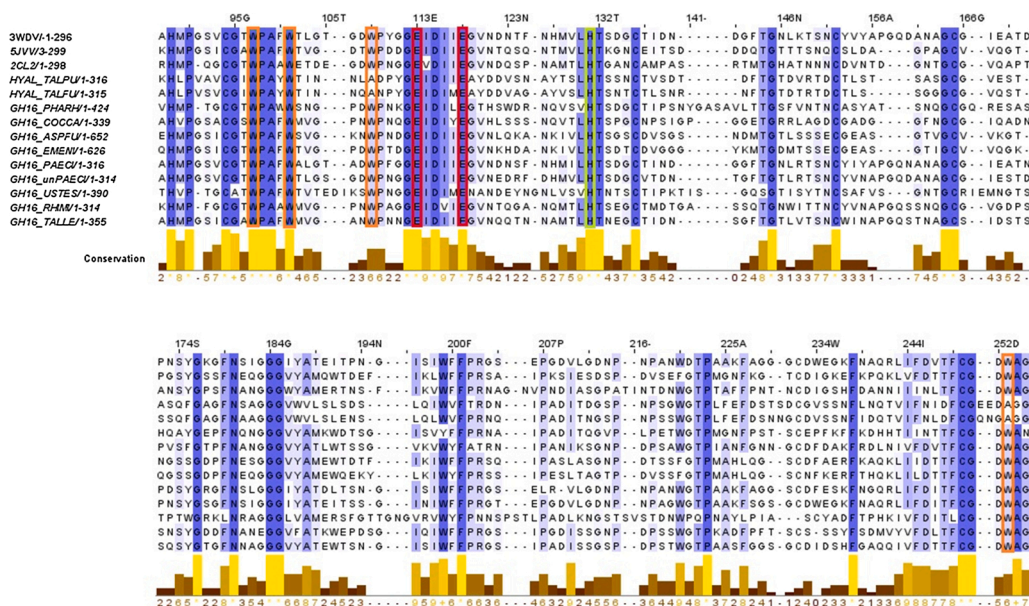


Fig. 2. Partial MSA of the GH16.1 subfamily using 14 characterized enzyme sequences. The aa residues are numbered according to the secondary structure of 3WDV (*Paecilomyces thermophila* J18 β -1,3-1,4-glucanase). Blue represents conserved residues; green frame represent polar residuum responsible for the hydrogen bonding of ligand in the 3WDV structure (H131); orange frames represent aromatic residues that provide CH- π stacking (W97, W101, W108, W253); red frames represent catalytic residues (E113, E118). Alignment was constructed using the ClustalW algorithm and visualized in Jalview software [202,203]. 3WDV (PDB ID); 5JVV (PDB ID); 2CL2 (PDB ID); GH16_PHARH (UniProt ID: O60019); GH16_COCCA (UniProt ID: O14421); GH16_ASPFU (UniProt ID: Q4 $\times$ 084); GH16_EMENI (UniProt ID: Q5BAP5); GH16_PAECI (UniProt ID: G4WNE9); GH16_unPAECI (UniProt ID: A0A386YJ14); GH16_USTES (UniProt ID: I7HBL1); GH16_RHIMI (UniProt ID: H9BZ70); GH16_TALLE (UniProt ID: A0A168SIC2).

residues are conserved throughout the whole dataset (Fig. 2). Concerning binding residues, the aromatic residues W97, W101, W108 and W253 (Fig. S5) are located around the active site in the binding cavity, providing stacking interaction [198]. W97 and W101 are involved in the binding of sugar ligands, because it was shown that carbohydrates interact preferably with aromatic residues by means of so-called CH- π interactions, with tryptophan's indole as the side chain being the most preferred [198,204,205]. Surprisingly, two residues (W108 and W253) participating in the ligand binding of the β -glucanases 3WDV and 5JVV are conserved in all characterized sequences of the GH16.1 subfamily, with the exception of two sequences of micromycete Hyals (Fig. 2) [198, 201]. The mutations of W108 and W253 revealed the importance of these residues for the ligand binding of the β -glucanases 3WDV and 5JVV [198,201]. Because of the importance of both aromatic residues for substrate binding and thus β -glucanase activity, the fact that residues W108 and W253 are missing in the sequences of micromycete Hyals could be related to the experimental finding of missing β -glucanase activity [29].

9. Conclusion

The rapid development of HA applications requires excellent knowledge of the precise degradation of HA by means of efficient tools such as Hyals and HA lyases. Up to now, many Hyals and HA lyases from different sources have been isolated and described. These enzymes belong to several carbohydrate-active enzyme families, possess different mechanisms for the degradation of HA, and share very low sequence identity. However, they exhibit at least one common ability - the degradation of HA. This review aimed to summarize knowledge about the biochemical properties, mechanisms of cleavage, and catalytic and binding amino acid residues of Hyals and HA lyases, as well as about their structures and substrate specificities. Precise information about individual Hyals and HA lyases is increasing thanks to rapidly evolving methods in biochemistry, molecular biology, bioengineering, and especially bioinformatics, such information crucial for the application of Hyals and HA lyases in different fields. Today, selected Hyals are isolated from vertebrates to serve as effective tools for the degradation of HA in the extracellular matrix, allowing increased tissue permeability and the increased efficiency of anesthetics and other therapeutical and

medicinal substances. One particularly important application is in the field of clinical and esthetic medicine, where vertebrate Hyals are employed in the fragmentation of HA-fillers. Meanwhile, the newly described and highly studied HA-depolymerizing proteins CEMIP and TMEM2 are frequently reported to show a tumor-promoting effect and are proposed to be promising diagnostic markers of different types of tumors. Further, research into animal venom Hyals has enabled the development of an anti-Hyal serum for envenoming therapy after animal bite or sting. In other research, the high specificity of Leech-type Hyals and *Streptomyces hyalurolyticus* HA lyase with respect to HA substrate is used in applications targeting the degradation of HA in a mixture with other GAGs. Bacterial and bacteriophage HA lyases serve as spreading factors, enabling them to penetrate host tissues and cells. At the present time, many bacterial strains have been reported to produced HA lyases in the context of pathogenesis and/or microbiome studies. Surprisingly, the group of Hyals produced by micromycetes remains a group of enzymes largely neglected by current research. Yet, at the same time, they are active in acidic environments and at high temperatures, which are excellent prerequisites for their biotechnological application.

Author contributions

Conceptualization – MŠ, KT, JJ; Bioinformatic analysis & Vizualization – MŠ; Writing & Editing – MŠ, JJ, KT; Funding acquisition – LK, VV; Critical reading & Reviewing – VV, LK; Formal analysis – KT, LK; Supervision – KT, LK, VV

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

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References

- [1] R. Stern, A.A. Asari, K.N. Sugahara, Hyaluronan fragments: an information-rich system, *Eur. J. Cell Biol.* 85 (8) (2006) 699–715.
- [2] A. Fallacara, E. Baldini, S. Manfredini, S. Vertuani, Hyaluronic acid in the third millennium, *Polymers (Basel)* 10 (7) (2018).
- [3] J. Casale, J.S. Crane, Biochemistry, Glycosaminoglycans, StatPearls, Treasure Island (FL), 2021.
- [4] T. Kobayashi, T. Chanmee, N. Itano, Hyaluronan: metabolism and function, *Biomolecules* 10 (11) (2020).
- [5] S. Garantziotis, R.C. Savani, Hyaluronan biology: a complex balancing act of structure, function, location and context, *Matrix Biol.* 78–79 (2019) 1–10.
- [6] I.C. King, P. Sorooshian, Hyaluronan in skin wound healing: therapeutic applications, *J. Wound Care* 29 (12) (2020) 782–787.
- [7] M. Prasathkumar, S. Sadhasivam, Chitosan/Hyaluronic acid/Alginate and an assorted polymers loaded with honey, plant, and marine compounds for progressive wound healing-Know-how, *Int. J. Biol. Macromol.* (2021).
- [8] N.G. Kotla, S.R. Bonam, S. Rasala, J. Wankar, R.A. Bohara, J. Bayry, Y. Rochev, A. Pandit, Recent advances and prospects of hyaluronan as a multifunctional therapeutic system, *J. Control. Release* 336 (2021) 598–620.
- [9] R.L. Pratt, Hyaluronan and the Fascial Frontier, *Int. J. Mol. Sci.* 22 (13) (2021).
- [10] I. Saha, V.K. Rai, Hyaluronic acid based microneedle array: recent applications in drug delivery and cosmetology, *Carbohydr. Polym.* 267 (2021) 118168.
- [11] W.H. Chang, P.Y. Liu, M.H. Lin, C.J. Lu, H.Y. Chou, C.Y. Nian, Y.T. Jiang, Y. H. Hsu, Applications of hyaluronic acid in ophthalmology and contact lenses, *Molecules* 26 (9) (2021).
- [12] G. Huerta Angeles, K. Nesporova, Hyaluronan and its derivatives for ophthalmology: recent advances and future perspectives, *Carbohydr. Polym.* 259 (2021) 117697.
- [13] M.S. Birajdar, H. Joo, W.G. Koh, H. Park, Natural bio-based monomers for biomedical applications: a review, *Biomater. Res.* 25 (1) (2021) 8.
- [14] J. Valcarcel, M.R. Garcia, U.R. Varela, J.A. Vazquez, Hyaluronic acid of tailored molecular weight by enzymatic and acid depolymerization, *Int. J. Biol. Macromol.* 145 (2020) 788–794.
- [15] M. Pavan, R. Beninato, D. Galesso, S. Panfilio, S. Vaccaro, L. Messina, C. Guarise, A new potential spreading factor: *Streptomyces koganeiensis* hyaluronidase. A comparative study with bovine testes hyaluronidase and recombinant human hyaluronidase of the HA degradation in ECM, *Biochim. Biophys. Acta* 1860 (4) (2016) 661–668.
- [16] K.S. Girish, K. Kemparaju, The magic glue hyaluronan and its eraser hyaluronidase: a biological overview, *Life Sci.* 80 (21) (2007) 1921–1943.
- [17] B.A. Bühren, H. Schrupf, N.P. Hoff, E. Bolke, S. Hilton, P.A. Gerber, Hyaluronidase: from clinical applications to molecular and cellular mechanisms, *Eur. J. Med. Res.* 21 (2016) 5.
- [18] E. Chain, E.S. Duthie, Identity of hyaluronidase and spreading factor, *Br. J. Exp. Pathol.* 21 (6) (1940) 324–338.
- [19] K. Meyer, M.M. Rapport, Hyaluronidases, *Adv. Enzymol. Relat. Subj. Biochem.* 13 (1952) 199–236.
- [20] R. Stern, M.J. Jedrzejewski, Hyaluronidases: their genomics, structures, and mechanisms of action, *Chem. Rev.* 106 (3) (2006) 818–839.
- [21] A. Linker, P. Hoffman, K. Meyer, The hyaluronidase of the leech: an endoglucuronidase, *Nature* 180 (4590) (1957) 810–811.
- [22] P. Jin, Z. Kang, N. Zhang, G. Du, J. Chen, High-yield novel leech hyaluronidase to expedite the preparation of specific hyaluronan oligomers, *Sci. Rep.* 4 (2014) 4471.
- [23] P. Hovingh, A. Linker, Hyaluronidase activity in leeches (Hirudinea), *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* 124 (3) (1999) 319–326.
- [24] M.L. Garron, M. Cygler, Structural and mechanistic classification of uronic acid-containing polysaccharide lyases, *Glycobiology* 20 (12) (2010) 1547–1573.
- [25] D. Ndeh, A. Basle, H. Strahl, E.A. Yates, U.L. McClurg, B. Henrissat, N. Terrapon, A. Cartmell, Metabolism of multiple glycosaminoglycans by *Bacteroides thetaiotaomicron* is orchestrated by a versatile core genetic locus, *Nat. Commun.* 11 (1) (2020) 646.
- [26] H. Yoshida, A. Nagaoka, A. Kusaka-Kikushima, M. Tobishi, K. Kawabata, T. Sayo, S. Sakai, Y. Sugiyama, H. Enomoto, Y. Okada, S. Inoue, KIAA1199, a deafness gene of unknown function, is a new hyaluronan binding protein involved in hyaluronan depolymerization, *Proc. Natl. Acad. Sci. U. S. A.* 110 (14) (2013) 5612–5617.
- [27] H. Yamamoto, Y. Tobisawa, T. Inubushi, F. Irie, C. Ohya, Y. Yamaguchi, A mammalian homolog of the zebrafish transmembrane protein 2 (TMEM2) is the long-sought-after cell-surface hyaluronidase, *J. Biol. Chem.* 292 (18) (2017) 7304–7313.
- [28] Y. Yamaguchi, H. Yamamoto, Y. Tobisawa, F. Irie, TMEM2: a missing link in hyaluronan catabolism identified? *Matrix Biol.* 78–79 (2019) 139–146.
- [29] M. Bakke, J. Kamei, A. Obata, Identification, characterization, and molecular cloning of a novel hyaluronidase, a member of glycosyl hydrolase family 16, from *Penicillium* spp, *FEBS Lett.* 585 (1) (2011) 115–120.
- [30] L. Bobkova, D. Smirnov, M. Krcmar, J. Kulhanek, M. Hermannova, L. Franke, V. Velebny, Discovery and characteristic of hyaluronidases from filamentous Fungi, *Curr. Biotechnol.* 7 (2018) 8.
- [31] D. Smirnov, M. Krcmar, J. Kulhanek, M. Hermannova, L. Bobkova, L. Franke, S. Pepeliaev, V. Velebny, Characterization of hyaluronan-degrading enzymes from yeasts, *Appl. Biochem. Biotechnol.* 177 (3) (2015) 700–712.
- [32] J.R. Baker, S. Dong, D.G. Pritchard, The hyaluronan lyase of *Streptococcus pyogenes* bacteriophage H4489A, *Biochem. J.* 365 (Pt 1) (2002) 317–322.
- [33] C. UniProt, UniProt: the universal protein knowledgebase in 2021, *Nucleic Acids Res.* 49 (D1) (2021) D480–D489.
- [34] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, *Nucleic Acids Res.* 42 (Database issue) (2014) D490–5.
- [35] M. Erickson, R. Stern, Chain gangs: new aspects of hyaluronan metabolism, *Biochem. Res. Int.* 2012 (2012) 893947.
- [36] R. Racine, M.E. Mummert, Hyaluronan Endocytosis: Mechanisms of Uptake and Biological Functions, *Mol. Reg. Endocytosis* (2012) 377–390.
- [37] B. Chowdhury, R. Hemming, S. Fayaz, B. Triggs-Raine, Hyaluronidase 2 (HYAL2) is expressed in endothelial cells, as well as some specialized epithelial cells, and is required for normal hyaluronan catabolism, *Histochem. Cell Biol.* 145 (1) (2016) 53–66.
- [38] L. Jadin, X. Wu, H. Ding, G.I. Frost, C. Onclinx, B. Triggs-Raine, B. Flamion, Skeletal and hematological anomalies in HYAL2-deficient mice: a second type of mucopolysaccharidosis IX? *FASEB J.* 22 (12) (2008) 4316–4326.
- [39] H. Yoshida, Y. Okada, Role of HYBID (Hyaluronan binding protein involved in hyaluronan depolymerization), alias KIAA1199/CEMIP, in hyaluronan degradation in normal and photoaged skin, *Int. J. Mol. Sci.* 20 (22) (2019).
- [40] Y. Yoshino, M. Goto, H. Hara, S. Inoue, The role and regulation of TMEM2 (transmembrane protein 2) in HYBID (hyaluronan (HA)-binding protein involved in HA depolymerization/ KIAA1199/CEMIP)-mediated HA depolymerization in human skin fibroblasts, *Biochem. Biophys. Res. Commun.* 505 (1) (2018) 74–80.
- [41] V. Atmuri, D.C. Martin, R. Hemming, A. Gutsol, S. Byers, S. Sahebjam, J. A. Thliveris, J.S. Mort, E. Carmona, J.E. Anderson, S. Dakshinamurti, B. Triggs-Raine, Hyaluronidase 3 (HYAL3) knockout mice do not display evidence of hyaluronan accumulation, *Matrix Biol.* 27 (8) (2008) 653–660.
- [42] R. Hemming, D.C. Martin, E. Slominski, J.I. Nagy, A.J. Halayko, S. Pind, B. Triggs-Raine, Mouse Hyal3 encodes a 45- to 56-kDa glycoprotein whose overexpression increases hyaluronidase 1 activity in cultured cells, *Glycobiology* 18 (4) (2008) 280–289.
- [43] A.B. Csoka, R. Stern, Hypotheses on the evolution of hyaluronan: a highly ironic acid, *Glycobiology* 23 (4) (2013) 398–411.
- [44] T. Kaneiwa, S. Mizumoto, K. Sugahara, S. Yamada, Identification of human hyaluronidase-4 as a novel chondroitin sulfate hydrolase that preferentially cleaves the galactosaminidic linkage in the trisulfated tetrasaccharide sequence, *Glycobiology* 20 (3) (2010) 300–309.
- [45] L. Bohaumilitsky, A.K. Huber, E.M. Stork, S. Wengert, F. Woelfl, H. Boehm, A trickster in disguise: Hyaluronan's ambivalent roles in the matrix, *Front. Oncol.* 7 (2017) 242.
- [46] M. Gmachl, S. Sagan, S. Ketter, G. Kreil, The human sperm protein PH-20 has hyaluronidase activity, *FEBS Lett.* 336 (3) (1993) 545–548.
- [47] E. Kim, D. Baba, M. Kimura, M. Yamashita, S. Kashiwabara, T. Baba, Identification of a hyaluronidase, Hyal5, involved in penetration of mouse sperm through cumulus mass, *Proc. Natl. Acad. Sci. U. S. A.* 102 (50) (2005) 18028–18033.
- [48] M. Kimura, E. Kim, W. Kang, M. Yamashita, M. Saigo, T. Yamazaki, T. Nakanishi, S. Kashiwabara, T. Baba, Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in fertilization, *Biol. Reprod.* 81 (5) (2009) 939–947.
- [49] S.H. Lv, S.F. Rong, B.G. Cai, S.M. Guan, Q.Q. Li, Property and current clinical applications of mammal hyaluronidase, *Eur. Rev. Med. Pharmacol. Sci.* 19 (20) (2015) 3968–3976.
- [50] A.B. Csoka, G.I. Frost, R. Stern, The six hyaluronidase-like genes in the human and mouse genomes, *Matrix Biol.* 20 (8) (2001) 499–508.
- [51] R. Nehme, R. Nasreddine, L. Orlic, C. Lopin-Bon, J. Hamacek, F. Piazza, Kinetic theory of hyaluronan cleavage by bovine testicular hyaluronidase in standard and crowded environments, *Biochim. Biophys. Acta Gen. Subj.* 1865 (3) (2021) 129837.
- [52] I. Kakizaki, H. Koizumi, F. Chen, M. Endo, Inhibitory effect of chondroitin sulfate oligosaccharides on bovine testicular hyaluronidase, *Carbohydr. Polym.* 121 (2015) 362–371.
- [53] I. Kakizaki, I. Nukatsuka, K. Takagaki, M. Majima, M. Iwafune, S. Suto, M. Endo, Effects of divalent cations on bovine testicular hyaluronidase catalyzed transglycosylation of chondroitin sulfates, *Biochem. Biophys. Res. Commun.* 406 (2) (2011) 239–244.
- [54] F. Chen, I. Kakizaki, M. Yamaguchi, K. Kojima, K. Takagaki, M. Endo, Novel products in hyaluronan digested by bovine testicular hyaluronidase, *Glycoconj. J.* 26 (5) (2009) 559–566.
- [55] M. Endo, I. Kakizaki, Synthesis of neoproteoglycans using the transglycosylation reaction as a reverse reaction of endo-glycosidases, *Proc. Jpn. Acad., Ser. B, Phys. Biol. Sci.* 88 (7) (2012) 327–344.
- [56] P. Hoffman, K. Meyer, A. Linker, Transglycosylation during the mixed digestion of hyaluronic acid and chondroitin sulfate by testicular hyaluronidase, *J. Biol. Chem.* 219 (2) (1956) 653–663.
- [57] S. Highsmith, J.H. Garvin Jr., D.M. Chipman, Mechanism of action of bovine testicular hyaluronidase. Mapping of the active site, *J. Biol. Chem.* 250 (18) (1975) 7473–7480.
- [58] H. Saitoh, K. Takagaki, M. Majima, T. Nakamura, A. Matsuki, M. Kasai, H. Narita, M. Endo, Enzymic reconstruction of glycosaminoglycan oligosaccharide chains using the transglycosylation reaction of bovine testicular hyaluronidase, *J. Biol. Chem.* 270 (8) (1995) 3741–3747.
- [59] A. Kaul, W.D. Short, X. Wang, S.G. Keswani, Hyaluronidases in human diseases, *Int. J. Mol. Sci.* 22 (6) (2021).

- [60] G.C. Weber, B.A. Buhren, H. Schrupp, J. Wohlrab, P.A. Gerber, Clinical applications of hyaluronidase, *Adv. Exp. Med. Biol.* 1148 (2019) 255–277.
- [61] N. Khan, Z.R. Niazi, F.U. Rehman, A. Akhtar, M.M. Khan, S. Khan, N. Baloch, S. Khan, Hyaluronidases: a therapeutic enzyme, *Protein Pept. Lett.* 25 (7) (2018) 663–676.
- [62] T. Searle, F.R. Ali, F. Al-Niaimi, Hyaluronidase in dermatology: uses beyond hyaluronic acid fillers, *J. Drugs Dermatol.* 19 (10) (2020) 993–998.
- [63] J. Boulanger, A. Ducharme, A. Dufour, S. Fortier, K. Almanric, p. Comité de l'évolution de la pratique des soins, o. Comité de l'évolution des pratiques en, Management of the extravasation of anti-neoplastic agents, Support. Care Cancer 23 (5) (2015) 1459–1471.
- [64] D.E. Vaughn, R.C. Yocum, D.B. Muchmore, B.J. Sugarman, A.M. Vick, I. P. Bilinsky, G.I. Frost, Accelerated pharmacokinetics and glucodynamics of prandial insulins injected with recombinant human hyaluronidase, *Diabetes Technol. Ther.* 11 (6) (2009) 345–352.
- [65] J.R. Thomas, M.S. Wallace, R.C. Yocum, D.E. Vaughn, M.F. Haller, J. Flament, The INFUSE-Morphine study: use of recombinant human hyaluronidase (rHuPH20) to enhance the absorption of subcutaneously administered morphine in patients with advanced illness, *J. Pain Symptom Manage.* 38 (5) (2009) 663–672.
- [66] J.R. Thomas, R.C. Yocum, M.F. Haller, J. Flament, The INFUSE-Morphine IIB study: use of recombinant human hyaluronidase (rHuPH20) to enhance the absorption of subcutaneous morphine in healthy volunteers, *J. Pain Symptom Manage.* 38 (5) (2009) 673–682.
- [67] G. Harb, F. Lebel, J. Battikha, J.W. Thackara, Safety and pharmacokinetics of subcutaneous ceftriaxone administered with or without recombinant human hyaluronidase (rHuPH20) versus intravenous ceftriaxone administration in adult volunteers, *Curr. Med. Res. Opin.* 26 (2) (2010) 279–288.
- [68] F. Speth, J.P. Haas, C.H. Hinze, Treatment with high-dose recombinant human hyaluronidase-facilitated subcutaneous immune globulins in patients with juvenile dermatomyositis who are intolerant to intravenous immune globulins: a report of 5 cases, *Pediatr. Rheumatol. Online J.* 14 (1) (2016) 52.
- [69] R.L. Wasserman, Recombinant human hyaluronidase-facilitated subcutaneous immunoglobulin infusion in primary immunodeficiency diseases, *Immunotherapy* 9 (12) (2017) 1035–1050.
- [70] K.W. Locke, D.C. Maneval, M.J. LaBarre, ENHANZE(R) drug delivery technology: a novel approach to subcutaneous administration using recombinant human hyaluronidase PH20, *Drug Deliv.* 26 (1) (2019) 98–106.
- [71] E.S. Hofinger, M. Spickenreither, J. Oschmann, G. Bernhardt, R. Rudolph, A. Buschauer, Recombinant human hyaluronidase Hyal-1: insect cells versus *Escherichia coli* as expression system and identification of low molecular weight inhibitors, *Glycobiology* 17 (4) (2007) 444–453.
- [72] Y. Goto, Y. Niwa, T. Suzuki, S. Uematsu, N. Dohmae, S. Simizu, N-glycosylation is required for secretion and enzymatic activity of human hyaluronidase1, *FEBS Open Bio* 4 (2014) 554–559.
- [73] M.W. Li, A.I. Yudin, K.R. Robertson, G.N. Cherr, J.W. Overstreet, Importance of glycosylation and disulfide bonds in hyaluronidase activity of macaque sperm surface PH-20, *J. Androl.* 23 (2) (2002) 211–219.
- [74] K.J. Chen, S. Sabrina, N.S. El-Safory, G.C. Lee, C.K. Lee, Constitutive expression of recombinant human hyaluronidase PH20 by *Pichia pastoris*, *J. Biosci. Bioeng.* 122 (6) (2016) 673–678.
- [75] M. Landau, Hyaluronidase caveats in treating filler complications, *Dermatol. Surg.* 41 (Suppl 1) (2015) S347–53.
- [76] S.S. Kwak, K.H. Yoon, J.H. Kwon, W.H. Kang, C.H. Rhee, G.H. Yang, D.J.M. Cruz, W.C. Son, Comparative analysis of hyaluronidase-mediated degradation among seven hyaluronic acid fillers in hairless mice, *Clin. Cosmet. Investig. Dermatol.* 14 (2021) 241–248.
- [77] B.A. Buhren, H. Schrupp, E. Bolke, K. Kammers, P.A. Gerber, Standardized in vitro analysis of the degradability of hyaluronic acid fillers by hyaluronidase, *Eur. J. Med. Res.* 23 (1) (2018) 37.
- [78] V. Rao, S. Chi, J. Woodward, Reversing facial fillers: interactions between hyaluronidase and commercially available hyaluronic-acid based fillers, *J. Drugs Dermatol.* 13 (9) (2014) 1053–1056.
- [79] B.S.F. Bravo, S. Bianco, J.T. de Bastos, R. de Melo Carvalho, Hyaluronidase: what is your fear? *J. Cosmet. Dermatol.* (2021).
- [80] S. Helm li, G. Racz, Hyaluronidase in neuroplasty: a review, *Pain Physician* 22 (6) (2019) 555–560.
- [81] K. Kemparaju, K.S. Girish, Snake venom hyaluronidase: a therapeutic target, *Cell Biochem. Funct.* 24 (1) (2006) 7–12.
- [82] K.S. Girish, H.P. Mohanakumari, S. Nagaraju, B.S. Vishwanath, K. Kemparaju, Hyaluronidase and protease activities from Indian snake venoms: neutralization by *Mimosa pudica* root extract, *Fitoterapia* 75 (3–4) (2004) 378–380.
- [83] A. Senff-Ribeiro, P. Henrique da Silva, O.M. Chaim, L.H. Gremski, K.S. Paludo, R. Bertoni da Silveira, W. Gremski, O.C. Mangili, S.S. Veiga, Biotechnological applications of brown spider (*Loxosceles* genus) venom toxins, *Biotechnol. Adv.* 26 (3) (2008) 210–218.
- [84] C.C. Horta, F. Magalhaes Bde, B.B. Oliveira-Mendes, A.O. do Carmo, C.G. Duarte, L.F. Felicori, R.A. Machado-de-Avila, C. Chavez-Olortegui, E. Kalapothakis, Molecular, immunological, and biological characterization of *Tityus serrulatus* venom hyaluronidase: new insights into its role in envenomation, *PLoS Negl. Trop. Dis.* 8 (2) (2014) e2693.
- [85] C. Guerra-Duarte, C.C. Rebello Horta, B.B. Ribeiro Oliveira-Mendes, B. de Freitas Magalhaes, F. Costal-Oliveira, S. Stransky, C. Fonseca de Freitas, D. Campolina, P. Pereira de Oliveira Pardal, R. Lira-da-Silva, R.A. Machado de Avila, E. Kalapothakis, C. Chavez-Olortegui, Determination of hyaluronidase activity in *Tityus* spp. Scorpion venoms and its inhibition by Brazilian antivenoms, *Toxicon* 167 (2019) 134–143.
- [86] B.B.R. Oliveira-Mendes, S.E.M. Miranda, D.F. Sales-Medina, B.F. Magalhaes, Y. Kalapothakis, R.P. Souza, V.N. Cardoso, A.L.B. de Barros, C. Guerra-Duarte, E. Kalapothakis, C.C.R. Horta, Inhibition of *Tityus serrulatus* venom hyaluronidase affects venom biodistribution, *PLoS Negl. Trop. Dis.* 13 (4) (2019), e0007048.
- [87] J. Frangieh, Y. Salma, K. Haddad, C. Mattei, C. Legros, Z. Fajloun, D. El Obeid, First characterization of the venom from *Apis mellifera syriaca*, a honeybee from the Middle East Region, *Toxins (Basel)* 11 (4) (2019).
- [88] Z. Markovic-Housley, G. Miglierini, L. Soldatova, P.J. Rizkallah, U. Muller, T. Schirmer, Crystal structure of hyaluronidase, a major allergen of bee venom, *Structure* 8 (10) (2000) 1025–1035.
- [89] S. An, L. Chen, J.F. Wei, X. Yang, D. Ma, X. Xu, X. Xu, S. He, J. Lu, R. Lai, Purification and characterization of two new allergens from the venom of *Vespa magnifica*, *PLoS One* 7 (2) (2012), e31920.
- [90] D.L. Justo Jacomini, F.D. Campos Pereira, J.R. Aparecido dos Santos Pinto, L. D. dos Santos, A.J. da Silva Neto, D.T. Giratto, M.S. Palma, R. de Lima Zollner, M. R. Brochetto Braga, Hyaluronidase from the venom of the social wasp *Polybia paulista* (Hymenoptera, Vespidae): cloning, structural modeling, purification, and immunological analysis, *Toxicon* 64 (2013) 70–80.
- [91] M. Madokoro, A. Ueda, A. Kiriake, K. Shiomi, Properties and cDNA cloning of a hyaluronidase from the stonefish *Synanceia verrucosa* venom, *Toxicon* 58 (4) (2011) 285–292.
- [92] A. Kiriake, M. Madokoro, K. Shiomi, Enzymatic properties and primary structures of hyaluronidases from two species of lionfish (Pterois antennata and Pterois volitans), *Fish Physiol. Biochem.* 40 (4) (2014) 1043–1053.
- [93] C. Moller, E. Clark, H. Safavi-Hemami, A. DeCaprio, F. Mari, Isolation and characterization of Conohyal-P1, a hyaluronidase from the injected venom of *Conus purpurascens*, *J. Proteomics* 164 (2017) 73–84.
- [94] E. Bala, R. Hazarika, P. Singh, M. Yasir, R. Shrivastava, A biological overview of Hyaluronidase: a venom enzyme and its inhibition with plants materials, *Mater Today-Proc* 5 (2) (2018) 6406–6412.
- [95] O.H. Del Brutto, Neurological effects of venomous bites and stings: snakes, spiders, and scorpions, *Handb. Clin. Neurol.* 114 (2013) 349–368.
- [96] K.S. Girish, K. Kemparaju, S. Nagaraju, B.S. Vishwanath, Hyaluronidase inhibitors: a biological and therapeutic perspective, *Curr. Med. Chem.* 16 (18) (2009) 2261–2288.
- [97] K.L. Chao, L. Muthukumar, O. Herzberg, Structure of human hyaluronidase-1, a hyaluronan hydrolyzing enzyme involved in tumor growth and angiogenesis, *Biochemistry* 46 (23) (2007) 6911–6920.
- [98] L. Zhang, A.G. Bharadwaj, A. Casper, J. Barkley, J.J. Barycki, M.A. Simpson, Hyaluronidase activity of human Hyal1 requires active site acidic and tyrosine residues, *J. Biol. Chem.* 284 (14) (2009) 9433–9442.
- [99] A.V. Maksimenko, A.D. Turashev, R.S. Beabekashvili, Stratification of chondroitin sulfate binding sites in 3D-model of bovine testicular hyaluronidase and effective size of glycosaminoglycan coat of the modified protein, *Biochemistry Mosc.* 80 (3) (2015) 284–295.
- [100] R. Stern, G. Kogan, M.J. Jedrzejak, L. Soltes, The many ways to cleave hyaluronan, *Biotechnol. Adv.* 25 (6) (2007) 537–557.
- [101] S.J. Williams, Carbohydrate enzymology, in: G. Roberts (Ed.), *Encyclopedia of Biophysics*, Springer, Berlin, Heidelberg, 2018.
- [102] S. Arming, B. Strobl, C. Wechselberger, G. Kreil, In vitro mutagenesis of PH-20 hyaluronidase from human sperm, *Eur. J. Biochem.* 247 (3) (1997) 810–814.
- [103] A.F. Wahby, S.M. Mahdy el, H.A. El-Mezayen, W.H. Salama, A.M. Abdel-Aty, A. S. Fahmy, Egyptian horned viper *Cerastes cerastes* venom hyaluronidase: purification, partial characterization and evidence for its action as a spreading factor, *Toxicon* 60 (8) (2012) 1380–1389.
- [104] D.E. Vivas-Ruiz, E.E. Gonzalez-Kozlova, J. Delgadillo, P.M. Palermo, G. A. Sandoval, F. Lazo, E. Rodriguez, C. Chavez-Olortegui, A. Yarleque, E. F. Sanchez, Biochemical and molecular characterization of the hyaluronidase from *Bothrops atrox* Peruvian snake venom, *Biochimie* 162 (2019) 33–45.
- [105] S. Abe, S. Usami, Y. Nakamura, Mutations in the gene encoding KIAA1199 protein, an inner-ear protein expressed in Deiters' cells and the fibrocytes, as the cause of nonsyndromic hearing loss, *J. Hum. Genet.* 48 (11) (2003) 564–570.
- [106] S. de Vega, H. Yoshida, Y. Okada, Expression and characterization of hyaluronan-binding protein involved in hyaluronan depolymerization: HYBID, alias KIAA1199 and CEMIP, *Methods Mol. Biol.* 2132 (2020) 129–138.
- [107] H. Yoshida, A. Nagaoka, S. Nakamura, Y. Sugiyama, Y. Okada, S. Inoue, Murine homologue of the human KIAA1199 is implicated in hyaluronan binding and depolymerization, *FEBS Open Bio* 3 (2013) 352–356.
- [108] Y. Yoshino, M. Ishisaka, K. Tsuruma, M. Shimazawa, H. Yoshida, S. Inoue, M. Shimoda, Y. Okada, H. Hara, Distribution and function of hyaluronan binding protein involved in hyaluronan depolymerization (HYBID, KIAA1199) in the mouse central nervous system, *Neuroscience* 347 (2017) 1–10.
- [109] Y. Yoshino, M. Shimazawa, S. Nakamura, S. Inoue, H. Yoshida, M. Shimoda, Y. Okada, H. Hara, Targeted deletion of HYBID (hyaluronan binding protein involved in hyaluronan depolymerization/KIAA1199/CEMIP) decreases dendritic spine density in the dentate gyrus through hyaluronan accumulation, *Biochem. Biophys. Res. Commun.* 503 (3) (2018) 1934–1940.
- [110] S. Sato, Y. Mizutani, Y. Yoshino, M. Masuda, M. Miyazaki, H. Hara, S. Inoue, Pro-inflammatory cytokines suppress HYBID (hyaluronan (HA) -binding protein involved in HA depolymerization/KIAA1199/CEMIP) -mediated HA metabolism in human skin fibroblasts, *Biochem. Biophys. Res. Commun.* 539 (2021) 77–82.
- [111] M.S. Jami, J. Hou, M. Liu, M.L. Varney, H. Hassan, J. Dong, L. Geng, J. Wang, F. Yu, X. Huang, H. Peng, K. Fu, Y. Li, R.K. Singh, S.J. Ding, Functional proteomic

- analysis reveals the involvement of KIAA1199 in breast cancer growth, motility and invasiveness, *BMC Cancer* 14 (2014) 194.
- [112] F. Shen, Z.H. Zong, Y. Liu, S. Chen, X.J. Sheng, Y. Zhao, CEMIP promotes ovarian cancer development and progression via the PI3K/AKT signaling pathway, *Biomed. Pharmacother.* 114 (2019) 108787.
- [113] A. Koga, N. Sato, S. Kohi, K. Yabuki, X.B. Cheng, M. Hisaoka, K. Hirata, KIAA1199/CEMIP/HYBID overexpression predicts poor prognosis in pancreatic ductal adenocarcinoma, *Pancreatol.* 17 (1) (2017) 115–122.
- [114] S. Matsuzaki, F. Tanaka, K. Mimori, K. Tahara, H. Inoue, M. Mori, Clinicopathologic significance of KIAA1199 overexpression in human gastric cancer, *Ann. Surg. Oncol.* 16 (7) (2009) 2042–2051.
- [115] Y. Xu, H. Xu, M. Li, H. Wu, Y. Guo, J. Chen, J. Shan, X. Chen, J. Shen, Q. Ma, J. Liu, M. Wang, W. Zhao, J. Hong, Y. Qi, C. Yao, Q. Zhang, Z. Yang, C. Qian, J. Li, KIAA1199 promotes sorafenib tolerance and the metastasis of hepatocellular carcinoma by activating the EGF/EGFR-dependent epithelial-mesenchymal transition program, *Cancer Lett.* 454 (2019) 78–89.
- [116] S. Tsuji, S. Nakamura, T. Yamada, S. de Vega, Y. Okada, S. Inoue, M. Shimazawa, H. Hara, HYBID derived from tumor cells and tumor-associated macrophages contribute to the glioblastoma growth, *Brain Res.* 1764 (2021) 147490.
- [117] L. Li, L.H. Yan, S. Manoj, Y. Li, L. Lu, Central role of CEMIP in Tumorigenesis and its potential as therapeutic target, *J. Cancer* 8 (12) (2017) 2238–2246.
- [118] X. Zhai, W. Wang, Y. Ma, Y. Zeng, D. Dou, H. Fan, J. Song, X. Yu, G. Du, Z. Jiang, H. Zhang, X. Zhang, B. Jin, Serum KIAA1199 is an advanced-stage prognostic biomarker and metastatic oncogene in cholangiocarcinoma, *Aging (Albany NY)* 12 (23) (2020) 23761–23777.
- [119] J. Ye, L. Qi, J. Liang, K. Zong, W. Liu, R. Li, R. Feng, W. Zhai, Lenvatinib induces anticancer activity in gallbladder cancer by targeting AKT, *J. Cancer* 12 (12) (2021) 3548–3557.
- [120] N.A. Evensen, Y. Li, C. Kuscus, J. Liu, J. Cathcart, A. Banach, Q. Zhang, E. Li, S. Joshi, J. Yang, P.I. Denoya, S. Pastorekova, S. Zucker, K.R. Shroyer, J. Cao, Hypoxia promotes colon cancer dissemination through up-regulation of cell migration-inducing protein (CEMIP), *Oncotarget* 6 (24) (2015) 20723–20739.
- [121] J. Liu, W. Yan, P. Han, D. Tian, The emerging role of KIAA1199 in cancer development and therapy, *Biomed. Pharmacother.* 138 (2021) 111507.
- [122] M. Oneyama, N. Sakamoto, N. Oue, Y. Kimura, Y. Hiroshima, I. Hashimoto, K. Hara, Y. Maezawa, K. Kano, T. Aoyama, H. Fujikawa, T. Yamada, H. Tamagawa, N. Yamamoto, T. Ogata, H. Cho, H. Ito, N. Yukawa, M. Shiozawa, T. Yoshikawa, S. Morinaga, Y. Rino, M. Masuda, Y. Miyagi, W. Yasui, T. Oshima, Clinical significance of KIAA1199 as a novel target for gastric cancer drug therapy, *Anticancer Res.* 39 (12) (2019) 6567–6573.
- [123] R.T. Schinzel, R. Higuchi-Sanabria, O. Shalem, E.A. Moehle, B.M. Webster, L. Joe, R. Bar-Ziv, P.A. Frankino, J. Durieux, C. Pender, S.S. Kumar, N. Savalia, H. Chi, M. Simic, N.T. Nguyen, A. Dillin, The Hyaluronidase, TMEM2, Promotes ER Homeostasis and Longevity Independent of the UPR(ER), *Cell* 179 (6) (2019), 1306–1318 e18.
- [124] X. Zhu, C. Xie, Y.M. Li, Z.L. Huang, Q.Y. Zhao, Z.X. Hu, P.P. Wang, Y.R. Gu, Z. L. Gao, L. Peng, TMEM2 inhibits hepatitis B virus infection in HepG2 and HepG2.2.15 cells by activating the JAK-STAT signaling pathway, *Cell Death Dis.* 7 (6) (2016) e2239.
- [125] H. Zhou, X. Jia, K. Hu, Z. Mo, W. Xu, L. Peng, K. Wang, X. Zhu, TMEM2 binds to CSNK2A3 to inhibit HBV infection via activation of the JAK/STAT pathway, *Exp. Cell Res.* 400 (1) (2021) 112517.
- [126] L. Gan, H. Yang, Z. Xiong, Z. Yang, T. Wang, G. Lyu, miR-518a-3p suppresses triple-negative breast cancer invasion and migration through regulation of TMEM2, *Technol. Cancer Res. Treat.* 19 (2020), 1533033820977523.
- [127] Y. Kudo, N. Sato, Y. Adachi, T. Amaike, A. Koga, S. Kohi, H. Noguchi, T. Nakayama, K. Hirata, Overexpression of transmembrane protein 2 (TMEM2), a novel hyaluronidase, predicts poor prognosis in pancreatic ductal adenocarcinoma, *Pancreatol.* 20 (7) (2020) 1479–1485.
- [128] F. Irie, Y. Tobisawa, A. Murao, H. Yamamoto, C. Ohyama, Y. Yamaguchi, The cell surface hyaluronidase TMEM2 regulates cell adhesion and migration via degradation of hyaluronan at focal adhesion sites, *J. Biol. Chem.* 296 (2021) 100481.
- [129] M. Kordowicz, D. Gussow, U. Hofmann, T. Pacuska, A. Gardas, in: M.P. GmbH (Ed.), *Hyaluronidase from the Hirudinaria manillensis Isolation, Purification and Recombinant Method of Production*, 2000.
- [130] M. Lv, M. Wang, W. Cai, W. Hao, P. Yuan, Z. Kang, Characterisation of separated end hyaluronan oligosaccharides from leech hyaluronidase and evaluation of angiogenesis, *Carbohydr. Polym.* 142 (2016) 309–316.
- [131] H. Huang, Q. Liang, Y. Wang, J. Chen, Z. Kang, High-level constitutive expression of leech hyaluronidase with combined strategies in recombinant *Pichia pastoris*, *Appl. Microbiol. Biotechnol.* 104 (4) (2020) 1621–1632.
- [132] Z. Kang, N. Zhang, Y. Zhang, Enhanced production of leech hyaluronidase by optimizing secretion and cultivation in *Pichia pastoris*, *Appl. Microbiol. Biotechnol.* 100 (2) (2016) 707–717.
- [133] P. Yuan, M. Lv, P. Jin, M. Wang, G. Du, J. Chen, Z. Kang, Enzymatic production of specifically distributed hyaluronan oligosaccharides, *Carbohydr. Polym.* 129 (2015) 194–200.
- [134] Y. Li, Z. Shi, Y. Shao, M. Wu, G. Li, T. Ma, Temperature-controlled molecular weight of hyaluronic acid produced by engineered *Bacillus subtilis*, *Biotechnol. Lett.* 43 (1) (2021) 271–277.
- [135] C. Wei, G. Du, J. Chen, Z. Kang, Construction of engineered *Streptococcus zooepidemicus* for the production of hyaluronic acid oligosaccharide, *Sheng Wu Gong Cheng Xue Bao* 35 (5) (2019) 805–815.
- [136] P. Jin, Z. Kang, P. Yuan, G. Du, J. Chen, Production of specific-molecular-weight hyaluronan by metabolically engineered *Bacillus subtilis* 168, *Metab. Eng.* 35 (2016) 21–30.
- [137] Y. Wang, L. Hu, H. Huang, H. Wang, T. Zhang, J. Chen, G. Du, Z. Kang, Eliminating the capsule-like layer to promote glucose uptake for hyaluronan production by engineered *Corynebacterium glutamicum*, *Nat. Commun.* 11 (1) (2020) 3120.
- [138] F. Levy-Adam, G. Abboud-Jarrous, M. Guerrini, D. Beccati, I. Vlodavsky, N. Ilan, Identification and characterization of heparin/heparan sulfate binding domains of the endoglycosidase heparanase, *J. Biol. Chem.* 280 (21) (2005) 20457–20466.
- [139] F. Levy-Adam, H.Q. Miao, R.L. Heinrikson, I. Vlodavsky, N. Ilan, Heterodimer formation is essential for heparanase enzymatic activity, *Biochem. Biophys. Res. Commun.* 308 (4) (2003) 885–891.
- [140] P. Jin, Z. Kang, J. Zhang, L. Zhang, G. Du, J. Chen, Combinatorial evolution of enzymes and synthetic pathways using one-step PCR, *ACS Synth. Biol.* 5 (3) (2016) 259–268.
- [141] L. Tao, F. Song, N.Y. Xu, D.X. Li, R.J. Linhardt, Z.Q. Zhang, New insights into the action of bacterial chondroitinase AC I and hyaluronidase on hyaluronic acid, *Carbohydr. Polym.* 158 (2017) 85–92.
- [142] Y. Park, S. Cho, R.J. Linhardt, Exploration of the action pattern of *Streptomyces hyaluronate* lyase using high-resolution capillary electrophoresis, *Biochim. Biophys. Acta* 1337 (2) (1997) 217–226.
- [143] I. Takazono, Y. Tanaka, Quantitative analysis of hyaluronic acid by high-performance liquid chromatography of streptomyces hyaluronidase digests, *J. Chromatogr.* 288 (1) (1984) 167–176.
- [144] K.N. Price, T. Al, D.C. Baker, C. Chisena, R.L. Cysyk, Isolation and characterization by electrospray-ionization mass spectrometry and high-performance anion-exchange chromatography of oligosaccharides derived from hyaluronic acid by hyaluronate lyase digestion: observation of some heretofore unobserved oligosaccharides that contain an odd number of units, *Carbohydr. Res.* 303 (3) (1997) 303–311.
- [145] W. Wang, J. Wang, F. Li, Hyaluronidase and chondroitinase, *Adv. Exp. Med. Biol.* 925 (2017) 75–87.
- [146] C. Marion, J.M. Stewart, M.F. Tazi, A.M. Burnaugh, C.M. Linke, S.A. Woodiga, S. J. King, *Streptococcus pneumoniae* can utilize multiple sources of hyaluronic acid for growth, *Infect. Immun.* 80 (4) (2012) 1390–1398.
- [147] D. Ndeh, J. Munoz Munoz, A. Cartmell, D. Bulmer, C. Wills, B. Henrissat, J. Gray, The human gut microbe *Bacteroides thetaiotaomicron* encodes the founding member of a novel glycosaminoglycan-degrading polysaccharide lyase family PL29, *J. Biol. Chem.* 293 (46) (2018) 17906–17916.
- [148] S. Nazipi, K. Stodkilde-Jorgensen, C. Scavenius, H. Bruggemann, The skin bacterium *Propionibacterium acnes* employs two variants of Hyaluronate Lyase with distinct properties, *Microorganisms* 5 (3) (2017).
- [149] X. Wang, Z. Wei, H. Wu, Y. Li, F. Han, W. Yu, Characterization of a hyaluronic acid utilization locus and identification of two hyaluronate lyases in a marine bacterium *Vibrio alginolyticus* LWW-9, *Front. Microbiol.* 12 (2021) 696096.
- [150] Y. Hirayama, M. Yoshimura, Y. Ozeki, I. Sugawara, T. Udagawa, S. Mizuno, N. Itano, K. Kimata, A. Tamaru, H. Ogura, K. Kobayashi, S. Matsumoto, Mycobacteria exploit host hyaluronan for efficient extracellular replication, *PLoS Pathog.* 5 (10) (2009) e1000643.
- [151] S.L. Kolar, P. Kyme, C.W. Tseng, A. Soliman, A. Kaplan, J. Liang, V. Nizet, D. Jiang, R. Murali, M. Arditi, D.M. Underhill, G.Y. Liu, Group B streptococcus Evades host immunity by degrading hyaluronan, *Cell Host Microbe* 18 (6) (2015) 694–704.
- [152] D.H. Jiang, J.R. Liang, P.W. Noble, Hyaluronan as an immune regulator in human diseases, *Physiol. Rev.* 91 (1) (2011) 221–264.
- [153] C. Zhu, J. Zhang, L. Li, J. Zhang, Y. Jiang, Z. Shen, H. Guan, X. Jiang, Purification and characterization of hyaluronate lyase from *Arthrobacter globiformis* A152, *Appl. Biochem. Biotechnol.* 182 (1) (2017) 216–228.
- [154] Y. Li, S. Zhang, H. Wu, X. Wang, W. Yu, F. Han, Biochemical characterization of a thermophilic hyaluronate lyase TcHly8C from *Thermasporomyces composti* DSM22891, *Int. J. Biol. Macromol.* 165 (Pt A) (2020) 1211–1218.
- [155] T. Furukawa, M. Arai, F. Garcia-Martin, M. Amano, H. Hinou, S. Nishimura, Glycoblotting-based high throughput protocol for the structural characterization of hyaluronan degradation products during enzymatic fragmentation, *Glycoconj. J.* 30 (2) (2013) 171–182.
- [156] H.M. Luu, A. Chen, I.S. Isayeva, Comparative stability of the bioresorbable ferric crosslinked hyaluronic acid adhesion prevention solutions, *J. Biomed. Mater. Res. B Appl. Biomater.* 101 (6) (2013) 1006–1013.
- [157] Y.K. Matsuno, N. Kakoi, M. Kinoshita, Y. Matsuzaki, J. Kumada, K. Kakehi, Electrophoresis studies on the contaminating glycosaminoglycans in commercially available hyaluronic acid products, *Electrophoresis* 29 (17) (2008) 3628–3635.
- [158] J. Sedlacek, M. Hermannova, J. Mrazek, R. Buffa, P. Liskova, D. Satinsky, V. Velebny, Insight into the distribution of amino groups along the chain of chemically deacetylated hyaluronan, *Carbohydr. Polym.* 225 (2019).
- [159] M. Simek, K. Nesporova, A. Kocurkova, T. Fogleva, G. Ambrozova, V. Velebny, L. Kubala, M. Hermannova, How the molecular weight affects the in vivo fate of exogenous hyaluronan delivered intravenously: a stable-isotope labelling strategy, *Carbohydr. Polym.* 263 (2021).
- [160] M. Simek, K. Lemr, M. Hermannova, V. Havlicek, Analysis of hyaluronan and its derivatives using chromatographic and mass spectrometric techniques, *Carbohydr. Polym.* 250 (2020).
- [161] H. Osago, T. Shibata, N. Hara, S. Kuwata, M. Kono, Y. Uchio, M. Tsuchiya, Quantitative analysis of glycosaminoglycans, chondroitin/dermatan sulfate, hyaluronic acid, heparan sulfate, and keratan sulfate by liquid chromatography-

- electrospray ionization-tandem mass spectrometry, *Anal. Biochem.* 467 (2014) 62–74.
- [162] X. Guo, Y. Shi, J. Sheng, F. Wang, A novel hyaluronidase produced by *Bacillus* sp. A50, *PLoS One* 9 (4) (2014), e94156.
- [163] A. Kurata, M. Matsumoto, T. Kobayashi, S. Deguchi, N. Kishimoto, Hyaluronate lyase of a deep-sea *Bacillus* niacini, *Mar. Biotechnol.* (N. Y.) 17 (3) (2015) 277–284.
- [164] X. Sun, Z. Wang, Y. Bi, Y. Wang, H. Liu, Genetic and functional characterization of the hyaluronate lyase HylB and the beta-N-acetylglucosaminidase HylZ in *Streptococcus zooepidemicus*, *Curr. Microbiol.* 70 (1) (2015) 35–42.
- [165] W. Han, W. Wang, M. Zhao, K. Sugahara, F. Li, A novel eliminase from a marine bacterium that degrades hyaluronan and chondroitin sulfate, *J. Biol. Chem.* 289 (40) (2014) 27886–27898.
- [166] S. Oiki, B. Mikami, Y. Maruyama, K. Murata, W. Hashimoto, A bacterial ABC transporter enables import of mammalian host glycosaminoglycans, *Sci. Rep.* 7 (1) (2017) 1069.
- [167] S. Oiki, Y. Nakamichi, Y. Maruyama, B. Mikami, K. Murata, W. Hashimoto, Streptococcal phosphotransferase system imports unsaturated hyaluronan disaccharide derived from host extracellular matrices, *PLoS One* 14 (11) (2019), e0224753.
- [168] S. Oiki, M. Sato, B. Mikami, K. Murata, W. Hashimoto, Substrate recognition by bacterial solute-binding protein is responsible for import of extracellular hyaluronan and chondroitin sulfate from the animal host, *Biosci. Biotechnol. Biochem.* 83 (10) (2019) 1946–1954.
- [169] M.J. Jedrzejewski, L.V. Mello, B.L. de Groot, S. Li, Mechanism of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase. Structures of complexes with the substrate, *J. Biol. Chem.* 277 (31) (2002) 28287–28297.
- [170] S.J. Kelly, K.B. Taylor, S. Li, M.J. Jedrzejewski, Kinetic properties of *Streptococcus pneumoniae* hyaluronate lyase, *Glycobiology* 11 (4) (2001) 297–304.
- [171] V.Q. Mai, T.T. Vo, M. Meere, Modelling hyaluronan degradation by streptococcus pneumoniae hyaluronate lyase, *Math. Biosci.* 303 (2018) 126–138.
- [172] E. Shimada, G. Matsumura, Degradation process of hyaluronic acid by *Streptomyces hyaluronidase*, *J. Biochem.* 88 (4) (1980) 1015–1023.
- [173] B. Steiner, S. Romero-Steiner, D. Cruce, R. George, Cloning and sequencing of the hyaluronate lyase gene from *Propionibacterium acnes*, *Can. J. Microbiol.* 43 (4) (1997) 315–321.
- [174] J.R. Baker, H. Yu, K. Morrison, W.F. Averett, D.G. Pritchard, Specificity of the hyaluronate lyase of group-B streptococcus toward unsulphated regions of chondroitin sulphate, *Biochem. J.* 327 (Pt 1) (1997) 65–71.
- [175] S. Li, S.J. Kelly, E. Lamani, M. Ferraroni, M.J. Jedrzejewski, Structural basis of hyaluronan degradation by *Streptococcus pneumoniae* hyaluronate lyase, *EMBO J.* 19 (6) (2000) 1228–1240.
- [176] M.J. Jedrzejewski, L. Chantalat, Structural studies of streptococcus agalactiae hyaluronate lyase, *Acta Crystallogr. D Biol. Crystallogr.* 56 (Pt 4) (2000) 460–463.
- [177] K. Ponnuraj, M.J. Jedrzejewski, Mechanism of hyaluronan binding and degradation: structure of *Streptococcus pneumoniae* hyaluronate lyase in complex with hyaluronic acid disaccharide at 1.7 Å resolution, *J. Mol. Biol.* 299 (4) (2000) 885–895.
- [178] L.V. Mello, B.L. De Groot, S. Li, M.J. Jedrzejewski, Structure and flexibility of *Streptococcus agalactiae* hyaluronate lyase complex with its substrate. Insights into the mechanism of processive degradation of hyaluronan, *J. Biol. Chem.* 277 (39) (2002) 36678–36688.
- [179] L.C. MacDonald, B.W. Berger, A polysaccharide lyase from *Stenotrophomonas maltophilia* with a unique, pH-regulated substrate specificity, *J. Biol. Chem.* 289 (1) (2014) 312–325.
- [180] M. Wu, N.P. McNulty, D.A. Rodionov, M.S. Khoroshkin, N.W. Griffin, J. Cheng, P. Latreille, R.A. Kerstetter, N. Terrapon, B. Henrissat, A.L. Osterman, J.J. Gordon, Genetic determinants of in vivo fitness and diet responsiveness in multiple human gut Bacteroides, *Science* 350 (6256) (2015) aac5992.
- [181] W. Helbert, L. Poulet, S. Drouillard, S. Mathieu, M. Loidice, M. Couturier, V. Lombard, N. Terrapon, J. Turchetto, R. Vincentelli, B. Henrissat, Discovery of novel carbohydrate-active enzymes through the rational exploration of the protein sequences space, *Proc. Natl. Acad. Sci. U. S. A.* 116 (13) (2019) 6063–6068.
- [182] B. Canard, T. Garnier, B. Saint-Joanis, S.T. Cole, Molecular genetic analysis of the nagH gene encoding a hyaluronidase of *Clostridium perfringens*, *Mol. Gen. Genet.* 243 (2) (1994) 215–224.
- [183] E. Ficko-Blean, A.B. Boraston, N-acetylglucosamine recognition by a family 32 carbohydrate-binding module from *Clostridium perfringens* NagH, *J. Mol. Biol.* 390 (2) (2009) 208–220.
- [184] B. Pluvinage, P.M. Massel, K. Burak, A.B. Boraston, Structural and functional analysis of four family 84 glycoside hydrolases from the opportunistic pathogen *Clostridium perfringens*, *Glycobiology* 30 (1) (2019) 49–57.
- [185] W.L. Hynes, L. Hancock, J.J. Ferretti, Analysis of a second bacteriophage hyaluronidase gene from *Streptococcus pyogenes*: evidence for a third hyaluronidase involved in extracellular enzymatic activity, *Infect. Immun.* 63 (8) (1995) 3015–3020.
- [186] D.P. Pires, H. Oliveira, L.D. Melo, S. Sillankorva, J. Azeredo, Bacteriophage-encoded depolymerases: their diversity and biotechnological applications, *Appl. Microbiol. Biotechnol.* 100 (5) (2016) 2141–2151.
- [187] N.L. Smith, E.J. Taylor, A.M. Lindsay, S.J. Charnock, J.P. Turkenburg, E. J. Dodson, G.J. Davies, G.W. Black, Structure of a group A streptococcal phage-encoded virulence factor reveals a catalytically active triple-stranded beta-helix, *Proc. Natl. Acad. Sci. U. S. A.* 102 (49) (2005) 17652–17657.
- [188] S.K. Singh, A.P. Bharati, N. Singh, P. Pandey, P. Joshi, K. Singh, K. Mitra, J. R. Gayen, J. Sarkar, M.S. Akhtar, The prophage-encoded hyaluronate lyase has broad substrate specificity and is regulated by the N-terminal domain, *J. Biol. Chem.* 289 (51) (2014) 35225–35236.
- [189] A.M. Lindsay, M. Zhang, Z. Mitchell, M.T.G. Holden, A.S. Waller, I.C. Sutcliffe, G. W. Black, The *Streptococcus equi* prophage-encoded protein SEQ2045 is a hyaluronan-specific hyaluronate lyase that is produced during equine infection, *Microbiology (Reading)* 155 (Pt 2) (2009) 443–449.
- [190] S.A. Halperin, P. Ferrieri, E.D. Gray, E.L. Kaplan, L.W. Wannamaker, Antibody response to bacteriophage hyaluronidase in acute glomerulonephritis after group A streptococcal infection, *J. Infect. Dis.* 155 (2) (1987) 253–261.
- [191] T.B. Broudy, V. Pancholi, V.A. Fischetti, Induction of lysogenic bacteriophage and phage-associated toxin from group A streptococci during coculture with human pharyngeal cells, *Infect. Immun.* 69 (3) (2001) 1440–1443.
- [192] J.H. Lee, L.D. Moore, S. Kumar, D.G. Pritchard, S. Ponnazhagan, C. Deivanayagam, Bacteriophage hyaluronidase effectively inhibits growth, migration and invasion by disrupting hyaluronan-mediated Erk1/2 activation and RhoA expression in human breast carcinoma cells, *Cancer Lett.* 298 (2) (2010) 238–249.
- [193] P. Mishra, M.S. Akhtar, V. Bhakuni, Unusual structural features of the bacteriophage-associated hyaluronate lyase (hlyp2), *J. Biol. Chem.* 281 (11) (2006) 7143–7150.
- [194] P. Mishra, R. Prem Kumar, A.S. Ethayathulla, N. Singh, S. Sharma, M. Perbandt, C. Betzel, P. Kaur, A. Srinivasan, V. Bhakuni, T.P. Singh, Polysaccharide binding sites in hyaluronate lyase–crystal structures of native phage-encoded hyaluronate lyase and its complexes with ascorbic acid and lactose, *FEBS J.* 276 (12) (2009) 3392–3402.
- [195] L. Messina, J.A. Gavira, S. Pernagallo, J.D. Unciti-Broceta, R.M. Sanchez Martin, J.J. Diaz-Mochon, S. Vaccaro, M. Conejero-Muriel, E. Pineda-Molina, S. Caruso, L. Musumeci, R. Di Pasquale, A. Pontillo, F. Sincinelli, M. Pavan, C. Secchieri, Identification and characterization of a bacterial hyaluronidase and its production in recombinant form, *FEBS Lett.* 590 (14) (2016) 2180–2189.
- [196] C. Martinez-Fleites, N.L. Smith, J.P. Turkenburg, G.W. Black, E.J. Taylor, Structures of two truncated phage-tail hyaluronate lyases from *Streptococcus pyogenes* serotype M1, *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* 65 (Pt 10) (2009) 963–966.
- [197] B. Bissaro, P. Monsan, R. Faure, M.J. O'Donohue, Glycosynthesis in a waterworld: new insight into the molecular basis of transglycosylation in retaining glycoside hydrolases, *Biochem. J.* 467 (1) (2015) 17–35.
- [198] Y.S. Cheng, C.H. Huang, C.C. Chen, T.Y. Huang, T.P. Ko, J.W. Huang, T.H. Wu, J. R. Liu, R.T. Guo, Structural and mutagenetic analyses of a 1,3-1,4-beta-glucanase from *Paeclomyces thermophila*, *Biochim. Biophys. Acta* 1844 (2) (2014) 366–373.
- [199] J. Vasur, R. Kawai, A.M. Larsson, K. Igarashi, M. Sandgren, M. Samejima, J. Stahlberg, X-ray crystallographic native sulfur SAD structure determination of laminarinase Lam16A from *Phanerochaete chrysosporium*, *Acta Crystallogr. D Biol. Crystallogr.* 62 (Pt 11) (2006) 1422–1429.
- [200] A.H. Viborg, N. Terrapon, V. Lombard, G. Michel, M. Czjzek, B. Henrissat, H. Brumer, A subfamily roadmap of the evolutionarily diverse glycoside hydrolase family 16 (GH16), *J. Biol. Chem.* 294 (44) (2019) 15973–15986.
- [201] Z. Qin, S. Yang, L. Zhao, X. You, Q. Yan, Z. Jiang, Catalytic mechanism of a novel glycoside hydrolase family 16 “Elongating” beta-transglycosylase, *J. Biol. Chem.* 292 (5) (2017) 1666–1678.
- [202] A.M. Waterhouse, J.B. Procter, D.M. Martin, M. Clamp, G.J. Barton, Jalview Version 2—a multiple sequence alignment editor and analysis workbench, *Bioinformatics* 25 (9) (2009) 1189–1191.
- [203] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.* 22 (22) (1994) 4673–4680.
- [204] K.L. Hudson, G.J. Bartlett, R.C. Diehl, J. Agirre, T. Gallagher, L.L. Kiessling, D. N. Woolfson, Carbohydrate-aromatic interactions in proteins, *J. Am. Chem. Soc.* 137 (48) (2015) 15152–15160.
- [205] V. Spiwok, CH/pi interactions in carbohydrate recognition, *Molecules* 22 (7) (2017).
- [206] Sudhir Kumar, MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms, *Mol Biol Evol* (2018), <https://doi.org/10.1093/molbev/msy096>.
- [207] Ivica Letunic, Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation, *Nucleic Acids Res.* (2021), <https://doi.org/10.1093/nar/gkab301>.

Supplementary material

Hyaluronidases and Hyaluronate lyases: from humans to bacteriophages.

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Tree scale: 1

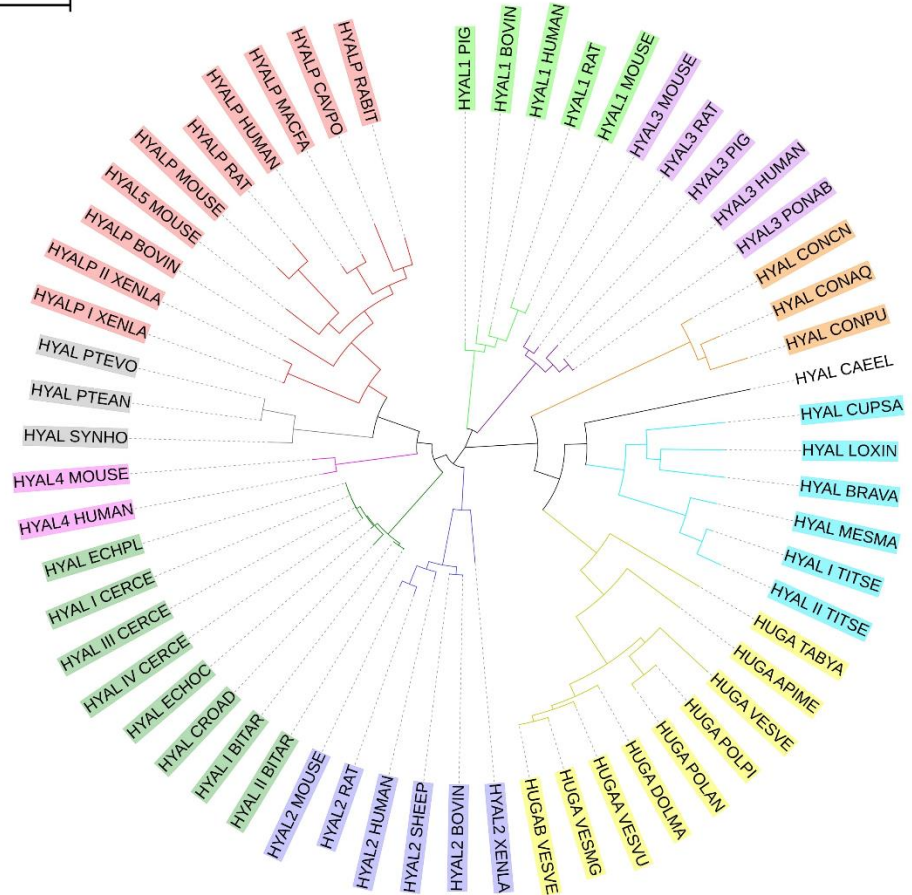


Fig. S1: A Phylogram of hyaluronidase sequences belonging to the vertebrate and venom hyaluronidase cluster. Alignment for phylogenetic tree was constructed using ClustalW algorithm and the maximum likelihood method in MEGA-X software and visualized by the iTOL tool. The length of branches represents the number of substitutions per site. Colours represent different subclusters of the GH56 family. Light green – HYAL1 of vertebrates. Purple – HYAL3 of vertebrates. Orange – venom Hyals of *Conus* sp. Uncoloured – Hyal of *Caenorhabditis elegans*. Cyan – venom Hyals of spiders and scorpions. Yellow – insect venom Hyals. Blue – HYAL2 of vertebrates. Dark green – venom Hyals of snakes. Pink – HYAL4 of vertebrates. Grey – fish venom Hyals. Red - HYALP and HYAL5 of vertebrates.

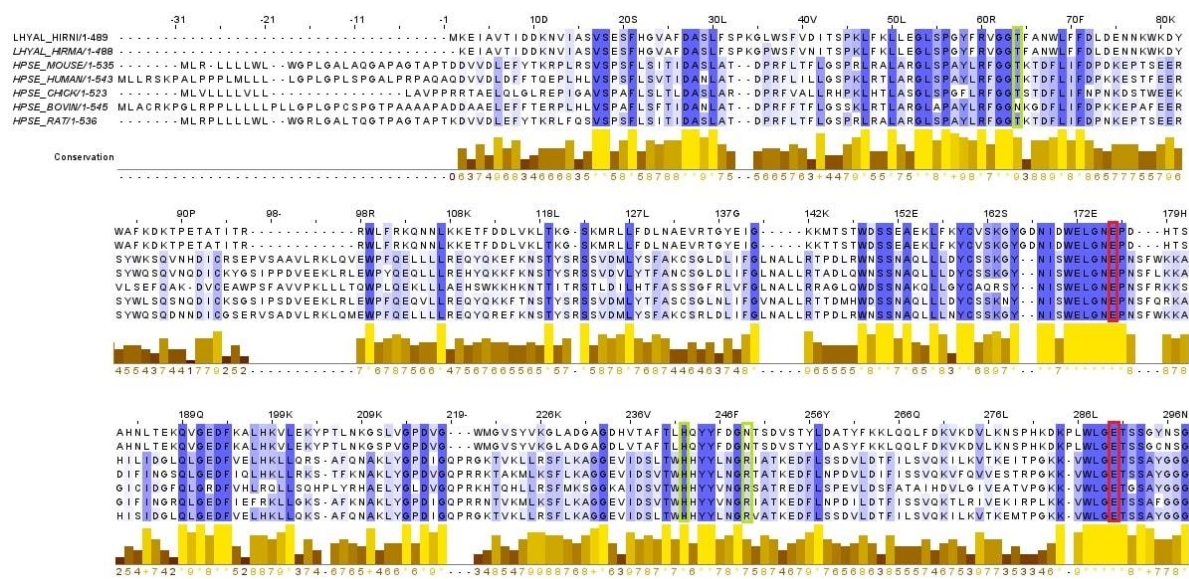


Fig. S3: Partial MSA of leech hyaluronidases and heparanases. Blue colour represents conserved residues; red frames represent catalytic residues (E176, E290); green frames represent heparan sulfate binding residues (T64, H242, R is substituted with N249 in leech hyaluronidase sequences). Alignment was constructed using ClustalW algorithm and visualized in Jalview software[1, 2].

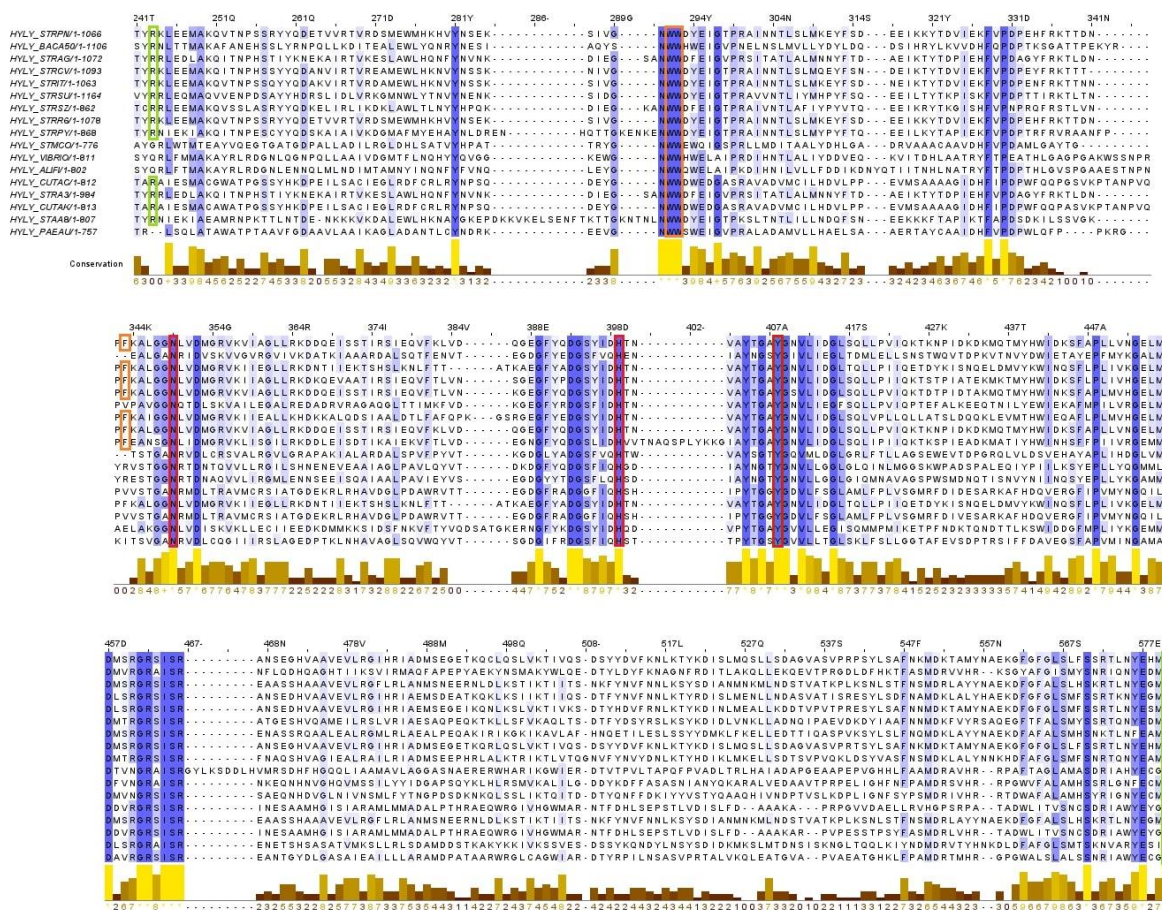


Fig. S4: Partial MSA of bacterial hyaluronate lyases belonging to polysaccharide lyase family 8. Blue represents conserved residues; green frames represent binding residues (R243, N580); orange frames represent aromatic residues that provides CH- π stacking (W291, W292, F342); red frames represent catalytic residues (N349, H399, Y408). Alignment was constructed using ClustalW algorithm and visualized in Jalview software [1, 2].

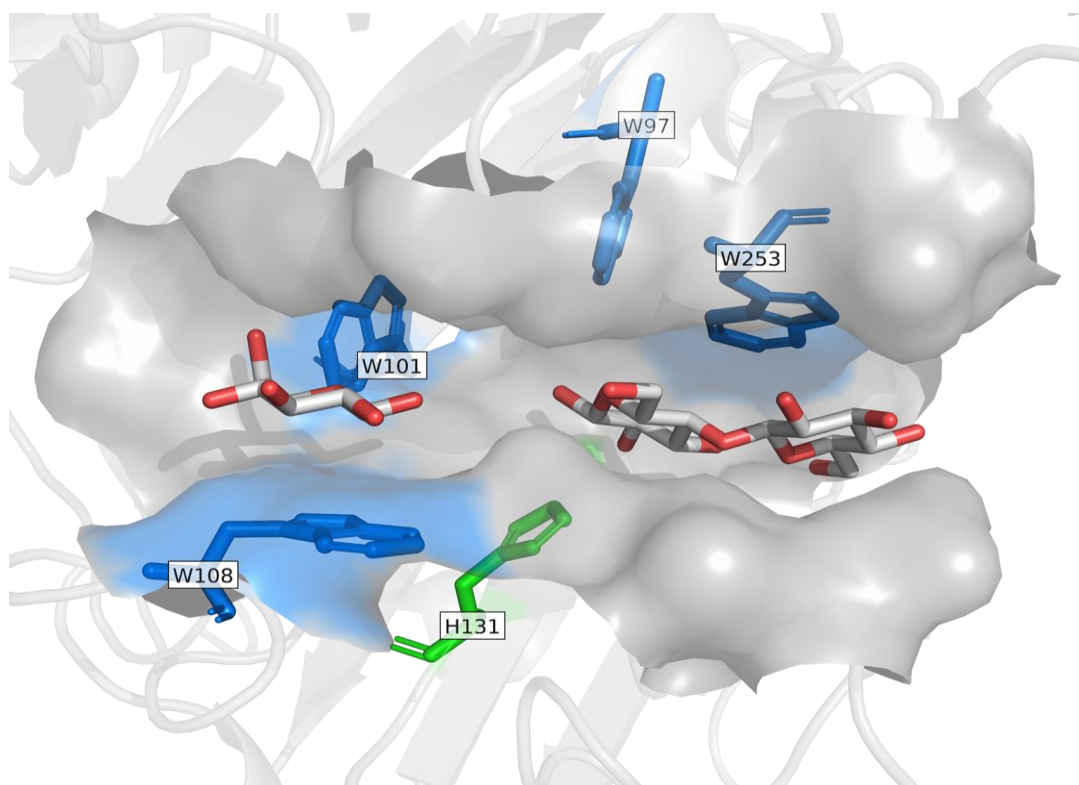


Fig. S5: Visualization of W97, W101, W108, H131 and W253 in 3WDV structure. W97, W101, W108 and W253 are represented as blue sticks; H131 as green sticks. Cellobiose and glucose as ligands. Visualized by PyMOL.

Supplement References

- [1] A.M. Waterhouse, J.B. Procter, D.M. Martin, M. Clamp, G.J. Barton, Jalview Version 2--a multiple sequence alignment editor and analysis workbench, *Bioinformatics* 25(9) (2009) 1189-91.
- [2] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res* 22(22) (1994) 4673-80.

Table S1: Annotated hyaluronidases and hyaluronate lyases

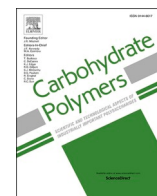
Enzyme abbreviation	Organism	EC number*	Family	Uniprot number	GeneBank number
HUGA_APIME	<i>Apis mellifera</i>	3.2.1.35	GH56	Q08169	AAA27730.1
HUGA_DOLMA	<i>Dolichovespula maculata</i>	3.2.1.35	GH56	P49371	AAA68279.1
HUGA_POLAN	<i>Polistes annularis</i>	3.2.1.35	GH56	Q9U6V9	AAD52616.1
HUGA_POLPI	<i>Polybia paulista</i>	3.2.1.35	GH56	P86687	P86687.1
HUGA_TABYA	<i>Tabanus yao</i>	3.2.1.35	GH56	E0XKJ9	ADM18346.1
HUGA_VESMG	<i>Vespa magnifica</i>	3.2.1.35	GH56	P86875	CBY83816.1
HUGAA_VESVU	<i>Vespula vulgaris</i>	3.2.1.35	GH56	P49370	AAB48073.1
HUGA_VESVE	<i>Vespa velutina</i>	3.2.1.35	GH56	C0HLL4	-
HUGAB_VESVE	<i>Vespa velutina</i>	3.2.1.35	GH56	C0HLL5	-
HYAL_BRAVA	<i>Brachypelma vagans</i>	3.2.1.35	GH56	-	AFS33217.1
HYAL_CAEEL	<i>Caenorhabditis elegans</i>	3.2.1.35	GH56	G5ECE8	CAA88874.1
HYAL_CONAQ	<i>Conus adamsonii</i>	3.2.1.35	GH56	I0CME8	AFH78529.1
HYAL_CONCN	<i>Conus consors</i>	3.2.1.35	GH56	I0CME7	AFH78528.1
HYAL_CONPU	<i>Conus purpurascens</i>	3.2.1.35	GH56	C0HKM3	C0HKM3.1
HYAL_CROAD	<i>Crotalus adamanteus</i>	3.2.1.35	GH56	J3S820	AFJ49188.1
HYAL_CUPSA	<i>Cupiennius salei</i>	3.2.1.35	GH56	-	CUH74573.1
HYAL_ECHOC	<i>Echis ocellatus</i>	3.2.1.35	GH56	A3QVN2	ABI33937.1
HYAL_ECHPL	<i>Echis pyramidum leakeyi</i>	3.2.1.35	GH56	A3QVN6	ABI33941.1
HYAL_I_BITAR	<i>Bitis arietans</i>	3.2.1.35	GH56	A3QVN9	ABI33944.1
HYAL_I_CERCE	<i>Cerastes cerastes</i>	3.2.1.35	GH56	A3QVN3	ABI33938.1
HYAL_I_TITSE	<i>Tityus serrulatus</i>	3.2.1.35	GH56	P85841	AHF72517.1
HYAL_II_BITAR	<i>Bitis arietans</i>	3.2.1.35	GH56	A3QVP0	ABI33945.1
HYAL_II_TITSE	<i>Tityus serrulatus</i>	3.2.1.35	GH56	W0HFN9	AHF72516.1
HYAL_III_CERCE	<i>Cerastes cerastes</i>	3.2.1.35	GH56	A3QVN4	ABI33939.1
HYAL_IV_CERCE	<i>Cerastes cerastes</i>	3.2.1.35	GH56	A3QVN5	ABI33940.1
HYAL_LOXIN	<i>Loxosceles intermedia</i>	3.2.1.35	GH56	R4J7Z9	AGH25912.1
HYAL_MESMA	<i>Mesobuthus martensii</i>	3.2.1.35	GH56	P86100	ACY69673.1
HYAL_PTEAN	<i>Pterois antennata</i>	3.2.1.35	GH56	-	BAM66299.1
HYAL_PTEVO	<i>Pterois volitans</i>	3.2.1.35	GH56	-	BAM66300.1
HYAL_SYNHO	<i>Synanceia horrida</i>	3.2.1.35	GH56	Q801Z8	AAO74499.1
HYAL1_BOVIN	<i>Bos taurus</i>	3.2.1.35	GH56	Q5E985	AAX09052.1
HYAL1_HUMAN	<i>Homo sapiens</i>	3.2.1.35	GH56	Q12794	AAM60770.1
HYAL1_MOUSE	<i>Mus musculus</i>	3.2.1.35	GH56	Q91ZJ9	AAC15949.1
HYAL1_PIG	<i>Sus scrofa</i>	3.2.1.35	GH56	Q6RHW4	AAR91599.1

HYAL1_RAT	<i>Rattus norvegicus</i>	3.2.1.35	GH56	Q76HN1	BAD14368.1
HYAL2_BOVIN	<i>Bos taurus</i>	3.2.1.35	GH56	Q8SQG8	AAI02043.1
HYAL2_HUMAN	<i>Homo sapiens</i>	3.2.1.35	GH56	Q12891	AAC62823.1
HYAL2_MOUSE	<i>Mus musculus</i>	3.2.1.35	GH56	O35632	AAK28482.1
HYAL2_RAT	<i>Rattus norvegicus</i>	3.2.1.35	GH56	Q9Z2Q3	AAD01980.1
HYAL2_SHEEP	<i>Ovis aries</i>	3.2.1.35	GH56	Q8SQG7	AAL78386.1
HYAL2_XENLA	<i>Xenopus laevis</i>	3.2.1.35	GH56	Q8UVY6	AAL55823.1
HYAL3_HUMAN	<i>Homo sapiens</i>	3.2.1.35	GH56	O43820	AAD04257.1
HYAL3_MOUSE	<i>Mus musculus</i>	3.2.1.35	GH56	Q8VEI3	AAL57175.1
HYAL3_PIG	<i>Sus scrofa</i>	3.2.1.35	GH56	Q6RHW2	AAR33037.1
HYAL3_PONAB	<i>Pongo abelii</i>	3.2.1.35	GH56	Q5REQ1	CAH89756.1
HYAL3_RAT	<i>Rattus norvegicus</i>	3.2.1.35	GH56	Q76HM9	AAH97259.1
HYAL4_HUMAN	<i>Homo sapiens</i>	3.2.1.35	GH56	Q2M3T9	AAC98883.1
HYAL4_MOUSE	<i>Mus musculus</i>	3.2.1.35	GH56	Q05A56	AAI25403.1
HYAL5_MOUSE	<i>Mus musculus</i>	3.2.1.35	GH56	Q812F3	EDL13813.1
HYALP_BOVIN	<i>Bos taurus</i>	3.2.1.35	GH56	Q2YDK3	AAI10184.1
HYALP_CAVPO	<i>Cavia porcellus</i>	3.2.1.35	GH56	P23613	CAA39768.1
HYALP_HUMAN	<i>Homo sapiens</i>	3.2.1.35	GH56	P38567	AAQ96882.1
HYALP_I_XENLA	<i>Xenopus laevis</i>	3.2.1.35	GH56	Q8UVT7	AAL67683.1
HYALP_II_XENLA	<i>Xenopus laevis</i>	3.2.1.35	GH56	Q9W6K5	AAD28281.1
HYALP_MACFA	<i>Macaca fascicularis</i>	3.2.1.35	GH56	P38568	EHH52472.1
HYALP_MOUSE	<i>Mus musculus</i>	3.2.1.35	GH56	P48794	AAA76603.1
HYALP_RABIT	<i>Oryctolagus cuniculus</i>	3.2.1.35	GH56	P38566	AAA88913.1
HYALP_RAT	<i>Rattus norvegicus</i>	3.2.1.35	GH56	Q62803	AAH81748.1
HYAL_TALPU	<i>Talaromyces purpureogenus</i> (<i>Penicillium purpureogenum</i>)	3.2.1.35	GH16	E7FI87	BAJ61836.1
HYAL_TALFU	<i>Talaromyces funiculosus</i> (<i>Penicillium funiculosum</i>)	3.2.1.35	GH16	E7FI86	BAJ61835.1
TMEM2_DANRE	<i>Danio rerio</i>	3.2.1.35	CEMIP	A3KPQ7	CAM56649.1
CEMIP_MOUSE	<i>Mus musculus</i>	3.2.1.35	CEMIP	Q8BI06	NP_109653.3
TMEM2_HUMAN	<i>Homo sapiens</i>	3.2.1.35	CEMIP	Q9UHN6	NP_037522.1
CEMIP_HUMAN	<i>Homo sapiens</i>	3.2.1.35	CEMIP	Q8WUJ3	NP_001280227.1
TMEM2_MOUSE	<i>Mus musculus</i>	3.2.1.35	CEMIP	Q5FWI3	NP_001028931.1
LHYAL_HIRNI	<i>Hirudo nipponia</i>	3.2.1.36	GH79	X4Y2L4	AHV78514.1
LHYAL_HIRMA	<i>Hirudinaria manillensis</i>	3.2.1.36	GH79	-	CAC22595.1
HYLY_BACA50	<i>Bacillus</i> sp. A50	4.2.2.1	PL8/CBM70	-	AHB61202.1
HYLY_STRAG	<i>Streptococcus agalactiae</i> 4755	4.2.2.1	PL8/CBM70	O86478	CAA75865.1

HYLY_STRA3	<i>Streptococcus agalactiae</i> serotype III (strain NEM316)	4.2.2.1	PL8/CBM70	Q53591	CAD46929.1
HYLY_STRCV	<i>Streptococcus constellatus</i> subsp. <i>constellatus</i> ATCC 27823	4.2.2.1	PL8/CBM70	Q934I5	AAK67165.1
HYLY_STRIT	<i>Streptococcus intermedius</i> ATCC 27335	4.2.2.1	PL8/CBM70	Q934I4	AAK67167.1
HYLY_STRPN	<i>Streptococcus pneumoniae</i> TIGR4	4.2.2.1	PL8/CBM70	Q54873	AAK74491.1
HYLY_STRSU	<i>Streptococcus suis</i> SEROTYPE 7	4.2.2.1	PL8/CBM70	Q8VLQ8	CAC87662.1
HYLY_ALIFI	<i>Aliivibrio fischeri</i> ES114	4.2.2.1	PL8	Q5DYT2	AAW88064.1
HYLY_STAA8	<i>Staphylococcus aureus</i> 8325-4	4.2.2.1	PL8	Q59801	AAA82984.1
HYLY_STRSZ	<i>Streptococcus equi</i> subsp. <i>zooepidemicus</i> CVCC23362	4.2.2.1	PL8	A8HJR5	ABV69920.1
HYLY_STRR6	<i>Streptococcus pneumoniae</i> (strain ATCC BAA-255 / R6)	4.2.2.1	PL8	Q8CWU3	AAK99090.1
HYLY_STRPY	<i>Streptococcus pyogenes</i>	4.2.2.1	PL8	Q9L7W9	AAF29533.1
HYLY_STMCO	<i>Streptomyces coelicolor</i> (strain ATCC BAA-471 / A3(2) / M145)	4.2.2.1	PL8	O86516	CAA19982.1
HYLY_VIBRIO	<i>Vibrio</i> sp. FC509	4.2.2.1	PL8	-	AIL54323.1
HYLY_CUTAC	<i>Cutibacterium acnes</i> (<i>Propionibacterium acnes</i>)	4.2.2.1	PL8	P0CZ00	P0CZ00.1
HYLY_PAEAU	<i>Paenarthrobacter aureus</i>	4.2.2.1, 4.2.2.5	PL8	P84141	-
HYLY_CUTAK	<i>Cutibacterium acnes</i> (strain DSM 16379 / KPA171202) (<i>Propionibacterium acnes</i>)	4.2.2.1	PL8	P0CZ01	AAT82132.1
HYLP2_STRPY_PHAGE	<i>Streptococcus pyogenes</i> phage H10403	4.2.2.1	PL16	Q54699	AAA86895.1
HYLP1_STRPY_PHAGE	<i>Streptococcus pyogenes</i> phage H4489A	4.2.2.1	PL16	P15316	AAA98101.1
HYAL_STRsp_PHAGE	<i>Streptococcus pyogenes</i> phage M1 GAS (370.1)	4.2.2.1	PL16	-	AAK33657.1
HYLY_STMKO	<i>Streptomyces koganeiensis</i> ATCC 31394	4.2.2.1	PL16	-	AKQ62598.1
HYLY_II_BACEL	<i>Bacteroides cellulosilyticus</i> WH2	4.2.2.1	PL30	-	ALJ59763.1
HYLY_I_BACEL	<i>Bacteroides cellulosilyticus</i> WH2	4.2.2.1	PL33	-	ALJ61728.1
HYAL_CLOPE	<i>Clostridium perfringens</i> (strain 13 / Type A)	3.2.1.35	GH84	P26831	AAA23259.1, BAB79897.1
HYLY_STEMA	<i>Stenotrophomonas maltophilia</i> (strain K279a)	4.2.2.1, 4.2.2.3, 4.2.2.14	PL5	B2FHL8	CAQ45011.1

*EC 3.2.1.35 hyaluronoglucosaminidase; EC 3.2.1.36 hyaluronoglucuronidase; EC 4.2.2.1 hyaluronate lyase; EC 4.2.2.3 mannuronate-specific alginate lyase; EC 4.2.2.5 chondroitin AC lyase; EC 4.2.2.14 glucuronan lyase

Article II



Molecular weight and gut microbiota determine the bioavailability of orally administered hyaluronic acid

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ABSTRACT

The ability of hyaluronan as a dietary supplement to increase skin moisture and relieve knee pain has been demonstrated in several clinical studies. To understand the mechanism of action, determining hyaluronan's bioavailability and *in vivo* fate is crucial. Here, we used ¹³C-hyaluronan combined with LC-MS analysis to compare the absorption and metabolism of oral hyaluronan in germ-free and conventional wild-type mice. The presence of *Bacteroides* spp. in the gut was crucial for hyaluronan absorption. Specific microorganisms cleave hyaluronan into unsaturated oligosaccharides (<3 kDa) which are partially absorbed through the intestinal wall. The remaining hyaluronan fragments are metabolized into short-chain fatty acids, which are only metabolites available to the host. The poor bioavailability (~0.2 %) of oral hyaluronan indicates that the mechanism of action is the result of the systematic regulatory function of hyaluronan or its metabolites rather than the direct effects of hyaluronan at distal sites of action (skin, joints).

1. Introduction

Hyaluronan (HA) is a simple repeating disaccharide polymer, consisting of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc), which is found in all vertebrate tissues as an essential component of the extracellular matrix. (Rodrigues et al., 2015) In the human body, HA is most abundant in the knee joint, articular cartilage, and skin, where it acts as a lubricant, shock absorber, and moisturizer (Gupta et al., 2019). HA metabolism is highly efficient and involves a number of enzymes. In humans, HA is synthesized at the cell surface by HA synthases 1–3 (HAS1–3) most commonly with a molecular weight (*M_w*) of 1–8 MDa (Cowman et al., 2015) and is degraded by different hyaluronidases (HYAL1, HYAL2, PH-20, CEMIP, CEMIP2) (Šindelář et al., 2021). Apart from homeostatic and filling functions, HA is implicated in cell proliferation, adhesion and migration, triggering a range of signalling

pathways by interacting with cell-surface receptors and binding molecules (e.g. CD44, layilin, LYVE-1) (Kobayashi et al., 2020).

HA had been used as a dietary supplement for many years for its anti-inflammatory properties and for its ability to increase skin moisture and to relieve knee chronic pain (Pan et al., 2021). Although many clinical studies have demonstrated the beneficial effect of orally-administered HA (Kawada et al., 2014; Oe et al., 2016), inconsistent information is available regarding its bioavailability and mechanism of action. It is suggested that a high-*M_w* of HA prevents its free transit through the intestinal wall. However, the gastrointestinal tract harbours a complex population of microorganisms, the gut microbiota, containing enzymes capable of cleaving HA into shorter fragments (Ndeh et al., 2020), which can then be more easily absorbed by the host intestinal epithelium (Sato et al., 2020). Gut microbiota may thus act as a crucial mediator of the absorption of orally administered HA. Furthermore, HA may serve as a

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substrate for gut microbiota (Pan et al., 2021) and thus modulate the composition of the microbial community (Mao et al., 2021), which may have a beneficial impact on the host. Considering the possible mechanisms, several suggestions have been made: a) HA is absorbed and has a direct effect at the site of action (skin, joints) (Oe et al., 2014), b) HA or its fragments in the intestine or organism trigger the specific cell response (Kolar et al., 2015), c) further metabolites of HA in the intestine or in the organism trigger the specific cell response. However, to identify the mechanism of action of orally administered HA, it is crucial to first determine its bioavailability and *in vivo* fate. The results of previous studies showed that HA (~1 MDa, ^{99m}Tc or ^{14}C labelled) orally administered to rats and dogs was distributed throughout the blood, skin, and joints (Balogh et al., 2008) and bioavailability was estimated to be over 16 % (Oe et al., 2014). Conversely, other studies reported that orally administered HA (~1 MDa, native) (Asari et al., 2010) or $^{99}\text{Tc}/^{14}\text{C}$ labelled (Laznicka et al., 2012) was not distributed in the body at all. These discrepancies may be attributed to artificial results of techniques employing radioactive or fluorescent probes (Svanovsky et al., 2008). To overcome these drawbacks, we recently introduced ^{13}C labelling in combination with sensitive LC-MS analysis in order to investigate HA metabolism after its application *via* an intravenous route (Šimek et al., 2021).

The aim of this study is to determine by means of unique ^{13}C -labeling technology how HA passes through the gastrointestinal tract, how it is metabolized and distributed in the body, and resolve the discrepant findings about its bioavailability. We hypothesize that bioavailability of HA depends on M_w , that is reducing during the pass of gastrointestinal tract. Additionally, we want to resolve if HA could really serve as a joint or skin nutrition or its clinically proved effects may be attributed to different therapeutic mechanism. The answers to these questions may provide new perspectives on the *in vivo* fate and mechanism of action of orally administered HA.

2. Material and methods

2.1. Materials

^{13}C -labelled form of Nutrihyal® (high- M_w HA) was prepared biotechnologically from D-glucose ($\text{U}-^{13}\text{C}$, 99 %) (Cambridge Isotope Laboratories, Tewksbury, USA) by Contipro (Dolní Dobrouč, Czechia). Acid hydrolysis was used for the preparation of low- M_w HA (15 kDa). The solution of high- M_w ^{13}C -HA (10 mg ml $^{-1}$) was acidified by sulfuric acid to pH 3.0 and then heated at 100 °C for 240 min. After cooling down to room temperature, solutions were neutralized by sodium hydroxide solution. Prior to lyophilization, the HA solution was purified and concentrated by active carbon filtration and ultrafiltration. ^{13}C -labelled unsaturated HA disaccharide (ΔAN_2) was prepared by enzymatic degradation of high- M_w ^{13}C -HA by lyase from *Streptococcus pneumoniae* provided by Contipro (Dolní Dobrouč, Czechia). The enzyme (133 UI mg $^{-1}$) was added to solution of high- M_w ^{13}C -HA (10 mg ml $^{-1}$) and samples were incubated for 3 h at 37 °C. Afterwards, the mixture was heated for 10 min at 100 °C and filtered using centrifugal filters with an M_w (cut off 2 kDa). The distribution of M_w , the efficiency of the stable isotope labelling of ^{13}C -HA and mass spectrum of ^{13}C - ΔAN_2 is displayed in S.Fig. 1. ^{13}C NMR analysis of high- M_w ^{13}C -HA was already published by (Šimek et al., 2021). The standards of even-numbered saturated (type AN_n) and unsaturated (type ΔAN_n) and odd-numbered (type AA_n and NN_n) oligosaccharides of HA (>95 %) were obtained from Contipro (Dolní Dobrouč, Czechia). The list of standards is summarized in S. Table 1. Oligosaccharides are labelled according to commonly used nomenclature (Blundell & Almond, 2006) where number (n) represents the number of building blocks of glucuronic acid (A) and N-acetylglucosamine (N). AN, NA, AA describe monosaccharide units at the ends of oligosaccharide chains, Δ stands for the double bond in the structure of the monosaccharide unit at the nonreducing end of the oligosaccharide chain.

Biotin-labelled HA (400 kDa M_w/M_n 1.67, with no fragments lower than 130 kDa) was prepared from native HA (Contipro, Dolní Dobrouč, Czechia) according to (Kessler et al., 2018). The M_w of HA and its distribution were determined by SEC-MALLS as described previously (Cožíková et al., 2017). A mixture of biotin-labelled HA oligosaccharides was prepared from the biotin-labelled HA by means of enzymatic depolymerization with bovine testicular hyaluronidase (EC 3.2.1.35, 2074 U mg $^{-1}$) (Finepharm, Jelenia Góra, Poland). 300 U mg $^{-1}$ of enzyme was added to the solution of biotin-labelled HA (1 mg ml $^{-1}$) in acetate buffer (0.05 M, pH 5.4) and the mixture was incubated for 24 h at 37 °C. Afterwards, the mixture was heated for 5 min at 100 °C and filtered using centrifugal filters with an M_w (cut off 2 kDa). Biotin-labelled oligosaccharides were analysed by LC-MS (S.Fig. 3). The main components of the biotin-labelled oligosaccharide mixture were AN4 and AN6 with one molecule of biotin attached. The abundance of other oligosaccharides was estimated to be <2 %. The content of free biotin was determined by LC-MS to be <0.01 %. The same procedure of enzymatic degradation was used for the preparation of mixture of ^{13}C -oligosaccharides which were derived from high- M_w ^{13}C -HA. ^{13}C -labelled oligosaccharides were analysed by MALDI-MS (S.Fig. 4) and by LC-MS (Fig. 3F).

2.2. Pharmacokinetic studies

Conventional female C57Bl/6 J mice (wild-type; WT), 4 weeks of age, were obtained from the laboratory of the Animal Breeding and Experimental Facility of the Faculty of Medicine (Masaryk University, Brno, Czechia) and Anlab (Prague, Czechia). The experiments were approved by the Animal Care Committee of the Czech Academy of Sciences (protocol n. 9/2019) and were in accordance with the EU and NIH Guide for the Care and Use of Laboratory Animals. The mice were co-housed and randomly mixed for 4 weeks in individually ventilated cages with *ad libitum* access to sterile food (25 kGy irradiated SSNIFF mouse breeding extrudate diet V1126-000) and sterile drinking water in the certified clean animal facility of the Institute of Biophysics of the Czech Academy of Sciences, Brno, Czechia with a controlled climate and light/dark cycle (12 h/12 h). The institute is authorized for the use of experimental animals (file number:16OZ22300/2019-18,134; ID:43049/2020-MZE-18134). After 4 weeks, the eight-week-old mice (18-20 g in weight) were randomly divided into experimental groups. Female C57Bl/6 (germ free; GF) mice (8-10 weeks), bred in the Laboratory of Gnotobiology (Nový Hrádek, Czechia) for >10 generations, were kept under sterile conditions in positive pressure Trexler-type plastic isolators on sterile Espe LTE E-002 bedding (Abedd, Kalnciems, Latvia), exposed to 12 h light/dark cycles at a temperature of 22 ± 2 °C and 40 %-60 % humidity, and supplied with autoclaved tap water and sterile food *ad libitum*. The experiments with GF mice were carried out at certified animal facility (ID: 66020/2020-MZE-18134, file number: 17OZ26090/2020-18,134), approved by the Animal Care Committee of the Czech Academy of Sciences (protocol n. 31/2020) and were in accordance with the EU and NIH Guide for the Care and Use of Laboratory Animals. Axenicity was assessed every two-weeks by confirming the absence of bacteria, mold, and yeast by the aerobic and anaerobic cultivation of mouse feces and swabs from the isolators in VL (Viande-Levure), Sabouraud-dextrose, and meat-peptone broth and by subsequent plating on blood, Sabouraud, and VL agar plates. The HA of six different forms – the oligosaccharides ^{13}C - ΔAN_2 , AN4, ΔAN_8 , and ΔAN_{16} , and 15 kDa ^{13}C -HA and 1600 kDa ^{13}C -HA was perorally administrated in a physiological saline solution by oral gavage (100 μl or 300 μl ; 10 mg ml $^{-1}$). The administered dose was 50 mg kg $^{-1}$; the high- M_w HA and ΔAN_2 were also dosed at 150 mg kg $^{-1}$. At nine time points – 0.5, 1, 2, 4, 8, 12, 24, 48 and 72 h after the application of HA (3 animals per each treatment and time point) –, mice were anesthetized by the inhalation of isoflurane, and blood, organs, and tissues were collected. The groups of mice (3 animals per each treatment and time point) were housed in metabolic cages for 24, 48, and 72 h and excrement was

sampled 4, 8, 24, 32, 48, 56 and 72 h after HA application. Plasma was separated by centrifugation (450 g/10 min). Organs and tissue were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

2.3. Long-term supplementation

Thirty conventional WT female C57Bl/6 J mice, 4 weeks of age, were obtained from Anlab (Prague, Czechia). The experiments were approved by the Animal Care Committee of the Czech Academy of Sciences (protocol n. 9/2019) and were in accordance with the EU and NIH Guide for the Care and Use of Laboratory Animals. The mice were co-housed and randomly mixed for 4 weeks in individually ventilated cages with *ad libitum* access to sterile food and sterile drinking water in the certified clean animal facility of the Institute of Biophysics of the Czech Academy of Sciences, Brno, Czechia with a controlled climate and light/dark cycle (12 h/12 h). After 4 weeks, eight-week-old mice (18–20 g in weight) were randomly divided into 10 groups ($n = 3$). Eight of the groups were assigned to the following supplementations (2 groups to 1 supplementation): HA high dose (2.5 mg ml^{-1} , 1600 kDa), HA low dose (0.25 mg ml^{-1} , 1600 kDa), pectin from citrus peel (2.5 mg ml^{-1} , Santa Cruz Biotechnology, Dallas, USA), and GlcNAc (2.5 mg ml^{-1} , Merck, Darmstadt, Germany), while the two remaining groups acted as control (drinking water). All supplements were administered as a sterile solution in drinking water. The bottles with drinking water plus the supplement were changed daily (HA high dose, HA low dose) or every other day (pectin, GlcNAc, water). The amount of consumed drinking water with supplement per cage was monitored daily by weighing on lab scales. After 28 days, mice were anesthetized by isoflurane inhalation and the content of the cecum was collected under sterile conditions, weighed, and frozen at -80°C .

2.4. In vitro degradation of ^{13}C -HA by cecal content from oMM¹² colonized mice

Oligo-Mouse-Microbiota (oMM¹²) strains were obtained from the DSMZ - German Collection of Microorganisms and Cell cultures GmbH. The bacterial strains were grown in an anaerobic chamber (Concept 400, Ruskinn Technology LTD, Bridgend, UK) filled with a gas mixture (7 % H_2 , 10 % CO_2 , 83 % N_2) (Linde, Dublin, Ireland) in Anaerobic Akkermansia medium, as described previously (Eberl et al., 2020). 1 ml mixture aliquots of grown strains were frozen at -80°C until used to establish oMM¹² gnotobiotic mouse line as described previously (Eberl et al., 2020). Six-week-old oMM¹²-colonized mice were sacrificed by cervical dislocation, and the cecum was removed and transferred to an anaerobic chamber (Concept 400, Ruskinn Technology LTD, Bridgend, UK) filled with a gas mixture (7 % H_2 , 10 % CO_2 , 83 % N_2) (Linde) at a temperature of 37°C . The content of the cecum was transferred to a 15 ml test tube and diluted 1:1 with PBS with 0.05 % L-cystein hydrochloride (pH 6.8, Merck, Darmstadt, Germany). Afterwards, a solution of high- M_w HA (10 mg ml^{-1} , 1600 kDa) was added to achieve an HA concentration of $500\text{ }\mu\text{g ml}^{-1}$. After throughout mixing, 50 μl of sample was withdrawn at 4 time points (15, 30, 60 and 120 min). Immediately after withdrawal, 200 μl of chilled methanol (-80°C) was added and samples were then frozen in liquid nitrogen and stored at -80°C until processed for LC-MS analysis (2.12). The experiments were carried out at certified animal facility (ID: 66020/2020-MZE-18134, file number: 17OZ26090/2020–18,134), approved by the Animal Care Committee of the Czech Academy of Sciences (protocol n. 31/2020) and were in accordance with the EU and NIH Guide for the Care and Use of Laboratory Animals.

2.5. Application of HA into jejunal loops

Female 8-week-old germ-free C57Bl/6 mice were deprived of food for 16 h before surgery (with free access to water). Mice were

anesthetized by the intraperitoneal administration of a ketamine/xylazine mixture. Afterwards, laparotomy was performed and 2 cm jejunal loops were created with nylon ligatures. ^{13}C -high- M_w HA, a ^{13}C -oligosaccharide mixture, 400 kDa biotin-labelled HA, and a biotin-labelled oligosaccharide mixture at a concentration of 10 mg ml^{-1} in PBS were injected into jejunal loops through a gauge needle (100 μl). After surgery, the jejunal loop was returned to the abdominal cavity, which was then sutured, and the mouse was placed on heated pad (37°C). After 2 h, the mouse was euthanized, the abdominal cavity was re-opened, and the jejunal loop was removed and placed in fixative solution (S.Met. 1). The distribution of ^{13}C -oligosaccharides was determined by MALDI-MS imaging (S.Met. 2) and distribution of biotin-labelled HA was determined by confocal microscope (S.Met. 1). The experiments were approved by the Animal Care Committee of the Czech Academy of Sciences (protocol n. 18/2019) and were in accordance with the EU and NIH Guide for the Care and Use of Laboratory Animals. The institute is authorized for the use of experimental animals (ID: 66020/2020-MZE-18134, file number: 17OZ26090/2020–18,134).

2.6. Cell line cultivation and ^{13}C -HA cell uptake

The Caco-2 human colon adenocarcinoma epithelial cell line was cultured according to the manufacturer's instructions in EMEM medium (30–2003, ATCC) supplemented with 10 % FBS (Capricorn Scientific, Ebsdorfergrund, Germany) and $1\times$ penicillin/streptomycin (Biosera, Cholet, France). Cells were seeded on a 6-well plate (350,000 cells/well) for 24 h (37°C , 5 % CO_2 , 95 % humidity) and afterwards media were exchanged for media containing ^{13}C -HA ($250\text{ }\mu\text{g ml}^{-1}$). After 48 h of cultivation, the media were collected and frozen (-80°C), along with the sterile PBS that was used for washing cells 5 times. To determine ^{13}C -HA content in the cells, 150 μl of cold demineralized water was added to each well and cells were scraped off and transferred to a test tube.

2.7. Quantification of ^{13}C -HA

Quantification of ^{13}C -HA was carried out according to the validated method described by (Šimek et al., 2019). Briefly, AN2 as internal standard together with actinase E (Merck Darmstadt, Germany) were added to the samples and samples were incubated for 3 h at 45°C . Then, HA lyase from *Streptococcus pneumoniae* (Contipro, Dolní Dobrouč, Czechia) was added and samples were incubated for 30 min at 37°C . Finally, a chloroform/methanol mixture (900 μl , 4:1, v/v) was added, the samples were centrifuged (10,000 g/2 min), and the aqueous phase was used for analysis. 1 μl of sample was injected into an Atlantis Premier BEH C18 AX column ($50\times 2.1\text{ mm}$, 1.7 μm) (Waters, Milford, USA) maintained at 25°C . The mobile phases consisted of 0.1 % formic acid in water (v/v) and methanol. The flow rate was set at 0.4 ml min^{-1} and the following gradient of methanol was applied: 0–40 % from 0.5 to 2.5 min, 40–70 % from 2.5 to 3.5 min, 70–0 % from 3.5 to 4 min, and 0 % for 4–5.5 min. An Acquity UPLC I-class chromatographic system was coupled with a Xevo TQ-XS mass spectrometer (Waters, Milford, USA) equipped with an electrospray ionization source.

2.8. Molecular weight of ^{13}C -HA and its distribution

The M_w distribution of ^{13}C -HA in stomach, small intestine, cecum, and colon contents was determined by size exclusion chromatography similarly as described previously (Šimek et al., 2021). 100 μl of sample was injected into two series-connected PL aquagel–OH 40 ($300\times 7.5\text{ mm}$, 8 μm) and PL aquagel–OH 20 ($300\times 7.5\text{ mm}$, 5 μm) columns (Agilent, Santa Clara, USA) which were eluted with 0.15 mol l^{-1} sodium chloride at 1 ml min^{-1} and 40°C . Twenty fractions (5th–25th minute of elution) were collected and ^{13}C -HA was quantified in each fraction by LC-MS (see Section 2.7).

2.9. Metabolomic analyses

Low- M_w metabolites from 100 μ l of cultivated cecum content, tissue homogenates, cell lysates, and cultivation media were extracted with 400 μ l of chilled (-80°C) methanol. Methanolic extracts were dried under a stream of nitrogen and dissolved in 100 μ l of 0.1 % formic acid or 60 % isopropanol (v/v) for analysis by means of reversed-phase chromatography (RPLC) or hydrophilic interaction chromatography (HILIC), respectively.

A Vanquish Horizon UHPLC system coupled with an Exploris 240 (Thermo Fisher Scientific) mass spectrometer equipped with an electrospray ionization source was used. RPLC was performed according to (Körver-Keularts et al., 2018). Basic HILIC was performed according to (Shi et al., 2019). Metabolites were detected in negative ion mode, utilizing an ion transfer tube temperature of 350°C , a sheath gas pressure of 60 psi, an auxiliary gas flow of 20, and a spray voltage of 3.5 kV or -2.5 kV. MS data (m/z 70–800) were collected using a dynamic exclusion list and a targeted inclusion list updated in the AcquireX data acquisition mode to achieve optimal metabolite identification in Compound Discoverer 3.3 software (Thermo Fisher Scientific). Metabolites were identified against mz cloud mass spectral library and in-house library of hyaluronan oligosaccharides (mass and retention time). Short chain fatty acids were analysed by GC–MS (S.Met. 6).

2.10. Analysis of oligosaccharides

For the quantification of unsaturated HA oligosaccharides (ΔAN 2,8,10,12,14,16; $\geq 95\%$), a mixture of internal standards (saturated HA oligosaccharides AN 2,8,10,12,14,16; 10 μ l; 10 $\mu\text{g ml}^{-1}$; $\geq 95\%$) was added to samples of intestinal content, plasma, and urine. Oligosaccharides were obtained from Contipro (Dolní Dobrouč, Czechia). Then, extraction with methanol was performed (see Section 2.9) and the obtained extracts were dissolved in 100 μ l of 10 mmol l^{-1} ammonium acetate (pH 5.0). The same buffer and acetonitrile were used as mobile phases for chromatographic separation performed on an Atlantis Premier BEH C18 AX column (50×2.1 mm, 1.7 μm) (Waters, Milford, USA), which was maintained at 60°C . The flow rate was set at 0.4 ml min^{-1} and the following gradient of acetonitrile was applied: 5 % from 0 to 0.5 min, 5–60 % from 0.5 to 5.0 min, 60 % for 5.0–6.0 min, 60–5 % from 6.0 to 6.3 min, and 5 % for 6.3–8.0 min. HA oligosaccharides were analysed on the same system used for the quantification of ^{13}C -HA (see Section 2.7). Measurements were performed in multiple reaction monitoring mode, whereas for each oligosaccharide two transitions were measured (S.Table 1). Typical fragmentations involved formation of glucuronic acid residue (saturated or unsaturated depending on type of oligosaccharides) and cleavage of GlcNAc from reducing end of oligosaccharide. Oligosaccharides were identified according to their retention time and presence of signal of both transitions.

2.11. Data analysis

Differences among samples, tissues and/or individual time points were analysed using linear models with interactions of all involved factors. Post-hoc tests for prespecified pairs were done, correction for multiple comparisons was applied. Generalized linear models were used to compare pharmacokinetic distributions. Level of significance was set to 0.05. All statistical tests were performed in R software.

3. Results and discussion

3.1. Fate of orally-administered HA in conventional WT mice

We used ^{13}C -labelled HA with stable isotopes directly incorporated in the HA structure (S.Fig. 1B) combined with LC-MS analysis (Šimek et al., 2019; Šimek et al., 2021) to determine the kinetics of the permeation of high- M_w HA through the gastrointestinal tract (GIT), its

bioavailability, and its distribution in the body. Stable isotope labelling in combination with LC-MS analysis allowed us to resolve *endo*- and exogenous HA, monitor the M_w changes in orally-given HA, and identify its metabolites. ^{13}C -high- M_w HA (1600 kDa) was administered to conventional wild-type (WT) mice by intragastric gavage; this step was followed by the determination of ^{13}C -HA contents in plasma, liver, kidneys, mesenteric lymph nodes, urine, feces, and various parts of the GIT (stomach, small intestine, cecum and colon) at nine different time points from 30 min to 72 h after application (S.Table 2 and 3).

We found that administered ^{13}C - high- M_w HA disappeared from GIT content within 24 h (Fig. 1A). Interestingly, ^{13}C -HA eliminated in feces and urine of WT mice within 72 h was only about 2.2 % and 0.2 % of the administered dose, respectively (Fig. 1B). Therefore, the majority of the high- M_w HA dose was processed within the mouse body. Analysis of the distribution of M_w by offline SEC(LC-MS) showed that the M_w of administered high- M_w ^{13}C -HA gradually decreased during passage through the GIT (Fig. 1D). In more detail, the M_w of ^{13}C -HA shifted from 1600 kDa to approximately 150–600 kDa in the stomach and small intestine and decreased further in both sections over time. Eight hours after administration, ^{13}C -HA was completely depolymerized into short fragments with $M_w < 10$ kDa. These low- M_w fragments were also the only forms of ^{13}C -HA present in the cecum and colon (Fig. 1E). The reduction in M_w could be caused by several factors. In the stomach, the slight shift in M_w is probably caused by the acidic environment, which partially depolymerizes the HA via scission of $\beta(1 \rightarrow 3)$ or $\beta(1 \rightarrow 4)$ glycosidic bonds (Han et al., 2019; Stern et al., 2007). Further depolymerization in the cecum and colon may be associated with enzymatic cleavage by hyaluronidases in the intestinal wall or HA lyases encoded by gut microbiota that abundantly populate this part of GIT (Yilmaz et al., 2021).

3.2. Origin and nature of HA fragments in the GIT

To identify the nature and origin of short HA fragments in the upper and lower parts of the GIT, an LC-MS analysis of GIT content was employed. Short HA fragments were identified as unsaturated even-numbered oligosaccharides (Fig. 1E), which can be only produced by bacterial and bacteriophage HA lyases (Sindelar et al., 2021). The contents of GIT of WT and GF mice were analysed also for other types of oligosaccharides such as odd-numbered oligosaccharides with GlcA or even-numbered oligosaccharides with GlcNAc at reducing end which can be produced by mammalian hyaluronidases (Sindelar et al., 2021) or by acid hydrolysis at high acid concentrations (Han et al., 2019). However, any of these oligosaccharides were not found in any part of GIT of WT or GF mice. Thus, it can be concluded that the activity of mammalian hyaluronidases nor the acid hydrolysis were not involved in the degradation process and formation of unsaturated fragments. The most abundant oligosaccharide was the unsaturated disaccharide ΔAN_2 , which accounted for $>80\%$ of HA present in colon contents (Fig. 1E, S. Table 4). The amount of other unsaturated oligosaccharides with 8–20 monosaccharide units accounted only for $<4\%$ of HA present in colon contents.

To confirm the bacterial origin of the observed depolymerization of HA in the GIT, ^{13}C -HA (1600 kDa) was orally administered to germ free (GF) mice and its pharmacokinetics and M_w distribution were inspected. The passage of HA through the GIT was expectedly slower (Fig. 2A); however, this cannot be attributed only to the different metabolism of HA. Passage and elimination kinetics in GF mice are generally slower due to the enlarged cecum (Luczynski et al., 2016) and thicker mucus layer (Johansson et al., 2015). However, even the metabolism of HA in the GIT of GF mice differed significantly. Surprisingly, administered HA crossed the GIT in an almost intact form, with no depolymerization in lower parts of the GIT (Fig. 2C). The LC-MS analysis also did not reveal any unsaturated or other HA oligosaccharides, which clearly indicates that the unsaturated oligosaccharides observed in WT mice came from the bacterial metabolism of HA. As mentioned before, HA can

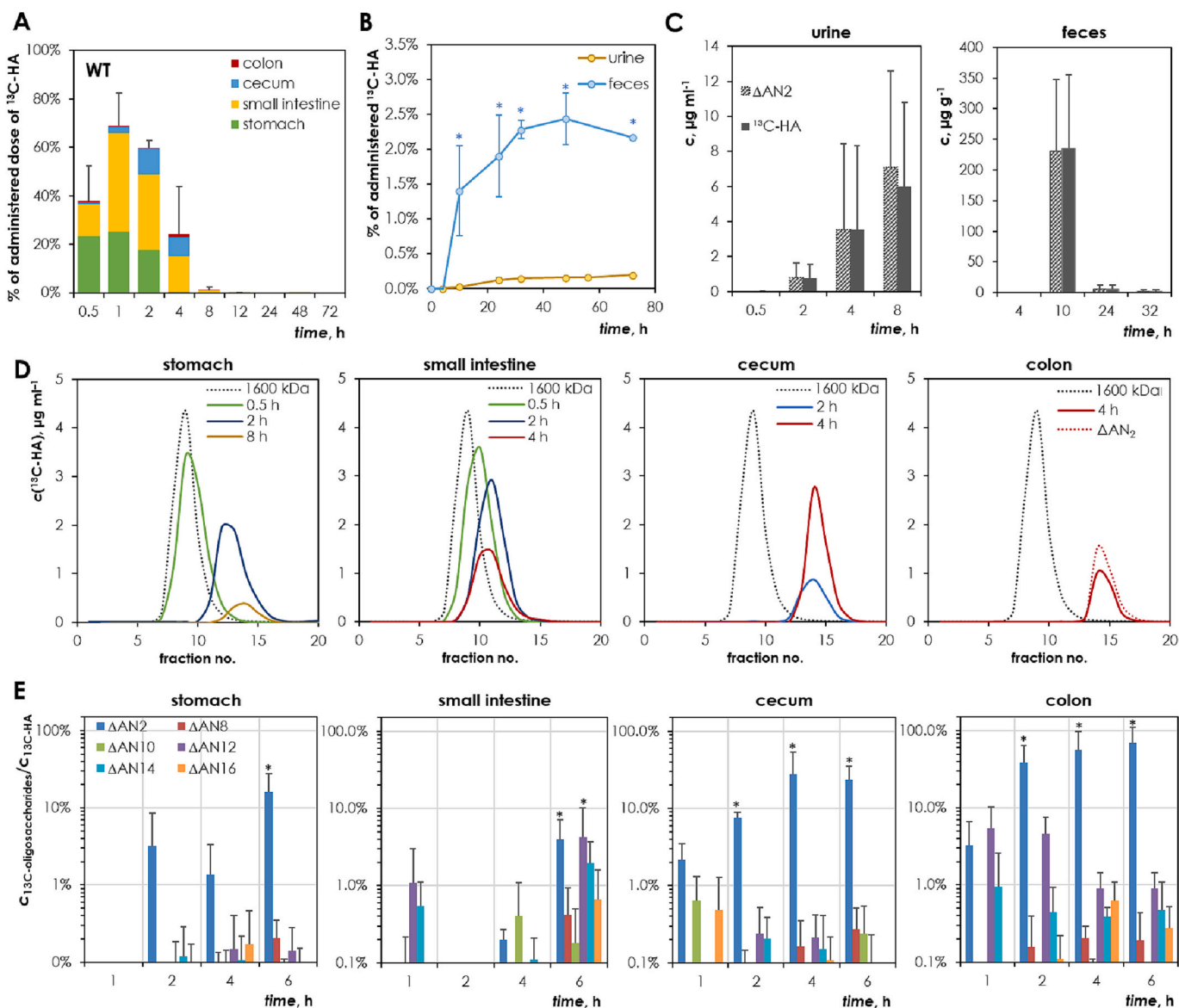


Fig. 1. Pharmacokinetics of high- M_w ^{13}C -HA administered orally to conventional WT mice. A) Amount of ^{13}C -HA in various parts of the GIT and at different time points, B) Elimination of ^{13}C -HA in urine and feces ($n = 3$; compared to samples of feces, $^*p < 0.05$), C) Concentration profiles of HA and unsaturated HA disaccharide in urine and feces, D) Changes in M_w distribution of orally administered ^{13}C -HA measured by SEC(LC-MS). The dotted lines are associated with a M_w distribution of 1600 kDa ^{13}C -HA before p.o. administration and standard of ΔAN_2 in PBS, solid lines represent the M_w distribution of ^{13}C -HA at various time points in various parts of the GIT. The shift of the fraction number to higher values indicates a decrease in M_w , E) Amount of unsaturated oligosaccharides in various parts of the GIT expressed as a ratio between the amount of oligosaccharide and the total amount of ^{13}C -HA ($n = 3$; compared to zero value, $^*p < 0.05$). AN describes the type of monosaccharide units at the ends of the oligosaccharide, the number describes the number of monosaccharide units, and Δ stands for the double bond in the structure of the monosaccharide unit at the end of oligosaccharide chain. Only the oligosaccharides ΔAN 2, 8, 10, 12, 14, and 16 were quantified due to the availability of standards; however, if these oligosaccharides were present in samples, longer oligosaccharides (ΔAN 18 and 20) were also present (S. Fig. 2).

also be depolymerized by acids (Stern et al., 2007); therefore, the acidic environment of the stomach needs to be considered. The acidic environment of the GF mouse stomach (pH 3–4) (McConnell et al., 2008) resulted in a slight decrease in M_w ; similar results were also obtained in an *in vitro* experiment with artificial gastric juice with a more human pH of 1.5, where a similar shift in M_w was observed (Fig. 2B). The decrease in M_w in the stomachs of WT mice (Fig. 1D) was more significant due to the presence of stomach microbiota (Wu et al., 2014) which could cause decrease of pH contributing to increased acid degradation of HA (Shimizu et al., 2021). However, the presence of unsaturated oligosaccharides (specific metabolites of bacterial degradation) in stomach (Fig. 1E) and absence of other types of oligosaccharides in stomach and artificial gastric juice indicates that higher depolymerization of HA in stomach of WT mice is caused by bacterial degradation.

To confirm the similarity between WT and GF mice models with respect to HA receptors and HA degrading enzymes in the GIT, the immunohistological analysis of HA-related proteins in the ileum, cecum, and colon was performed. It was found that the distribution and abundance of HA receptors and enzymes was similar in WT and GF mice (S. Fig. 5). Therefore, the observed differences in the pharmacokinetics of HA in WT and GF may be attributed only to anatomical differences and the presence of gut microbiota.

3.3. Penetration of HA fragments through the intestinal wall

To determine which HA fragments can penetrate through the intestinal wall and be available to the host, HA of six different forms (oligosaccharides (ΔAN 2, AN4, ΔAN 8, ΔAN 16), low- M_w HA, high- M_w HA)

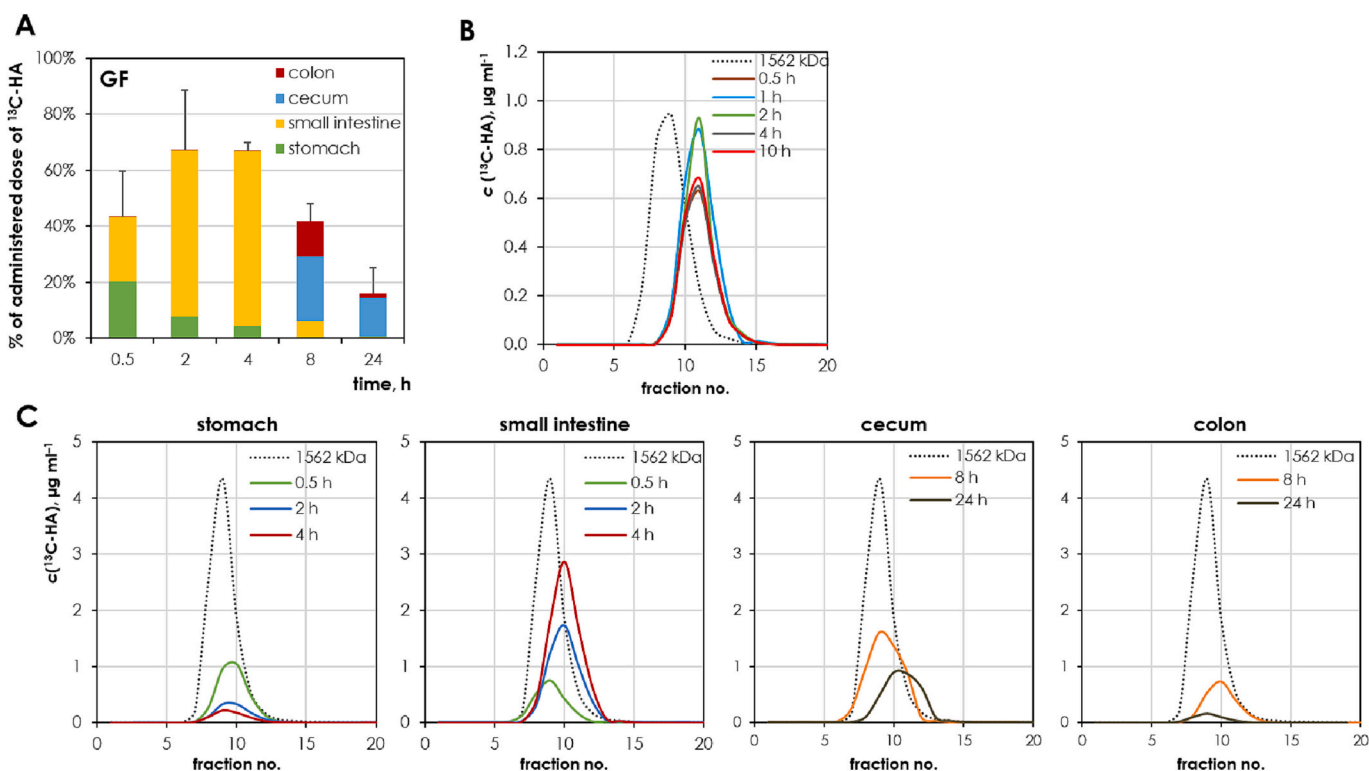


Fig. 2. Pharmacokinetics of high- M_w ^{13}C -HA administered orally to GF mice. A) Amount of ^{13}C -HA in various parts of the GIT at different time points, B) Changes in the M_w distribution of ^{13}C -HA during degradation by artificial gastric juice (pH 1.5), C) Changes in the M_w distribution of orally-administered ^{13}C -HA. The dotted lines are associated with the M_w distribution of administered ^{13}C -HA in PBS; solid lines represent the M_w distribution of ^{13}C -HA at various time points in various parts of the GIT. The shift of the fraction number to higher values indicates a decrease in M_w .

with M_w s (0.38, 0.78, 1.52, 3.03, 15.0 and 1600 kDa) were orally administered to WT mice. The analysis of HA in urine showed that the amount of HA in urine, and, thus, respectively, the bioavailability of oral HA, is inversely related to its M_w (Fig. 3A). Additionally, using LC-MS oligosaccharide analysis, the presence of $\Delta\text{AN}16$ (3.03 kDa) in urine after its oral administration was confirmed (S.Fig. 6). This suggest that fragments with $M_w \leq \sim 3$ kDa can pass through the intestinal wall, be available to the host, and subsequently be eliminated in urine. These data also correlate with the *in vitro* study by (Hisada et al., 2008), in which the authors observed an inverse relation between the permeability of HA through the Caco-2 cell monolayer and its M_w . Similar results have also been reported by (Sato et al., 2020), the authors observing a significant difference between the absorption of HA fragments of 2 kDa and 8 kDa.

Despite these results indicating that the intestinal absorption of native high- M_w HA is not possible, there are several studies suggesting the possibility of high- M_w HA uptake through the lymphatics (de Souza et al., 2019; Kawada et al., 2014). To resolve this question, biotin- and ^{13}C -labelled middle and high- M_w HA (0.4 and 1.6 MDa) and an oligosaccharide mixture (AN4, AN6, AN8 ($M_w < 1.5$ kDa)) were injected into jejunal loops of GF mice (Štěpánková et al., 1996). After 2 h, the penetration of HA through the intestinal wall was evaluated by confocal microscopy and MALDI-MS imaging, while the absorption of ^{13}C -HA into portal blood, blood from cardiac puncture, and mesenteric lymphatic nodes was determined by LC-MS. Due to the absence of intestinal microbiota, the intestinal absorption depended only on the M_w of the administered HA. MALDI-MS imaging together with confocal microscopy revealed the distribution of oligosaccharides in intestinal epithelium 2 h after their administration (Fig. 3B,C); however, biotin-labelled 400 kDa HA did not penetrate into the epithelium and remained intact inside the jejunal loop. This was also confirmed by SEC (LC-MS) analysis of ^{13}C -HA (Fig. 3E). After the administration of

oligosaccharides, the ^{13}C -HA concentration in portal blood and mesenteric lymph nodes was $151.2 \pm 70.8 \text{ ng ml}^{-1}$ and $321.3 \pm 179.0 \text{ ng g}^{-1}$, respectively. The concentration of ^{13}C -HA after the administration of high- M_w HA was below the limit of quantification (10 ng ml^{-1}). These data correspond to our pharmacokinetic studies with GF and WT mice (S.Table 3). Similar *in vitro* observations were made by (Kim et al., 2018) on mouse intestinal organoids, where 35 kDa HA in contrast to 2000 kDa HA penetrated into epithelial cells. Thus, high- M_w HA is not absorbed in its intact form and microbial degradation into short fragments ($< \sim 3$ kDa) is necessary for its absorption from the gut.

HA and its fragments can bind to several HA binding receptors including CD44, LYVE-1, and layilin. Their presence in the intestine was confirmed by several studies (Gracz et al., 2013; Kim & de la Motte, 2020; Kim et al., 2018; Soroosh et al., 2016). Additionally, intestinal epithelial cells contain hyaluronidases (Hyal1, Hyal2 and CEMIP2) (Bouga et al., 2010; Chowdhury et al., 2016; Uhlén et al., 2015). To investigate whether orally-administered HA could be utilized by intestinal epithelium, an *in vitro* study with Caco-2 cells was performed. The Caco-2 cells, commonly used as a model for intestinal absorption, were cultivated for 24 h in medium containing HA of eight different forms: unsaturated ($\Delta\text{AN}2$, $\Delta\text{AN}8$, $\Delta\text{AN}16$) and saturated (AN2, AN8, AN16) oligosaccharides, and low- M_w and high- M_w HA (15 and 1600 kDa). The change in HA concentration at the end of cultivation was negligible, and only a small proportion of the applied HA was found intracellularly ($< 0.1\%$). The uptake was inversely related to the M_w of HA and a significant difference between unsaturated oligosaccharides ($\sim 50\text{--}90\%$) and their saturated analogues (Fig. 3D) was observed. The ability of intestinal mucosa to utilize HA was also tested on jejunal loops of GF mice. However, no decrease in the M_w of applied high- M_w HA (Fig. 3E), no changes in the lengths of the applied oligosaccharides (Fig. 3F), and no HA metabolites in the jejunal loop content or tissue were observed. The abundance and distribution of HA receptors and enzymes in

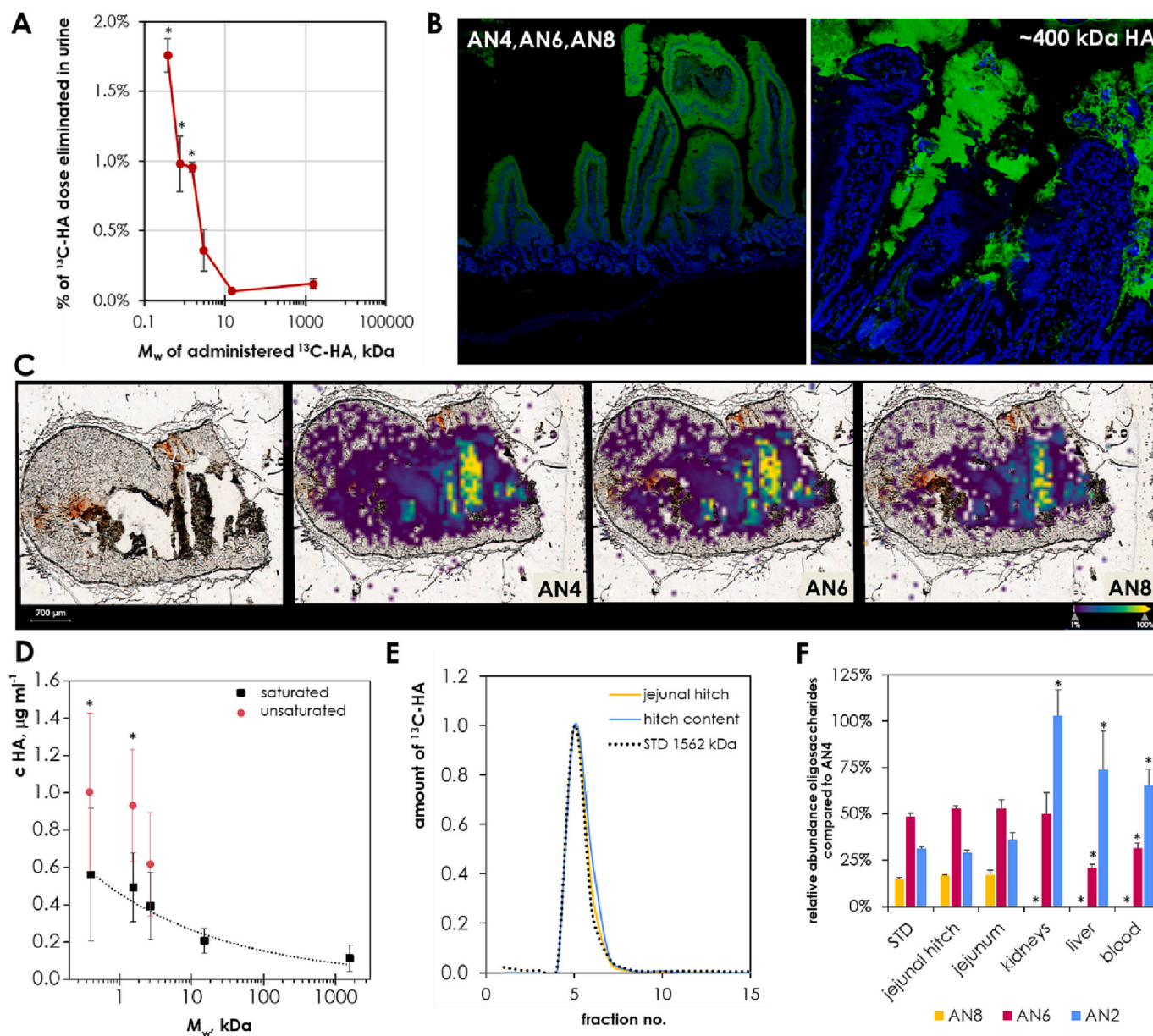


Fig. 3. Absorption and metabolism of oral HA by intestinal epithelium. A) Amount of ^{13}C -HA eliminated in urine as fragments of ^{13}C -HA in dependence on M_w of ^{13}C -HA administered to WT mice ($n = 3$; compared to results for 1600 kDa, $*p < 0.05$); B) Penetration of biotin-labelled HA in jejunal loops of GF mice 2 h after a mixture of HA oligosaccharides (AN4,AN6,AN8) or middle weight molecular weight HA (M_w 400 kDa) was injected into the loops (green – biotin-HA, blue – cell nuclei) – the specificity of biotin labelling was verified by LC-MS (S.Fig. 3); C) MALDI-MS imaging of ^{13}C -HA oligosaccharides 2 h after their administration into the jejunal loop. The distribution of oligosaccharide signal intensity from lowest to highest is represented by a color series (purple-blue-green-yellow). The specificity of ^{13}C labelling was verified by MALDI-MS (S.Fig. 4); D) Intracellular concentration of HA after cultivation of Caco-2 cells for 24 h in medium containing saturated or unsaturated oligosaccharides (AN- and Δ AN-type 2,8-,16- mers-), or low- or high- M_w HA at 0.25 mg ml^{-1} ($n = 3$; $*p < 0.05$); E) M_w distribution of ^{13}C -HA after its incubation for 2 h in jejunal loops of GF mice; F) Relative abundance of saturated oligosaccharides (AN-type 2,6,8-mers) compared to AN4 in oligosaccharide mixture administered to jejunal loops of GF mice and in tissue after 2 h of incubation ($n = 3$; compared to standard mixture, $*p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

jejunum is similar to their abundance and distribution in the rest of the intestine (S.Fig. 5); therefore, HA from intestinal contents is not metabolized by intestinal epithelium, but only by gut microbiota into unsaturated oligosaccharides, which are transported by lymph and the blood stream into the body. The concentrations of HA in portal blood and mesenteric lymph nodes were similar; however, the lymphatic flow rate is much slower (0.2 %) than that of portal blood (Sato et al., 2020). Therefore, it can be concluded that the absorption of HA in blood capillaries is the dominant route of transportation of unsaturated oligosaccharides from the intestine into the body.

3.4. Distribution of absorbed HA in the body

The bioavailability of administered high- M_w HA was practically zero for GF mice, since the concentration of ^{13}C -HA in plasma, urine, and organs was below the limit of detection ($<3 \text{ ng ml}^{-1}$) (S.Table 2, 3). In contrast, the plasma concentration of ^{13}C -HA for WT mice was significantly higher ($c_{\text{MAX}} 140 \text{ ng ml}^{-1}$). The concentration of ^{13}C -HA in other parts of the body was negligible (S.Table 3). The highest concentration of ^{13}C -HA was measured in urine ($c_{\text{MAX}} 6.0 \text{ } \mu\text{g ml}^{-1}$). The low concentration of ^{13}C -HA in plasma and the relatively high concentration in urine can be explained by the rapid elimination of low- M_w HA fragments

by glomerulus filtration (Šimek et al., 2021). The bioavailability of HA is therefore represented by the amount of ^{13}C -HA absorbed and eliminated in urine, which represented only 0.2 % of the administered dose for high- M_w HA (Fig. 3A). Additionally, $\Delta\text{AN}2$ was the only form of absorbed HA present in urine after the administration of high- M_w (1600 kDa) (Fig. 1C) or low- M_w (15 kDa) HA. These results clearly indicate that the depolymerization of HA by gut microbiota into unsaturated oligosaccharides is essential for its absorption into the body; however, even after this process, the bioavailability of HA is still very low.

After 24 h, only about 3 % of the administered dose of high- M_w HA was recovered from the feces, urine, and bodies of WT mice (Fig. 1B, S. Table 2). Therefore, > 97 % of the administered HA was either completely metabolized by the gut microbiota or depolymerized by the gut microbiota to unsaturated oligosaccharides and subsequently absorbed and metabolized in the body to low- M_w metabolites. To determine which was the case, GF and WT mice were fed ^{13}C - $\Delta\text{AN}2$ (Fig. 4). The quantification of $\Delta\text{AN}2$ in plasma, urine, and body organs showed that the amount of absorbed $\Delta\text{AN}2$ for WT and GF mice was similar and very low (<3 % of the administered dose) (Fig. 4B), while only about 1 % of administered $\Delta\text{AN}2$ was recovered in feces of WT mice (Fig. 4C). Additionally, no ^{13}C eukaryotic metabolites were observed in the body after the administration of high- M_w ^{13}C -HA, or ^{13}C - $\Delta\text{AN}2$. This indicates that regardless of the form of administered HA the majority of dosed HA is metabolized in the GIT and its absorption into the body is very limited. This conclusion correlates with the fact that no metabolization of HA fragments in intestinal epithelium was observed (Fig. 3E, F) and that after the administration of longer oligosaccharides (Fig. 3A), these were absorbed and eliminated in urine predominantly in their intact form (S.Fig. 6).

These findings correlate with previously-published data on the bioavailability of oral HA (Asari et al., 2010; Laznicek et al., 2012; Ma et al., 2015) and oral chondroitin sulphate (Volpi, 2002; Volpi, 2003).

Conversely, other studies have reported that HA (~1 MDa, ^{99m}Tc or ^{14}C labelled), orally administered to rats and dogs, was distributed throughout the blood, skin, and joints (Balogh et al., 2008) and that its bioavailability was estimated to be over 16 % (Oe et al., 2014). These discrepancies in the context of our new findings probably result from non-ideal labelling strategies on the part of techniques employing radioactive probes which could not distinguish between free label, labelled high- M_w HA, and its labelled metabolites. In contrast, our stable isotope labelling of HA combined with LC-MS or SEC analysis provides an excellent approach to elucidating HA's *in vivo* fate.

To test the hypothesis that absorbed HA can penetrate deeper into the organism – for example, into skin and joints, where it can act as an anti-inflammatory, lubricating, and humidifier agent – and since we did not observe any significant absorption with a dosage of 50 mg kg^{-1} , we applied high- M_w ^{13}C -HA and ^{13}C - $\Delta\text{AN}2$ to WT mice at the highest possible dose of 150 mg kg^{-1} . After the administration of ^{13}C - $\Delta\text{AN}2$, the ^{13}C - $\Delta\text{AN}2$ was clearly visible in skin and knee joints (Fig. 4D); in contrast, the administration of a high dose of high- M_w HA resulted in only a negligible increase above the baseline. Thus, if HA is administered at high doses and the gut microbiota can efficiently cleave HA, oral HA in the form of $\Delta\text{AN}2$, may reach the skin and joints, albeit in very limited amounts (< 5 ng g^{-1}). Thus, this commonly discussed mechanism of action is very unlikely, since HA absorption is very limited. This is further amplified by the fact that beneficial effects of oral HA are reported for much lower doses (< 3 mg kg day^{-1}) (Oe et al., 2016) than were used in our pharmacokinetic study.

3.5. Bacterial metabolization of HA

The action of gut microbiota has been shown to be crucial for the bioavailability of orally administered HA. The presence of $\Delta\text{AN}2$ in urine (Fig. 1C) after the oral administration of high- M_w HA indicates

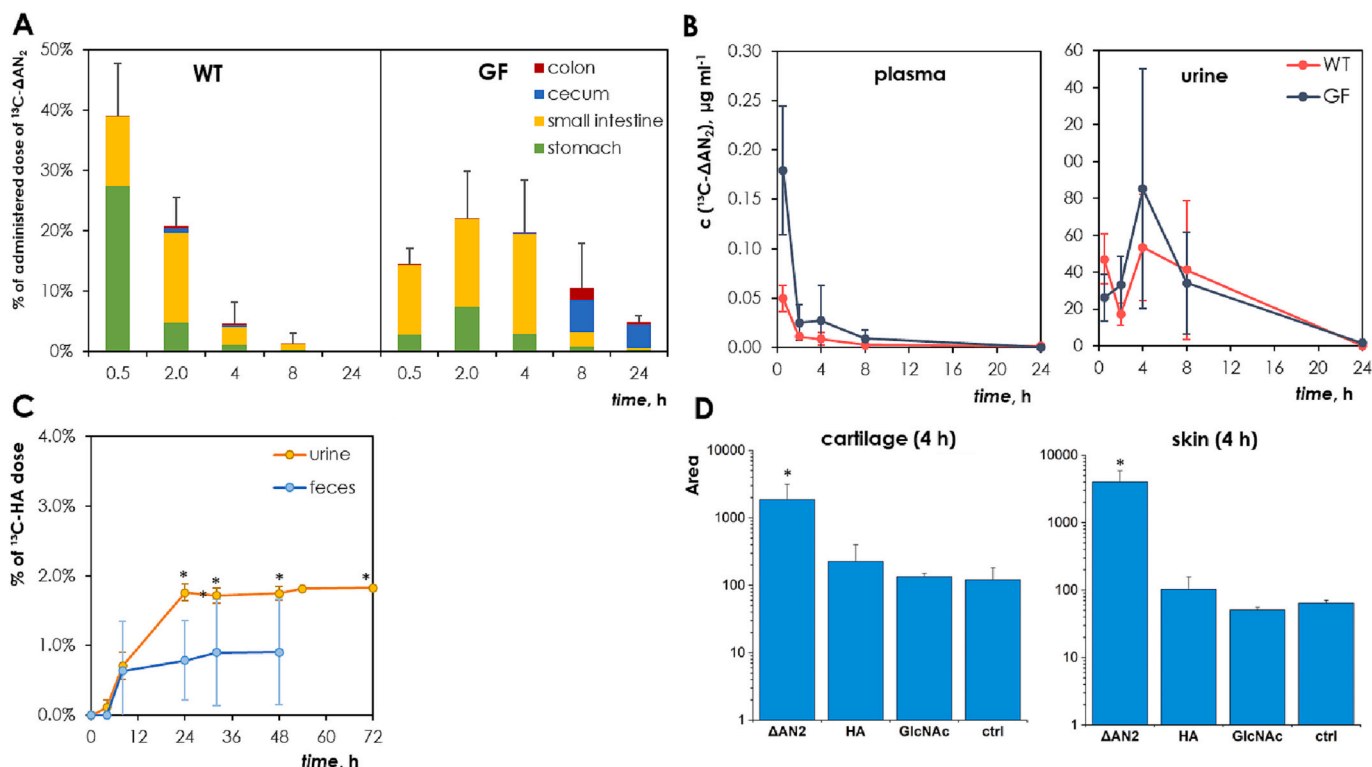


Fig. 4. Pharmacokinetics of orally administered unsaturated HA disaccharide (^{13}C - $\Delta\text{AN}2$). A) Amount of $\Delta\text{AN}2$ in various parts of GIT and at different time points after its oral administration to WT and GF mice (50 mg kg^{-1}), B) Absorption of orally administered $\Delta\text{AN}2$ to plasma and urine of WT and GF mice (50 mg kg^{-1}), C) Elimination of $\Delta\text{AN}2$ in urine and feces after its oral administration to WT mice (50 mg kg^{-1} , samples from metabolic cages; $n = 3$, compared to samples of feces, * $p < 0.05$), D) Distribution of $\Delta\text{AN}2$ into distal tissues 4 h after administration of a high dose of HA and $\Delta\text{AN}2$ (150 mg kg^{-1}). Samples labelled as GlcNAc and ctrl refer to mice which received *N*-acetylglucosamine or water, respectively ($n = 3$; compared to ctrl, * $p < 0.05$).

that $\Delta\text{AN}2$ is the product of the cleavage of extracellular or cell-surface bacterial lyases (Ndeh et al., 2020; Oiki et al., 2017). However, there are number of known enzymes which could also participate in the bacterial degradation of HA. To determine other metabolites and reveal possible metabolic changes in the intestine, the contents of the small intestine and cecum of mice colonized with the defined oMM¹² bacterial mixture

were cultivated with high- M_w ^{13}C -HA. The quantification of ^{13}C -HA by LC-MS revealed that the ^{13}C -HA was completely metabolized in cultivated cecal content within 120 min (Fig. 5A). In contrast, ^{13}C -HA in the content of the small intestine was not degraded even after 4 h (data not shown). The oMM¹² mixture contains five bacterial phyla that are naturally abundant in the murine GIT, such as *Actinobacteria*,

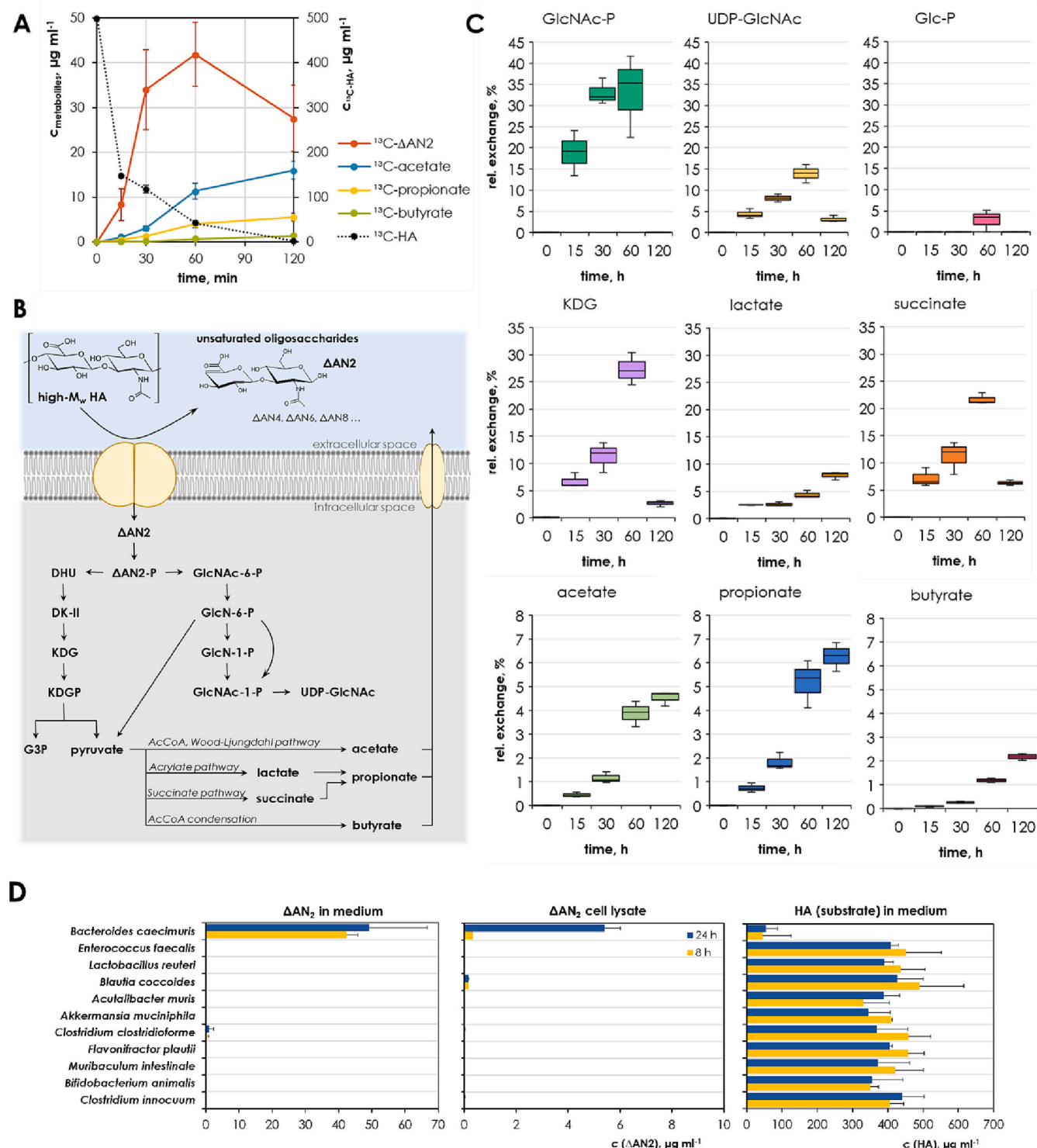


Fig. 5. *In vitro* degradation of ^{13}C -HA by oMM¹² microbiota. A) Kinetics of ^{13}C -HA degradation and the formation of its metabolites, B) Schematic diagram of bacterial degradation of HA, C) Relative exchanges of ^{13}C in selected metabolites, D) Degradation of HA by isolated bacterial strains of oMM¹² mixture into unsaturated HA disaccharide ($\Delta\text{AN}2$) (S.Met. 3). ($\Delta\text{AN}2\text{-P}$: 2-Acetamido-2-deoxy-6-phospho-3-O-(4-deoxyhex-4-enopyranuronosyl)hexopyranose, DHU: 4-deoxy-1-threo-5-hexosulose-uronic acid, DK-II: 3-deoxy-d-glycero-2,5-hexodiulosonic acid, KDG: 2-keto-3-deoxy-d-gluconic acid, KDGP: 2-keto-3-deoxy-6-phosphogluconic acid, G3P: glyceraldehyde-3-phosphate, UDP-GlcNAc: uridine diphosphate *N*-acetylglucosamine, GlcNAc: *N*-acetylglucosamine).

Bacteroidetes, *Firmicutes*, *Proteobacteria* and *Verrucomicrobia* (Eberl et al., 2020). *Bacteroides caecimuris* was the only HA cleaving bacteria from the synthetic microbial community of the oMM¹² mixture (Fig. 5D) and its abundance in the cecum of oMM¹² mice is significantly higher than in the small intestine (Yilmaz et al., 2021). This corresponds to our *in vivo* data for WT mice (Fig. 1D), suggesting that bacteria-degrading HA are present primarily in the mouse cecum. Generally, 20 % of the *Bacteroides* genome is dedicated to complex glycan metabolism. *Bacteroides* spp. are prominent degraders of glycosaminoglycans, polysaccharides and N-glycans; thus, their presence in the intestine is closely related to HA degradation (McKee et al., 2021).

Simultaneously with the decrease in ¹³C-HA concentration in the cecal content of oMM¹² mice, the production of ΔAN2 was observed (Fig. 5A, S.Fig. 7). Interestingly, no other unsaturated oligosaccharides were found, even though the ability of *Bacteroides* surface lyase to produce longer unsaturated oligosaccharides was described (Ndeh et al., 2020). Nevertheless, the ¹³C-label was further identified in several low-*M_w* metabolites (Fig. 5C). According to the proposed metabolic pathway for gram-positive and gram-negative bacteria (Oiki et al., 2017), ΔAN2 undergoes phosphorylation on GlcNAc and subsequent cleavage into unsaturated GlcA and *N*-acetylglucosamine-phosphate (GlcNAc-P). A high level of incorporation (>25 %) was identified in C₆H₁₀O₆, which corresponds to the metabolite of unsaturated GlcA. Similarly, the high incorporation of the ¹³C-label was observed in GlcNAc-P (>30 %) and uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) (>12 %). The high level of incorporation and the number of eight ¹³C atoms within GlcNAc-P and UDP-GlcNAc indicates that these molecules are formed by direct conversion from ΔAN2 (Fig. 5B), which is in accordance with the suggested metabolic pathway. The metabolism of HA into UDP-GlcNAc implies not only that the HA serves as a source of energy but also that its metabolites could serve as building blocks for the synthesis of complex glycans, which was also recently proven for human liver endothelial cells (Šimek et al., 2021).

The significant incorporation of ¹³C was also observed within the structures of short chain fatty acids (SCFA) and hydroxy acids (lactate, succinate) (Fig. 5C, S.Fig. 8). SCFA are end products of anaerobic bacterial fermentation and can be formed via different metabolic pathways according to the bacterial phyla. The level of ¹³C-incorporation within the structure of succinate (~20 %) is significantly higher than that within lactate (~7 %); simultaneously, a significant proportion of ¹³C-succinate and ¹³C-propionate molecules contains three ¹³C atoms (S. Fig. 8). These data mean that the majority of propionate is formed via the succinate pathway characteristic for *Bacteroides* spp. (Reichardt et al., 2014). A half of ¹³C-butyrate molecules have 2–4 atoms of ¹³C, which corresponds to its formation by the butyryl-CoA pathway; the other half contain only 1 atom of ¹³C, which corresponds to its formation via the fixation of ¹³C-carbon dioxide (S.Fig. 8). These data signify that HA cannot only be cleaved but also utilized by *Bacteroides* spp. and completely metabolized into SCFA and CO₂.

These findings suggest that bacterial metabolites arising from administered HA can be absorbed into the body and have beneficial effects for the host. For example, SCFA can strengthen the function of the gut barrier and have important immunomodulatory functions (Parada Venegas et al., 2019), while UDP-GlcNAc is a substrate in multiple protein glycosylation pathways, thereby widely impacting the proteome (Biwi et al., 2018). To investigate the bioavailability of the identified HA metabolites, metabolomic analyses of plasma and liver of WT mice after *p.o.* administration of high-*M_w* ¹³C-HA and ¹³C-ΔAN2 (150 mg kg⁻¹) were performed. LC-MS analysis revealed that the ¹³C-label was not incorporated into these metabolites (e.g. GlcNAc, UDP-GlcNAc) or these metabolites (e.g. KDG, DHU) were not found. These data indicate that intermediate metabolites of the intracellular metabolism of the bacterial degradation of HA are not available to the host. However, this may not be the case with SCFA, end products of the bacterial metabolism of HA, which are effectively absorbed in the colon (Parada Venegas et al., 2019). Significant metabolism of ¹³C-HA into ¹³C-SCFA was observed

in the cecum and colon (S.Fig. 9), which means that HA supplementation may lead to a significant increase in SCFA contributing to intestinal homeostasis (Parada Venegas et al., 2019).

3.6. Modulation of the cecal microbial community by the long-term administration of HA

The presence of gut microbiota, especially in the cecum, seems to be crucial for the metabolism and absorption of orally administered HA. However, the interactions between HA and gut microbiota are not fully understood and have not been precisely described. Several *in vitro* and *in vivo* studies were conducted to study the modulation of microbial communities by the administration of HA (Bellar et al., 2019; Chaaban et al., 2021; Mao et al., 2021; Pan et al., 2021), but their conclusions are not consistent.

To determine whether a 4-week supplementation with high-*M_w* HA affects the cecal microbiota composition of healthy WT mice, DNA isolated from cecal contents was amplified in the V3-V4 region of the 16S-rRNA gene, followed by sequencing of the amplified region (S.Met. 4). Together with HA, the effects of GlcNAc and pectin supplementation were studied. The obtained genomics data contained 3,180,588 raw reads; after the removal of unqualified sequences, a total of 2,140,547 raw reads with an average length of 408 bases remained. Principal co-ordinates analysis showed that the cecal microbiota of HA-treated mice were not altered compared to the control group (S.Fig. 10). Mothur method *get.communitytype* predicted only one metacommunity cluster. No statistical significance in the alpha diversity between groups was observed. Analysis of molecular variance analysis revealed only a slight shift in gut microbiota composition for GlcNAc treatment. Homogeneity of molecular variance analysis did not confirm any significant difference across the tested groups. These results are consistent with literature. The oral administration (140 mg day⁻¹) of 35 kDa HA to healthy human subjects for 4 weeks was found to have no significant effect on microbiome alpha- and beta diversity, and no significantly different operational taxonomic units were revealed throughout the time course (day-0, day-8, and day-28) (Bellar et al., 2019). Similar results were observed by (Mao et al., 2021) with healthy mice and mice with DSS-induced colitis. Once-a-day supplementation with 35 kDa HA did not lead to any significant changes in gut microbiota populations; however, a significant increase in gut microbial diversity was observed.

Overall, the relative abundances of bacterial genera revealed high between- and within-group variation (Fig. 6A) and correspond to high within-group variability in the bacterial degradation products of HA (Fig. 1E). Despite exhibiting high within-group variability, the genera with confirmed HA degradation activity – namely, *Bacteroides* and *Enterococcus* (Kawai et al., 2018) – showed a marked increase in abundance for HA treated groups (Fig. 6B). However, the difference was not statistically significant due to the large sample diversity. Although not visible in taxonomic classification, an increase in some OTUs of the less frequent genera *Anaeroplasm*, *Clostridium_XIVb*, and *Parabacteroides* was observed despite there being no information about their ability to cleave HA found in databases. Similarly, the abundance of *Akkermansia muciniphila* increased after the oral application of HA (Lee et al., 2020; Mao et al., 2021), despite the fact that its ability to degrade HA was not observed in our study (Fig. 5D).

4. Conclusion

In this study, we revealed in detail the pharmacokinetic behavior of orally-administered high-*M_w* HA in conventional WT and GF mice in order to provide a new perspective on the *in vivo* fate of orally administered HA. As we hypothesized, high-*M_w* HA is depolymerized during the pass of GIT. However, the main finding is that the depolymerization of orally-administered HA by gut microorganisms is essential for ensuring its bioavailability and is fully dependent on gut microbiota, since in GF animals high-*M_w* HA is not absorbed at all. Our results clearly

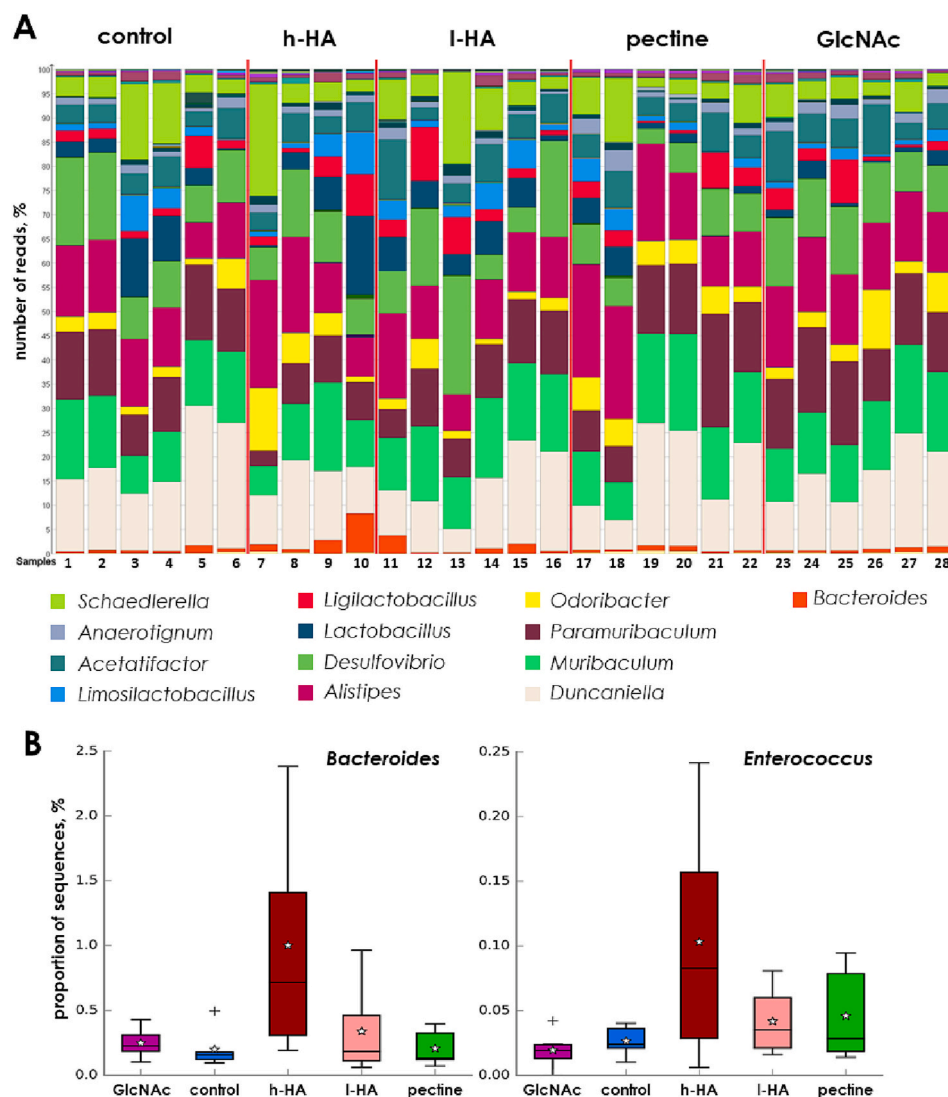


Fig. 6. Modulation of cecal microbiota by long-term HA-supplementation. A) Structural comparison of cecal microbiota at the genus level, B) Relative abundance of *Bacteroides* and *Enterococcus* in mice cecum after four-week supplementation with high doses of high- M_w HA (h-HA, 1600 kDa, 760 mg kg⁻¹ day⁻¹), low doses of high- M_w HA (l-HA, 1600 kDa, 90 mg kg⁻¹ day⁻¹), pectin (640 mg kg⁻¹ day⁻¹) and GlcNAc (650 mg kg⁻¹ day⁻¹).

show that the gut microbiota of conventional WT mice metabolize >97 % of administered high- M_w HA, and around 0.2 % is absorbed into the body of WT mice. Through the action of gut microbiota, M_w of high- M_w HA decreased into HA fragments of several kDa, and unsaturated oligosaccharides (mostly disaccharide) were formed. In contrast, only a slight decrease in the M_w of HA (1600 kDa → ~150–600 kDa) caused by acid hydrolysis in the stomach was observed for GF mice; nor were any unsaturated oligosaccharides typical for the activity of microbial HA lyases observed in the same mice. We identified ΔAN2 (0.38 kDa) as the dominant form of HA absorbed into the body, although the M_w cut-off for absorption of HA from the GIT was around 3 kDa. The degradation of HA by the well-defined gut microbiota mixture oMM¹² was tested *in vitro* and several biologically active metabolites including SCFA and UDP-GlcNAc were identified. However, metabolomic analyses showed that only unsaturated oligosaccharides and SCFA were available to the host *in vivo*. The *Bacteroides* genus metabolized high- M_w HA to ΔAN2 and SCFA *in vitro*. *In vivo*, long-term HA supplementation revealed an increase of *Bacteroides* abundance in the cecal content of conventional WT mice. These data show that *Bacteroides* could be an important player in the bioavailability of orally-administered high- M_w HA. Moreover, the limited absorption of orally-administered HA and its rapid metabolism by gut microbiota indicate that the *in vivo* fate of HA is not

related to the M_w of the administered HA (15–1600 kDa) and that the orally-administered HA do not serve as a nutrition for joint and skin but its mechanism of action is probably the result of the systematic regulatory function of HA or its metabolites. However, to fully understand the mechanism of action of orally-applied HA directed at diseases of the extracellular matrix in distal tissues (skin, joints), further investigation of the interactions of oral HA with HA receptors and of the biological properties of unsaturated oligosaccharides is needed.

CRediT authorship contribution statement

Matěj Šimek: Conceptualization, Supervision, Visualization, Formal analysis, Data curation, Writing – original draft, Project administration. **Kristýna Turková:** Investigation, Data curation, Writing – original draft, Writing – review & editing. **Martin Schwarzer:** Visualization, Investigation, Conceptualization, Writing – review & editing. **Kristina Nešporová:** Conceptualization, Supervision. **Lukáš Kubala:** Funding acquisition, Supervision, Methodology, Resources, Writing – original draft, Project administration. **Martina Hermannová:** Supervision, Project administration. **Tereza Foglová:** Investigation. **Barbora Šafránková:** Investigation, Data curation. **Martin Šindelář:** Methodology, Data curation, Visualization. **Dagmar Šrůtková:** Investigation.

Sofia Chatzigeorgiou: Investigation. **Tereza Novotná:** Investigation. **Tomáš Hudcovic:** Methodology, Investigation. **Vladimír Velebný:** Funding acquisition, Supervision, Resources.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carbpol.2023.120880>.

References

- Asari, A., Kanemitsu, T., & Kurihara, H. (2010). Oral administration of high molecular weight hyaluronan (900 kDa) controls immune system via toll-like receptor 4 in the intestinal epithelium. *Journal of Biological Chemistry*, 285(32), 24751–24758. <https://doi.org/10.1074/jbc.M110.104950>
- Balogh, L., Polyak, A., Mathe, D., Kiraly, R., Thuroczy, J., Terez, M., Janoki, G., Ting, Y., Bucci, L. R., & Schauss, A. G. (2008). Absorption, uptake and tissue affinity of high-molecular-weight hyaluronan after oral administration in rats and dogs. *Journal of Agricultural and Food Chemistry*, 56(22), 10582–10593. <https://doi.org/10.1021/jf8017029>
- Bellar, A., Kessler, S. P., Obery, D. R., Sangwan, N., Welch, N., Nagy, L. E., Dasarthy, S., & de la Motte, C. (2019). Safety of hyaluronan 35 in healthy human subjects: A pilot study. *Nutrients*, 11(5), 5. <https://doi.org/10.3390/nu11051135>
- Biwi, J., Biot, C., Guerardel, Y., Vercoutter-Edouart, A.-S., & Lefebvre, T. (2018). The many ways by which O-GlcNAcylation may orchestrate the diversity of complex glycosylations. *Molecules*, 23(11), 11. <https://doi.org/10.3390/molecules23112858>
- Blundell, C. D., & Almond, A. (2006). Enzymatic and chemical methods for the generation of pure hyaluronan oligosaccharides with both odd and even numbers of monosaccharide units. *Analytical Biochemistry*, 353(2), 236–247. <https://doi.org/10.1016/j.ab.2006.03.013>
- Bouga, H., Tsouros, I., Bounias, D., Kyriakopoulou, D., Stavropoulos, M. S., Papageorgakopoulou, N., Theodoris, D. A., & Vynios, D. H. (2010). Involvement of hyaluronidases in colorectal cancer. *BMC Cancer*, 10(1), 499. <https://doi.org/10.1186/1471-2407-10-499>
- Chaaban, H., Burge, K., Eckert, J., Trammell, M., Dyer, D., Keshari, R. S., Silasi, R., Regmi, G., Lupu, C., Good, M., McElroy, S. J., & Lupu, F. (2021). Acceleration of small intestine development and remodeling of the microbiome following hyaluronan 35 kDa treatment in neonatal mice. *Nutrients*, 13(6), 6. <https://doi.org/10.3390/nu13062030>
- Chowdhury, B., Hemming, R., Faiyaz, S., & Triggs-Raine, B. (2016). Hyaluronidase 2 (HYAL2) is expressed in endothelial cells, as well as some specialized epithelial cells, and is required for normal hyaluronan catabolism. *Histochemistry and Cell Biology*, 145(1), 53–66.
- Cowman, M. K., Lee, H.-G., Schwertfeger, K. L., McCarthy, J. B., & Turley, E. A. (2015). The content and size of hyaluronan in biological fluids and tissues. *Frontiers in Immunology*, 6. <https://doi.org/10.3389/fimmu.2015.00261>
- Čožiková, D., Šilová, T., Moravcová, V., Šmejkalová, D., Pepeliaev, S., Velebný, V., et al. (2017). Preparation and extensive characterization of hyaluronan with narrow molecular weight distribution. *Carbohydrate Polymers*, 160, 134–142. <https://doi.org/10.1016/j.carbpol.2016.12.045>
- de Souza, A. B., Chaud, M. V., & Santana, M. H. A. (2019). Hyaluronic acid behavior in oral administration and perspectives for nanotechnology-based formulations: A review. *Carbohydrate Polymers*, 222, Article 115001. <https://doi.org/10.1016/j.carbpol.2019.115001>
- Eberl, C., Ring, D., Münch, P. C., Beutler, M., Basic, M., Slack, E. C., Schwarzer, M., Srukova, D., Lange, A., Frick, J. S., Bleich, A., & Stecher, B. (2020). Reproducible colonization of germ-free mice with the oligo-mouse-microbiota in different animal facilities. *Frontiers in Microbiology*, 10. <https://doi.org/10.3389/fmicb.2019.02999>
- Gracz, A. D., Fuller, M. K., Wang, F., Li, L., Stelzner, M., Dunn, J. C. Y., Martin, M. G., & Magness, S. T. (2013). Brief report: CD24 and CD44 mark human intestinal epithelial cell populations with characteristics of active and facultative stem cells. *Stem Cells*, 31(9), 2024–2030. <https://doi.org/10.1002/stem.1391>
- Gupta, R. C., Lall, R., Srivastava, A., & Sinha, A. (2019). Hyaluronic acid: Molecular mechanisms and therapeutic trajectory. *Frontiers in Veterinary Science*, 6, 192. <https://doi.org/10.3389/fvets.2019.00192>
- Han, W., Song, L., Wang, Y., Lv, Y., Chen, X., & Zhao, X. (2019). Preparation, characterization, and inhibition of hyaluronan acid oligosaccharides in triple-negative breast cancer. *Biomolecules*, 9(9), 9. <https://doi.org/10.3390/biom9090436>
- Hisada, N., Satsu, H., Mori, A., Totsuka, M., Kamei, J., Nozawa, T., & Shimizu, M. (2008). Low-molecular-weight hyaluronan permeates through human intestinal Caco-2 cell monolayers via the paracellular pathway. *Bioscience, Biotechnology, and Biochemistry*, 72(4), 1111–1114.
- Johansson, M. E. V., Jakobsson, H. E., Holmén-Larsson, J., Schütte, A., Ermund, A., Rodríguez-Piñeiro, A. M., Arike, L., Wising, C., Svensson, F., Bäckhed, F., & Hansson, G. C. (2015). Normalization of host intestinal mucus layers requires long-term microbial colonization. *Cell Host & Microbe*, 18(5), 582–592. <https://doi.org/10.1016/j.chom.2015.10.007>
- Kawada, C., Yoshida, T., Yoshida, H., Matsuoka, R., Sakamoto, W., Odanaka, W., Sato, T., Yamasaki, T., Kanemitsu, T., Masuda, Y., & Urushibata, O. (2014). Ingested hyaluronan moisturizes dry skin. *Nutrition Journal*, 13(1), 70. <https://doi.org/10.1186/1475-2891-13-70>
- Kawai, K., Kamochi, R., Oiki, S., Murata, K., & Hashimoto, W. (2018). Probiotics in human gut microbiota can degrade host glycosaminoglycans. *Scientific Reports*, 8(1), 10674. <https://doi.org/10.1038/s41598-018-28886-w>
- Kessler, S. P., Obery, D. R., Nickerson, K. P., Petrey, A. C., McDonald, C., & de la Motte, C. A. (2018). Multifunctional role of 35 kilodalton hyaluronan in promoting defense of the intestinal epithelium. *Journal of Histochemistry & Cytochemistry*. <https://doi.org/10.1369/0022155417746775>
- Kim, Y., & de la Motte, C. A. (2020). The role of hyaluronan treatment in intestinal innate host defense. *Frontiers in Immunology*, 11, 569. <https://doi.org/10.3389/fimmu.2020.00569>
- Kim, Y., West, G. A., Ray, G., Kessler, S. P., Petrey, A. C., Fiocchi, C., McDonald, C., Longworth, M. S., Nagy, L. E., & de la Motte, C. A. (2018). Layilin is critical for mediating hyaluronan 35kDa-induced intestinal epithelial tight junction protein ZO-1 in vitro and in vivo. *Matrix Biology*, 66, 93–109. <https://doi.org/10.1016/j.matbio.2017.09.003>
- Kobayashi, T., Chanmee, T., & Itano, N. (2020). Hyaluronan: Metabolism and function. *Biomolecules*, 10(11), 11. <https://doi.org/10.3390/biom10111525>
- Kolar, S. L., Kyme, P., Tseng, C. W., Soliman, A., Kaplan, A., Liang, J., Nizet, V., Jiang, D., Murali, R., Arditi, M., Underhill, D. M., & Liu, G. Y. (2015). Group B streptococcus evades host immunity by degrading hyaluronan. *Cell Host & Microbe*, 18(6), 694–704. <https://doi.org/10.1016/j.chom.2015.11.001>
- Körver-Keularts, I. M. L. W., Wang, P., Waterval, H. W. A. H., Kluijtmans, L. A. J., Wevers, R. A., Langhans, C.-D., Scott, C., Habets, D. D. J., & Bierau, J. (2018). Fast and accurate quantitative organic acid analysis with LC-QTOF/MS facilitates screening of patients for inborn errors of metabolism. *Journal of Inherited Metabolic Disease*, 41(3), 415–424. <https://doi.org/10.1007/s10545-017-0129-0>
- Laznicka, M., Laznickova, A., Cozikova, D., & Velebný, V. (2012). Preclinical pharmacokinetics of radiolabeled hyaluronan. *Pharmacological Reports*, 64(2), 428–437. [https://doi.org/10.1016/S1734-1140\(12\)70784-3](https://doi.org/10.1016/S1734-1140(12)70784-3)
- Lee, Y., Sugihara, K., Gilliland, M. G., Jon, S., Kamada, N., & Moon, J. J. (2020). Hyaluronic acid–bilirubin nanomedicine for targeted modulation of dysregulated intestinal barrier, microbiome and immune responses in colitis. *Nature Materials*, 19(1), 118–126. <https://doi.org/10.1038/s41563-019-0462-9>
- Luczynski, P., McVey Neufeld, K.-A., Oriach, C. S., Clarke, G., Dinan, T. G., & Cryan, J. F. (2016). Growing up in a bubble: Using germ-free animals to assess the influence of the gut microbiota on brain and behavior. *The International Journal of Neuropsychopharmacology*, 19(8), pyw020. <https://doi.org/10.1093/ijnp/pyw020>
- Ma, W., Wang, J., Guo, Q., & Tu, P. (2015). Quantitative subcellular study of doxorubicin in MCF-7/Adr cells using liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B*, 1007, 18–22. <https://doi.org/10.1016/j.jchromb.2015.11.002>
- Mao, T., Su, C.-W., Ji, Q., Chen, C.-Y., Wang, R., Vijaya Kumar, D., Lan, J., Jiao, L., & Shi, H. N. (2021). Hyaluronan-induced alterations of the gut microbiome protects mice against Citrobacter rodentium infection and intestinal inflammation. *Gut Microbes*, 13(1), 1972757. <https://doi.org/10.1080/19490976.2021.1972757>
- McConnell, E. L., Basit, A. W., & Murdan, S. (2008). Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *Journal of Pharmacy and Pharmacology*, 60(1), 63–70. <https://doi.org/10.1211/jpp.60.1.0008>
- McKee, L. S., La Rosa, S. L., Westereng, B., Eijlsink, V. G., Pope, P. B., & Larsbrink, J. (2021). Polysaccharide degradation by the Bacteroidetes: Mechanisms and nomenclature. *Environmental Microbiology Reports*, 13(5), 559–581. <https://doi.org/10.1111/1758-2229.12980>
- Ndeh, D., Baslé, A., Strahl, H., Yates, E. A., McClurg, U. L., Henrissat, B., Terrapon, N., & Cartmell, A. (2020). Metabolism of multiple glycosaminoglycans by Bacteroides thetaiotaomicron is orchestrated by a versatile core genetic locus. *Nature Communications*, 11(1), 1. <https://doi.org/10.1038/s41467-020-14509-4>
- Oe, M., Mitsugi, K., Odanaka, W., Yoshida, H., Matsuoka, R., Seino, S., Kanemitsu, T., & Masuda, Y. (2014). Dietary hyaluronic acid migrates into the skin of rats. *The Scientific World Journal*, 2014, Article 378024. <https://doi.org/10.1155/2014/378024>
- Oe, M., Tashiro, T., Yoshida, H., Nishiyama, H., Masuda, Y., Maruyama, K., Koikeda, T., Maruya, R., & Fukui, N. (2016). Oral hyaluronan relieves knee pain: A review. *Nutrition Journal*, 15, 11. <https://doi.org/10.1186/s12937-016-0128-2>

- Oiki, S., Mikami, B., Maruyama, Y., Murata, K., & Hashimoto, W. (2017). A bacterial ABC transporter enables import of mammalian host glycosaminoglycans. *Scientific Reports*, 7(1), 1069. <https://doi.org/10.1038/s41598-017-00917-y>
- Pan, L., Ai, X., Fu, T., Ren, L., Shang, Q., Li, G., & Yu, G. (2021). In vitro fermentation of hyaluronan by human gut microbiota: Changes in microbiota community and potential degradation mechanism. *Carbohydrate Polymers*, 269, Article 118313. <https://doi.org/10.1016/j.carbpol.2021.118313>
- Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., Harmsen, H. J. M., Faber, K. N., & Hermoso, M. A. (2019). Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in Immunology*, 10. <https://doi.org/10.3389/fimmu.2019.00277>
- Reichardt, N., Duncan, S. H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K. P., Flint, H. J., & Louis, P. (2014). Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *The ISME Journal*, 8(6), 1323–1335. <https://doi.org/10.1038/ismej.2014.14>
- Rodrigues, M. T., Carvalho, P. P., Gomes, M. E., & Reis, R. L. (2015). Chapter 11—Biomaterials in preclinical approaches for engineering skeletal tissues. In A. Atala, & J. G. Allickson (Eds.), *Translational Regenerative Medicine* (pp. 127–139). Academic Press. <https://doi.org/10.1016/B978-0-12-410396-2.00011-6>
- Sato, Y., Joumura, T., Takekuma, Y., & Sugawara, M. (2020). Transfer of orally administered hyaluronan to the lymph. *European Journal of Pharmaceutics and Biopharmaceutics*, 154, 210–213. <https://doi.org/10.1016/j.ejpb.2020.07.007>
- Shi, X., Wang, S., Jasbi, P., Turner, C., Hrovat, J., Wei, Y., Liu, J., & Gu, H. (2019). Database-assisted globally optimized targeted mass spectrometry (dGOT-MS): Broad and reliable metabolomics analysis with enhanced identification. *Analytical Chemistry*, 91(21), 13737–13745. <https://doi.org/10.1021/acs.analchem.9b03107>
- Shimizu, K., Seiki, I., Goto, Y., & Murata, T. (2021). Measurement of the intestinal pH in mice under various conditions reveals alkalization induced by antibiotics. *Antibiotics*, 10(2), 180.
- Šimek, M., Hermannová, M., Šmejkalová, D., Foglová, T., Souček, K., Binó, L., & Velebný, V. (2019). LC-MS/MS study of in vivo fate of hyaluronan polymeric micelles carrying doxorubicin. *Carbohydrate Polymers*, 181–189. <https://doi.org/10.1016/j.carbpol.2018.12.104>
- Šimek, M., Nešporová, K., Kocurková, A., Foglová, T., Ambrožová, G., Velebný, V., Kubala, L., & Hermannová, M. (2021). How the molecular weight affects the in vivo fate of exogenous hyaluronan delivered intravenously: A stable-isotope labelling strategy. *Carbohydrate Polymers*, 263, Article 117927. <https://doi.org/10.1016/j.carbpol.2021.117927>
- Sindelar, M., Jilkova, J., Kubala, L., Velebný, V., & Turkova, K. (2021). Hyaluronidases and hyaluronate lyases: From humans to bacteriophages. *Colloids and Surfaces B: Biointerfaces*, 208, Article 112095. <https://doi.org/10.1016/j.colsurfb.2021.112095>
- Soroosh, A., Albeiroti, S., West, G. A., Willard, B., Fiocchi, C., & de la Motte, C. A. (2016). Crohn's disease fibroblasts overproduce the novel protein KIAA1199 to create proinflammatory hyaluronan fragments. *Cellular and Molecular Gastroenterology and Hepatology*, 2(3), 358–368.e4. <https://doi.org/10.1016/j.jcmgh.2015.12.007>
- Štěpánková, R., Taskalová-hogenová, H., Šinkora, J., Jodl, J., & Frič, P. (1996). Changes in jejunal mucosa after long-term feeding of germfree rats with gluten. *Scandinavian Journal of Gastroenterology*, 31(6), 551–557. <https://doi.org/10.3109/00365529609009127>
- Stern, R., Kogan, G., Jedrzejewski, M. J., & Šoltés, L. (2007). The many ways to cleave hyaluronan. *Biotechnology Advances*, 25(6), 537–557. <https://doi.org/10.1016/j.biotechadv.2007.07.001>
- Svanovsky, E., Velebný, V., Laznickova, A., & Laznicek, M. (2008). The effect of molecular weight on the biodistribution of hyaluronic acid radiolabeled with¹¹¹In after intravenous administration to rats. *European Journal of Drug Metabolism and Pharmacokinetics*, 33(3), 149–157. <https://doi.org/10.1007/BF03191112>
- Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szegedy, C. A.-K., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Pontén, F., ... (2015). Tissue-based map of the human proteome. *Science*, 347(6220), 1260419. <https://doi.org/10.1126/science.1260419>
- Volpi, N. (2002). Oral bioavailability of chondroitin sulfate (Condrosulf®) and its constituents in healthy male volunteers. *Osteoarthritis and Cartilage*, 10(10), 768–777. <https://doi.org/10.1053/joca.2002.0824>
- Volpi, N. (2003). Oral absorption and bioavailability of ichthyic origin chondroitin sulfate in healthy male volunteers. *Osteoarthritis and Cartilage*, 11(6), 433–441. [https://doi.org/10.1016/S1063-4584\(03\)00051-7](https://doi.org/10.1016/S1063-4584(03)00051-7)
- Wu, W. M., Yang, Y. S., & Peng, L. H. (2014). Microbiota in the stomach: New insights. *Journal of Digestive Diseases*, 15(2), 54–61. <https://doi.org/10.1111/1751-2980.12116>
- Yilmaz, B., Mooser, C., Keller, I., Li, H., Zimmermann, J., Bosshard, L., Fuhrer, T., Gomez de Agüero, M., Trigo, N. F., Tschanz-Lischer, H., Limenitakis, J. P., Hardt, W.-D., McCoy, K. D., Stecher, B., Excoffier, L., Sauer, U., Ganai-Vonarburg, S. C., & Macpherson, A. J. (2021). Long-term evolution and short-term adaptation of microbiota strains and sub-strains in mice. *Cell Host & Microbe*, 29(4), 650–663.e9. <https://doi.org/10.1016/j.chom.2021.02.001>

MOLECULAR WEIGHT AND GUT MICROBIOTA DETERMINE THE BIOAVAILABILITY OF ORALLY ADMINISTERED HYALURONIC ACID

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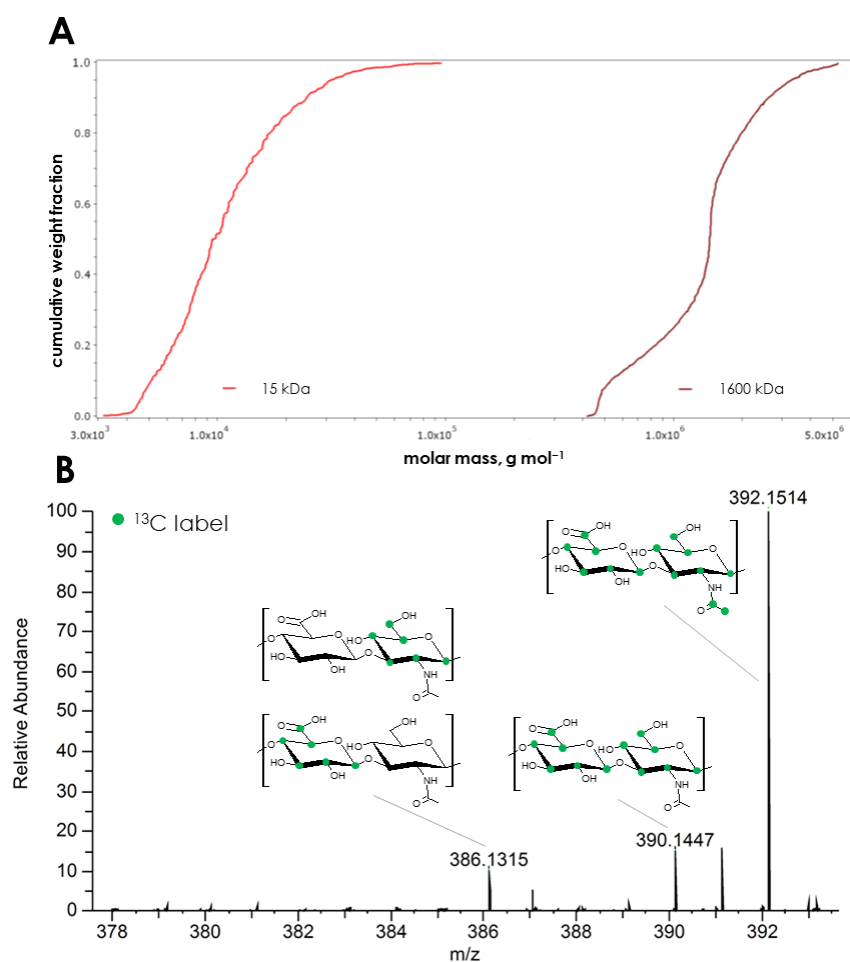
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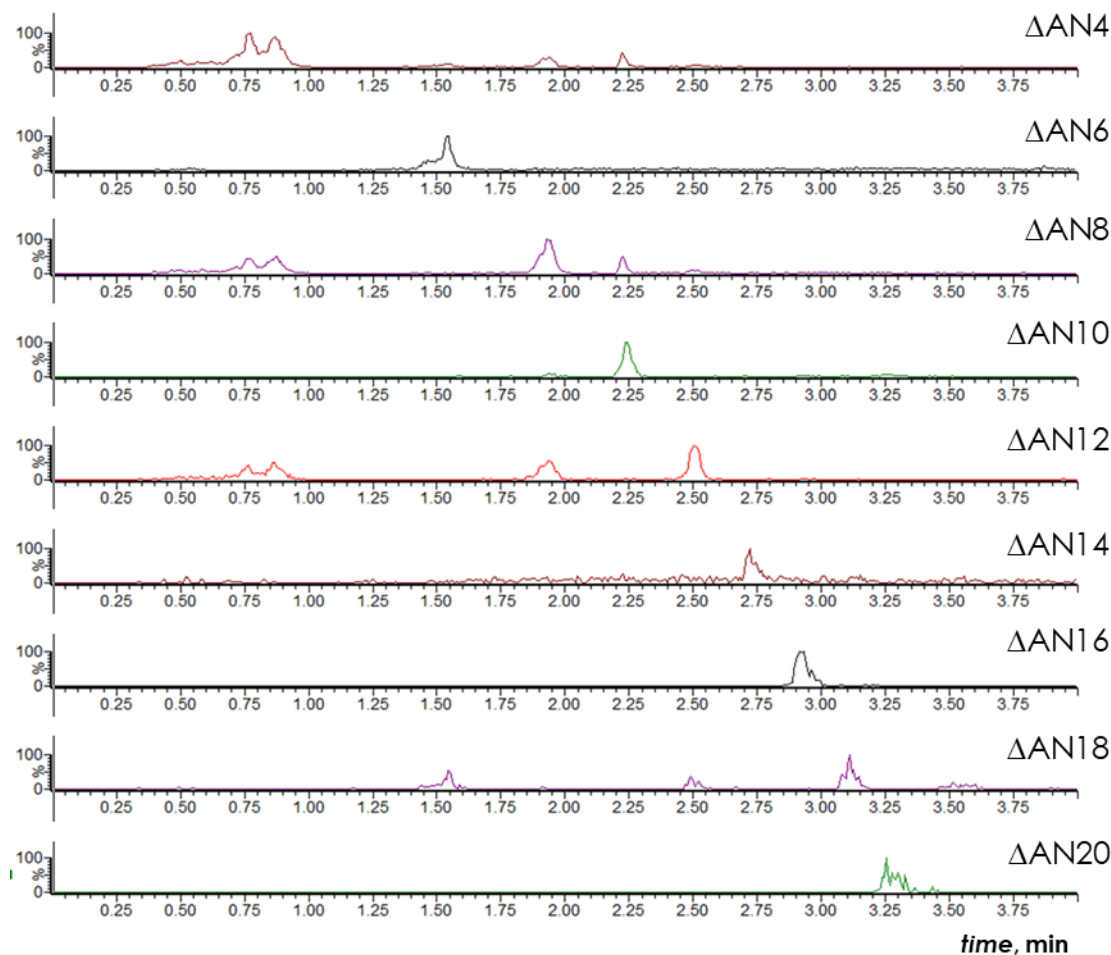
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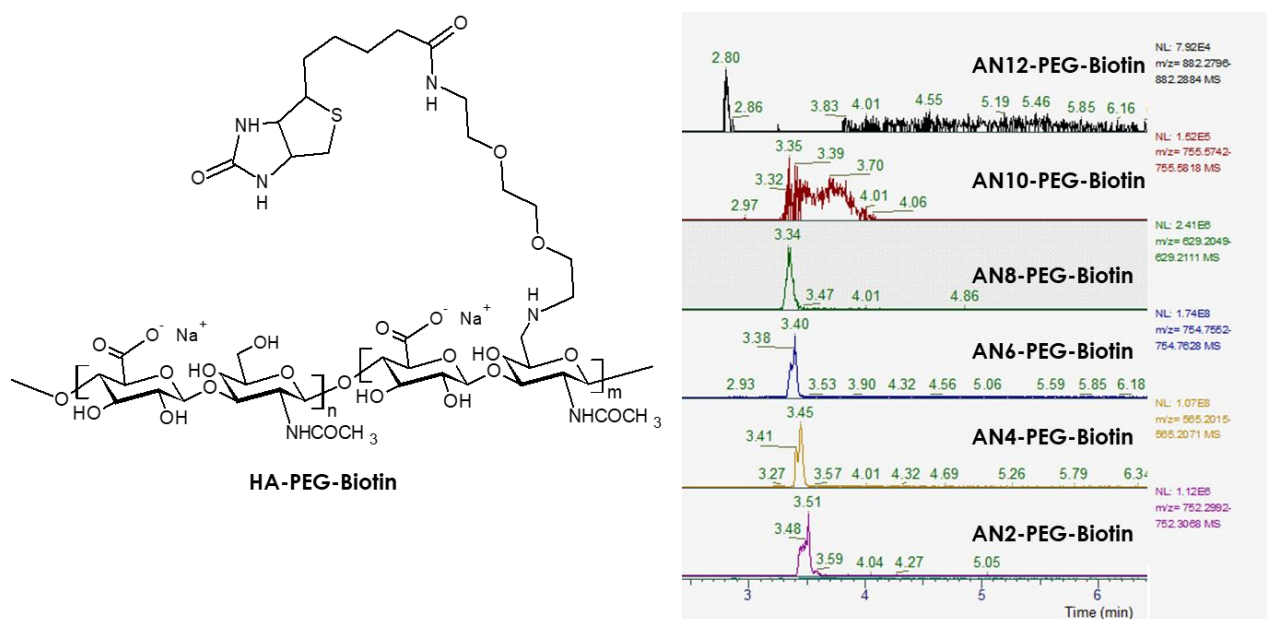
1. SUPPLEMENTARY FIGURES



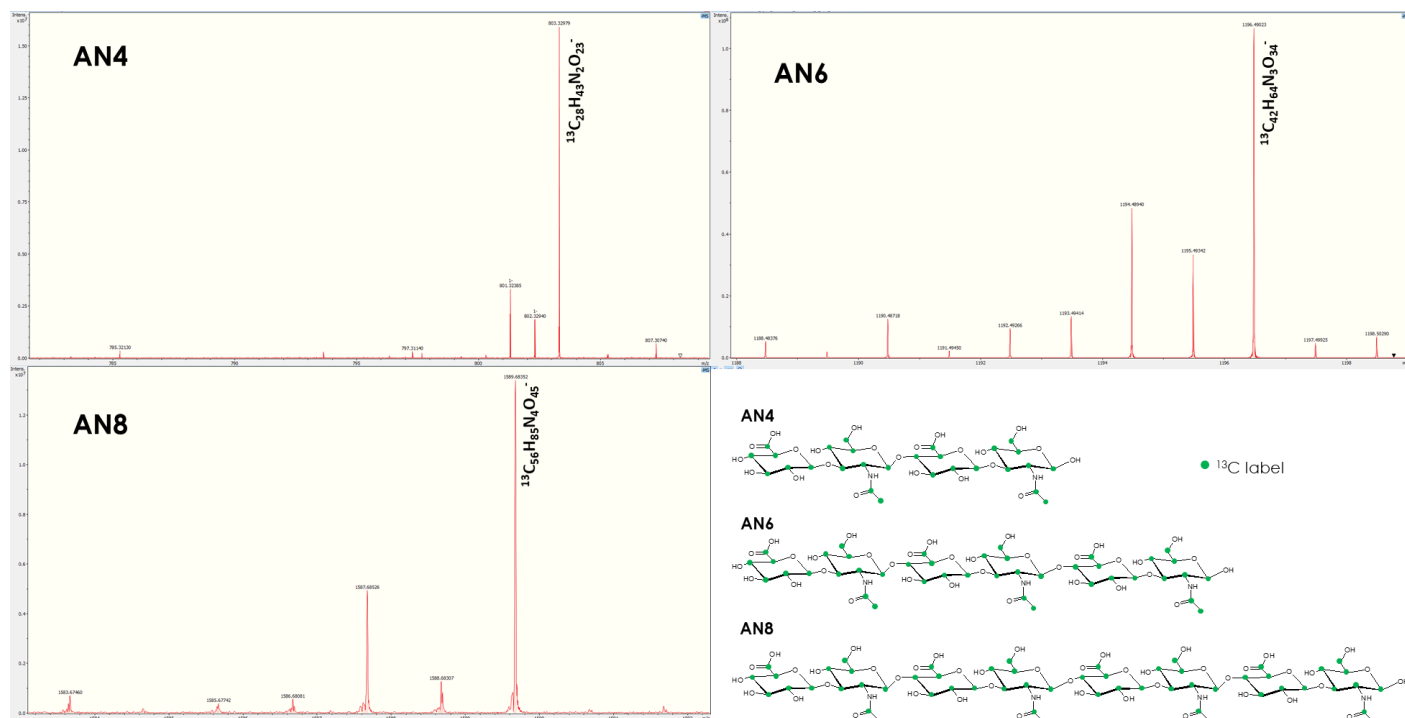
S.Fig. 1 Characterization of ¹³C-labelled hyaluronic acid. A) Molecular weight distributions of the used ¹³C-labelled hyaluronic acid, B) Mass spectrum of unsaturated disaccharide derived from ¹³C-hyaluronic acid after total enzymatic degradation by hyaluronan lyase from *Streptococcus pneumoniae*. The abundances of differently labelled disaccharides represents the distributions of differently labelled monomers, whose structures are displayed. The dominant form of ¹³C-labelled hyaluronic acid was the molecule with all carbon positions substituted with the heavy ¹³C isotope.



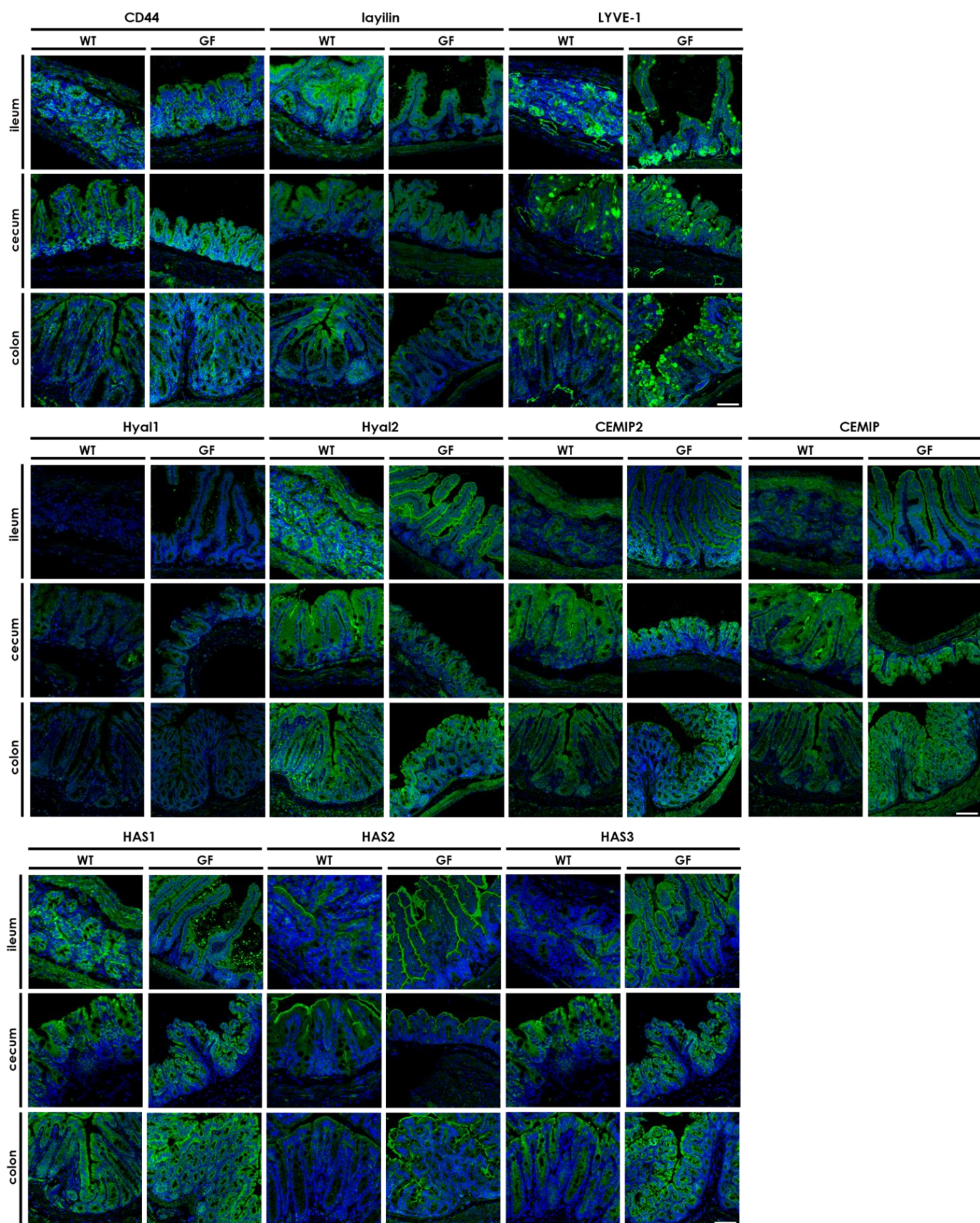
S.Fig. 2 Representative LC-MS chromatogram for unsaturated oligosaccharides (*DP* 4-20) in colon content 4 hours after oral administration of high- M_w ^{13}C -HA. Some chromatograms contain multiple signals, because of the same values of m/z for some multiply charged oligosaccharides e. g. $[\Delta\text{AN}8\text{-}2\text{H}]^{2-}$ and $[\Delta\text{AN}12\text{-}3\text{H}]^{3-}$ see S.Tab.1.



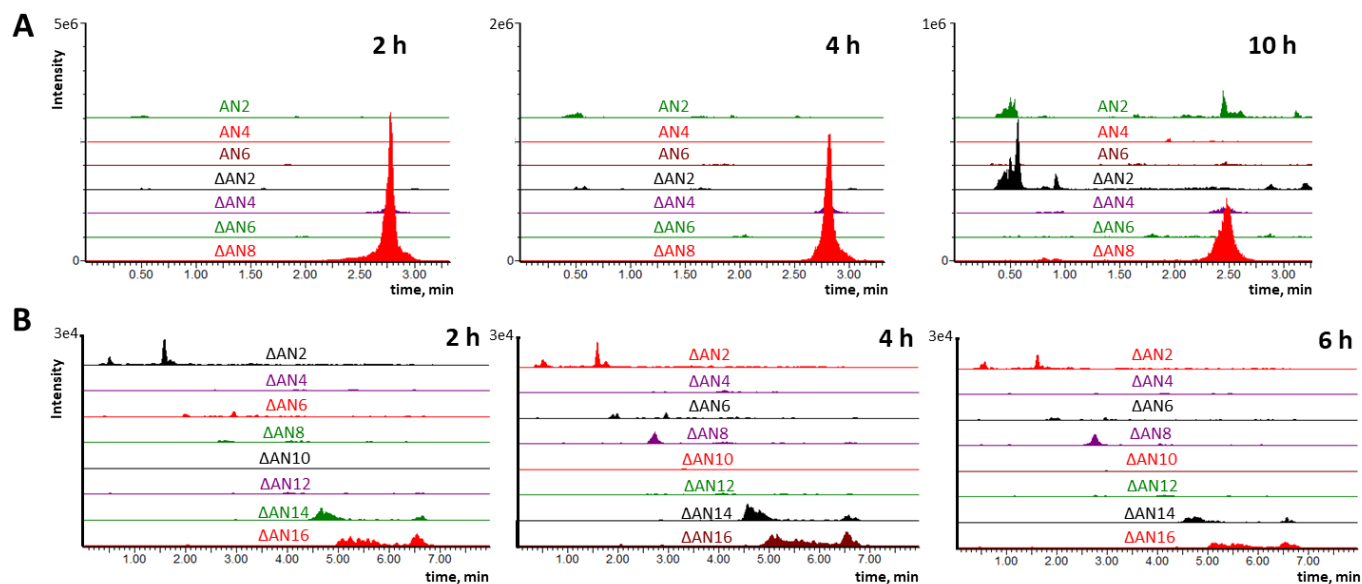
S.Fig. 3 Structure of biotin-labelled HA, and LC-MS analysis of biotin-labelled oligosaccharides of HA.



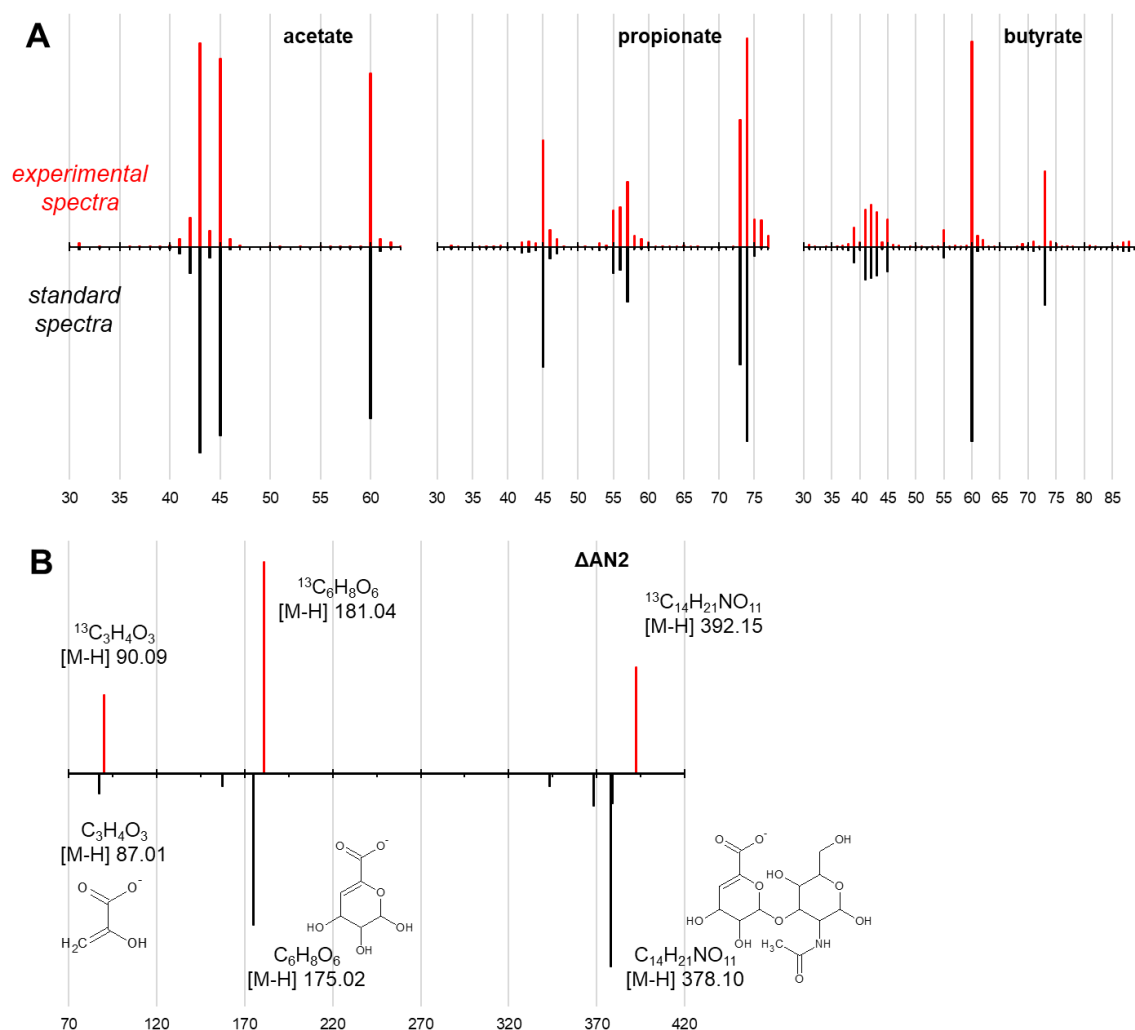
S.Fig. 4 Mass spectra of HA oligosaccharides. Molecules with all carbon positions substituted by heavy ^{13}C atoms were the dominant forms of AN4, AN6 and AN8 present in the oligosaccharide mixture. Spectra of oligosaccharides were measured by MALDI-MS (1 μl spots of oligosaccharide mixture $10\text{ }\mu\text{g ml}^{-1}$ diluted with 9-aminoacridone matrix (1:1), negative ion mode).



S.Fig. 5 Distribution of HA-related proteins in the ileum, cecum and distal colon of WT and GF mice. Green – protein of interest, blue – nuclei. Scale bars represent 50 μ m. Details of histochemistry staining are described in supplementary methods (**S.Met. 5**).

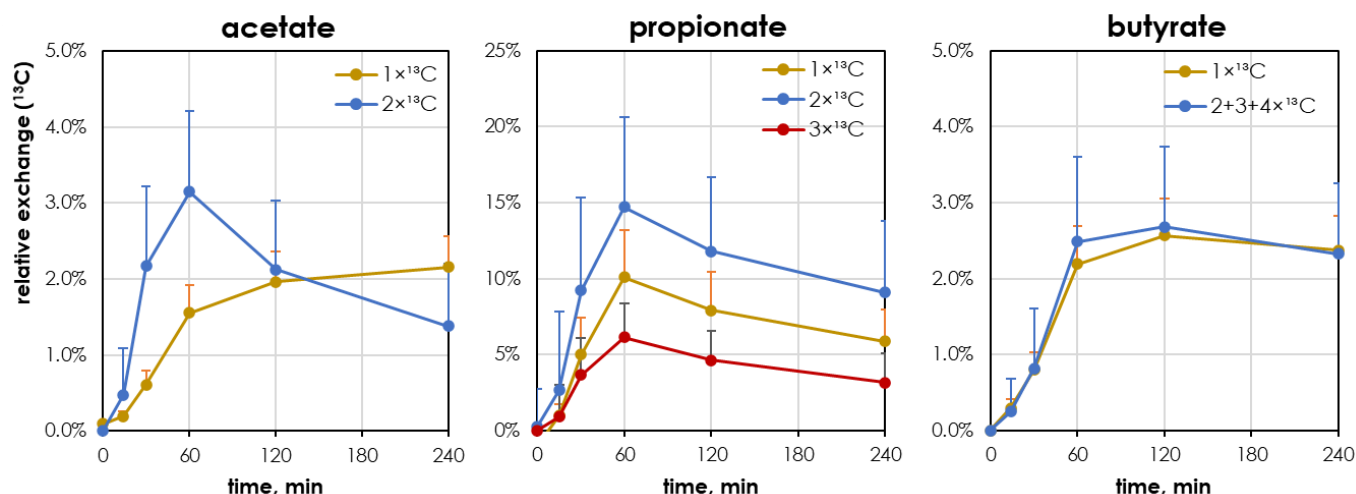


S.Fig. 6 LC-MS chromatograms of oligosaccharides in urine. A) Chromatograms of saturated and unsaturated oligosaccharides in urine 2, 4 and 10 hours after oral administration of unsaturated octasaccharide (Δ AN8, 1.52 kDa), B) Chromatograms of unsaturated oligosaccharides in 2, 4 and 6 hours after oral administration of unsaturated hexadecasaccharide (Δ AN16, 3.03 kDa).

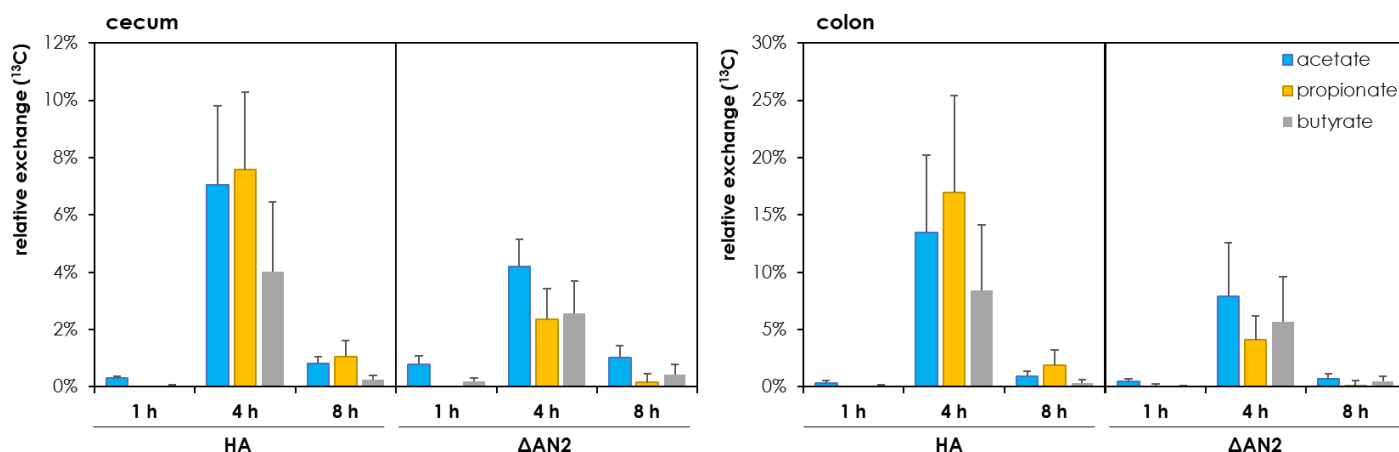


S.Fig. 7 Fragmentation spectra of short chain fatty acids and unsaturated hyaluronan disaccharide in sample of cecal content (mice inoculated with oMM12 microbiota) cultivated in *vitro* with ^{13}C -HA (1600 kDa) for 120 min. A) fragmentation spectra of short-

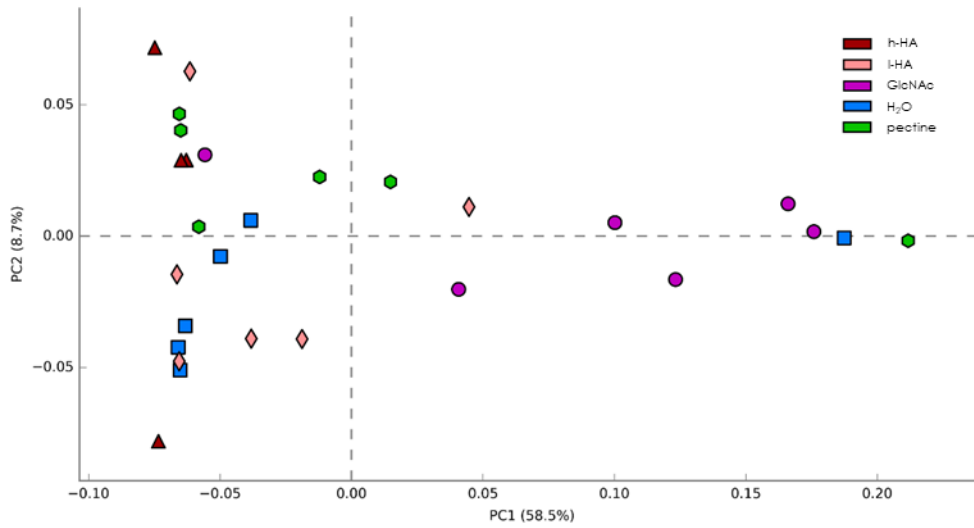
chain fatty acids (GC-MS, electron ionisation), B) CID fragmentation spectrum of unsaturated HA disaccharide (LC-MS, collision induced dissociation).



S.Fig. 8 The kinetics of ^{13}C -label incorporation within the structures of short chain fatty acids during the degradation of high- M_w ^{13}C -HA by cecal content of oMM12 mice. Relative exchange represents the ratio between signals of molecules containing 1-4 ^{13}C atoms and signals of molecules without any ^{13}C atoms. Values of relative exchange were corrected for the natural $^{12}\text{C}/^{13}\text{C}$ ratio.



S.Fig. 9 ^{13}C -SCFA production in the cecum and colon after oral administration of ^{13}C -labelled unsaturated HA disaccharide (ΔAN2) and high- M_w HA (1600 kDa). Relative exchange represents the ratio between summary signal of molecules containing 1-4 ^{13}C atoms and signal of molecules without any ^{13}C atoms. Values of relative exchange were corrected for natural $^{12}\text{C}/^{13}\text{C}$ ratio.



S.Fig. 10 Principal coordinates analysis of the cecal microbiota structure after four-week supplementation with high doses of high- M_w HA (h-HA, 1600 kDa, 760 mg kg⁻¹ day⁻¹), low doses of high- M_w HA (l-HA, 1600 kDa, 90 mg kg⁻¹ day⁻¹), pectin (640 mg kg⁻¹ day⁻¹) and GlcNAc (650 mg kg⁻¹ day⁻¹).

2. SUPPLEMENTARY TABLES

S.Tab. 1 List of MRM transitions for native (¹²C) and isotope labelled (¹³C) HA oligosaccharides with optimized values of collision energy (CE) and cone voltage (CV). Number represents the number of building blocks of glucuronic acid (A) and *N*-acetylglucosamine (N). AN, NA, AA describe monosaccharide units at the ends of oligosaccharide chains, Δ stands for the double bond in the structure of the monosaccharide unit at the nonreducing end of the oligosaccharide chain.

oligosaccharide	type	<i>z</i>	<i>m/z</i>	fragment 1	fragment 2	CE F1, eV	CE F2, eV	CV, V
ΔAN2	¹² C	1-	378.1	175	87	24	10	22
	¹³ C		392.1	181	90	24	10	22
AN2	¹² C	1-	396.2	113	193	26	16	40
	¹³ C		410.2	118	199	26	16	40
ΔAA3	¹² C	1-	554.1	175	-	42	-	18
	¹³ C		574.2	181	-	42	-	18
AA3	¹² C	1-	572.2	113	193	70	42	18
	¹³ C		592.2	118	199	70	42	18
NN3	¹² C	1-	599.2	175	396	16	6	8
	¹³ C		621.3	181	412	26	6	8
ΔAN4	¹² C	1-	757.6	175	554.5	38	28	72
	¹³ C		785.3	181	574	38	28	72
AN4	¹² C	1-	775.2	193	572.1	38	28	72
	¹³ C		803.3	199	594.2	38	28	72
ΔAA5	¹² C	1-	933.2	175	-	42	-	18
	¹³ C		967.4	181	-	42	-	18
AA5	¹² C	1-	951.3	113	193	70	42	18
	¹³ C		985.4	118	199	70	42	18
NN5	¹² C	2-	488.7	175	387	16	6	8
	¹³ C		506.7	181	401	16	6	8
ΔAN6	¹² C	2-	568	175	465.9	30	16	42
	¹³ C		588.7	181	482.6	30	16	42
AN6	¹² C	2-	576.8	193	475.2	30	16	42
	¹³ C		597.7	199	492.7	30	16	42
ΔAA7	¹² C	2-	646.7	175	-	24	-	10
	¹³ C		670.8	181	-	24	-	10
AA7	¹² C	2-	664.8	175	193	24	30	10
	¹³ C		688.8	181	199	24	30	10
NN7	¹² C	2-	678.3	175	576.5	30	20	40

	¹³ C		703.3	181	597.5	30	20	40
ΔAN8	¹² C	2-	757.5	175	655.8	40	18	54
	¹³ C		785.3	181	679	40	18	54
AN8	¹² C	2-	766.4	193	664.7	40	18	54
	¹³ C		794.3	199	689.3	40	18	54
ΔAA9	¹² C	2-	836.2	175	-	46	-	18
	¹³ C		867.3	181	-	46	-	18
AA9	¹² C	2-	854.4	113	193	60	46	18
	¹³ C		885.3	118	199	60	46	18
NN9	¹² C	2-	867.9	175	766.1	38	24	24
	¹³ C		899.9	181	794.1	38	24	24
ΔAN10	¹² C	2-	946.8	175	844.7	44	26	18
	¹³ C		981.9	181	875.6	44	28	18
AN10	¹² C	2-	956	193	854.4	44	26	18
	¹³ C		990.9	199	885.8	44	26	18
ΔAA11	¹² C	2-	1025.8	175	-	48	-	18
	¹³ C		1063.9	181	-	48	-	18
AA11	¹² C	2-	1044	113	193	68	48	18
	¹³ C		1081.9	118	199	68	48	18
NN11	¹² C	3-	704.7	175	636.8	30	20	44
	¹³ C		730.7	181	660.1	30	20	44
ΔAN12	¹² C	3-	757.5	695.2	175	20	44	38
	¹³ C		785.1	90	181	20	44	38
AN12	¹² C	3-	763.5	695.7	113	20	44	38
	¹³ C		791.3	721.3	118	20	44	38
ΔAA13	¹² C	3-	803.9	175	-	24	-	17
	¹³ C		834	181	-	24	-	17
AA13	¹² C	3-	821.9	113	175	45	24	17
	¹³ C		851.9	118	181	45	24	17
NN13	¹² C	3-	830.9	175	762.9	30	20	44
	¹³ C		861.7	181	791.6	30	20	44
ΔAN14	¹² C	3-	884.1	175	816.2	38	22	12
	¹³ C		916.2	181	875	38	22	12
AN14	¹² C	3-	889.6	193	821.6	38	22	12
	¹³ C		922.4	199	852.3	38	22	12
ΔAA15	¹² C	3-	930.3	175	-	35	-	30
	¹³ C		965	181	-	35	-	30
AA15	¹² C	3-	948.3	113	880.2	50	35	30
	¹³ C		983	118	181	50	35	30
NN15	¹² C	3-	957.3	175	889.2	40	30	55
	¹³ C		992.7	181	922.7	40	30	55
ΔAN16	¹² C	3-	1010.6	113	942.7	64	28	38
	¹³ C		1047.2	181	976.5	40	24	26
AN16	¹² C	3-	1016.3	113	948.5	64	28	38
	¹³ C		1053.4	118	983.4	64	28	38
ΔAA17	¹² C	3-	1074.6	175	-	40	-	50
	¹³ C		1114.1	181	-	40	-	50
AA17	¹² C	3-	1074.6	113	175	60	40	50
	¹³ C		1114.1	118	181	60	40	50
NN17	¹² C	3-	1083.7	175	1015.6	45	35	60
	¹³ C		1123.8	181	1053.8	45	35	60
ΔAN18	¹² C	3-	1136.3	175	1068.3	50	28	92
	¹³ C		1178.2	181	1107.5	50	28	92
AN18	¹² C	3-	1142.7	113	1074.9	64	30	66
	¹³ C		1184.5	118	1114.4	64	30	66
ΔAA19	¹² C	3-	1183	175	-	50	-	60
	¹³ C		1227.1	175	-	50	-	60
AA19	¹² C	3-	1201	113	175	70	50	60
	¹³ C		1245.2	118	181	70	50	60
NN19	¹² C	3-	1210	175	1142	50	40	65
	¹³ C		1254.8	181	1184.8	50	40	65
ΔAN20	¹² C	3-	1262.7	113	1194.7	68	26	76
	¹³ C		1309.2	118	1238.5	68	26	76
AN20	¹² C	3-	1269.2	113	1201.5	74	32	88
	¹³ C		1315.5	118	1245.5	74	32	88

S.Tab. 2 Distribution of orally-administered ^{13}C -HA expressed as percent of applied dose present in various parts of gastrointestinal tract, organs, and body fluids after oral administration of ^{13}C - ΔAN2 , high- M_w ^{13}C -HA (1600 kDa) and low- M_w ^{13}C -HA (15 kDa) to wild type or germ free mice ($n=3$).

time, h	stomach content	small int. content	cecal content	colon content	stomach	small int.	cecum	colon	plasma	urine	liver	kidneys	spleen	lungs	knee	cartilage	node
^{13}C-ΔAN2 (wild type)																	
0.5	27.4 $\pm$ 0	11.55 $\pm$ 4.56	0.05 $\pm$ 0.01	0.02 $\pm$ 0.01	1.31 $\pm$ 0.61	4.35 $\pm$ 0.37	0.1 $\pm$ 0.08	0.32 $\pm$ 0.5	0.01 $\pm$ 0	0.38 $\pm$ 0.28	0.15 $\pm$ 0.05	0.06 $\pm$ 0.03	0 $\pm$ 0	0 $\pm$ 0	-	-	-
1	28.14 $\pm$ 10.91	28.49 $\pm$ 10.93	8.73 $\pm$ 14.61	0.01 $\pm$ 0.02	0.47 $\pm$ 0.21	5.02 $\pm$ 3.08	1.4 $\pm$ 2.26	0.06 $\pm$ 0.04	0 $\pm$ 0	0.52 $\pm$ 0.06	0.03 $\pm$ 0.02	0.04 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	-	-	-
2	4.73 $\pm$ 1.88	14.91 $\pm$ 2	0.71 $\pm$ 0.53	0.01 $\pm$ 0.01	0.15 $\pm$ 0.02	2.71 $\pm$ 1.35	0.2 $\pm$ 0.21	0.18 $\pm$ 0.23	0 $\pm$ 0	0.09 $\pm$ 0.11	0 $\pm$ 0	0.01 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
4	1.11 $\pm$ 0.39	2.96 $\pm$ 2.74	0.28 $\pm$ 0.2	0.01 $\pm$ 0.02	0.08 $\pm$ 0.07	0.76 $\pm$ 0.41	0.1 $\pm$ 0.06	0.07 $\pm$ 0.08	0 $\pm$ 0	0.06 $\pm$ 0.05	0.01 $\pm$ 0	0.01 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
8	0.3 $\pm$ 0.31	0.87 $\pm$ 1.35	0.07 $\pm$ 0.08	0 $\pm$ 0	0.01 $\pm$ 0	0.38 $\pm$ 0.54	0.03 $\pm$ 0.03	0.01 $\pm$ 0.01	0 $\pm$ 0	0.11 $\pm$ 0.05	0.01 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
12	0.01 $\pm$ 0.01	0.07 $\pm$ 0.03	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.01 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
24	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
48	0.01 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
72	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
1600 kDa ^{13}C-HA (wild type)																	
0.5	17.17 $\pm$ 0.02	8.03 $\pm$ 6.22	0.19 $\pm$ 0.2	0.24 $\pm$ 0.33	6.14 $\pm$ 2.17	5.31 $\pm$ 0.92	0.28 $\pm$ 0.22	0.58 $\pm$ 0.59	0 $\pm$ 0	0 $\pm$ 0	0.2 $\pm$ 0.35	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
1	18.85 $\pm$ 4.43	21.19 $\pm$ 15.41	2.09 $\pm$ 2.92	0.05 $\pm$ 0.04	6.41 $\pm$ 5.23	19.5 $\pm$ 14.63	0.36 $\pm$ 0.2	0.25 $\pm$ 0.25	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
2	14.34 $\pm$ 6.71	20.96 $\pm$ 4.42	9.66 $\pm$ 9.96	0.28 $\pm$ 0.26	3.33 $\pm$ 3.61	10.17 $\pm$ 6.08	0.91 $\pm$ 0.62	0.08 $\pm$ 0.05	0 $\pm$ 0	0 $\pm$ 0.09	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
4	0.2 $\pm$ 0.24	9.43 $\pm$ 13.36	5.34 $\pm$ 7.31	0.71 $\pm$ 0.75	0.31 $\pm$ 0.28	5.09 $\pm$ 5.75	2.42 $\pm$ 2.2	0.58 $\pm$ 0.62	0 $\pm$ 0	0.05 $\pm$ 0.08	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
8	0.06 $\pm$ 0.11	0.42 $\pm$ 0.58	0.01 $\pm$ 0.02	0 $\pm$ 0	0.02 $\pm$ 0.04	0.41 $\pm$ 0.51	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.09 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
12	0.01 $\pm$ 0.03	0.02 $\pm$ 0.03	0 $\pm$ 0	0 $\pm$ 0	0.01 $\pm$ 0.02	0.12 $\pm$ 0.11	0 $\pm$ 0	0 $\pm$ 0	0.02 $\pm$ 0	0 $\pm$ 0	0.05 $\pm$ 0.08	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
24	0 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.06 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
48	0 $\pm$ 0	0.01 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.07 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
72	0 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.07 $\pm$ 0.03	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
^{13}C-ΔAN2 (germ free)																	
0.5	13.95 $\pm$ 4.76	11.51 $\pm$ 1.75	0.02 $\pm$ 0	0.01 $\pm$ 0.01	0.46 $\pm$ 0.17	7.75 $\pm$ 2.79	0.00 $\pm$ 0.01	0.00 $\pm$ 0.01	0.02 $\pm$ 0	0.22 $\pm$ 0.11	0.02 $\pm$ 0	0.03 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
2	12.47 $\pm$ 6.32	15.19 $\pm$ 1.09	0.02 $\pm$ 0	0 $\pm$ 0	0.13 $\pm$ 0.02	2.78 $\pm$ 0.93	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0 $\pm$ 0	0.36 $\pm$ 0.5	0.02 $\pm$ 0.01	0.01 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
4	2.85 $\pm$ 2.01	16.71 $\pm$ 6.38	0.05 $\pm$ 0.07	0.14 $\pm$ 0.24	0.13 $\pm$ 0.2	4.08 $\pm$ 1.76	0.05 $\pm$ 0.07	0.03 $\pm$ 0.03	0 $\pm$ 0	0.21 $\pm$ 0.16	0.01 $\pm$ 0.01	0.02 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
8	0.8 $\pm$ 0.79	2.32 $\pm$ 0.9	5.5 $\pm$ 4.61	1.87 $\pm$ 1.10	0.1 $\pm$ 0.08	0.43 $\pm$ 0.27	0.73 $\pm$ 0.34	0.07 $\pm$ 0.04	0 $\pm$ 0	0.35 $\pm$ 0.38	0.01 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
24	0.33 $\pm$ 0.41	0.2 $\pm$ 0.22	3.89 $\pm$ 0.22	0.48 $\pm$ 0.15	0 $\pm$ 0	0.08 $\pm$ 0.05	0.33 $\pm$ 0.14	0.01 $\pm$ 0.01	0 $\pm$ 0	0.01 $\pm$ 0.01	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
1600 kDa ^{13}C-HA (germ free)																	
0.5	20.11 $\pm$ 10.23	11.72 $\pm$ 3.61	0 $\pm$ 0	0.01 $\pm$ 0.01	0.17 $\pm$ 0.14	11.23 $\pm$ 2.48	0.01 $\pm$ 0.01	0.04 $\pm$ 0.05	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
2	7.23 $\pm$ 5.64	51.15 $\pm$ 26.42	0 $\pm$ 0	0.01 $\pm$ 0	0.38 $\pm$ 0.59	8.3 $\pm$ 1.32	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
4	4.5 $\pm$ 3.73	56.86 $\pm$ 6.68	0 $\pm$ 0	0.01 $\pm$ 0.01	0.04 $\pm$ 0.04	5.37 $\pm$ 0.3	0 $\pm$ 0	0.11 $\pm$ 0.19	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
8	0.16 $\pm$ 0.08	5.33 $\pm$ 4.97	21.4 $\pm$ 9.03	12.27 $\pm$ 1.83	0 $\pm$ 0	0.51 $\pm$ 0.39	1.77 $\pm$ 0.47	0.38 $\pm$ 0.11	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
24	0.18 $\pm$ 0.12	0.16 $\pm$ 0.28	13.29 $\pm$ 8.96	1.56 $\pm$ 0.24	0 $\pm$ 0	0.09 $\pm$ 0.04	0.69 $\pm$ 0.12	0.09 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
15 kDa ^{13}C-HA (wild type)																	
1	8.53 $\pm$ 2.64	24.43 $\pm$ 2.61	3.32 $\pm$ 5.01	0.56 $\pm$ 0.9	-	-	-	-	0 $\pm$ 0	-	-	-	-	-	-	-	-
2	4.61 $\pm$ 1.76	22.63 $\pm$ 10.48	13.3 $\pm$ 3	4.04 $\pm$ 2.35	-	-	-	-	0 $\pm$ 0	-	-	-	-	-	-	-	-
4	7.12 $\pm$ 4.1	1.9 $\pm$ 0.99	7.6 $\pm$ 9.75	2.03 $\pm$ 1.17	-	-	-	-	0 $\pm$ 0	-	-	-	-	-	-	-	-
6	2.15 $\pm$ 1.13	3.26 $\pm$ 1.91	0.39 $\pm$ 0.17	1.93 $\pm$ 2.02	-	-	-	-	0 $\pm$ 0	-	-	-	-	-	-	-	-
24	0.04 $\pm$ 0.01	0.11 $\pm$ 0.03	0.11 $\pm$ 0	0.03 $\pm$ 0.01	-	-	-	-	0 $\pm$ 0	-	-	-	-	-	-	-	-

S.Tab. 3 Concentrations ($\mu\text{g g}^{-1}$) of ^{13}C -HA in various parts of gastrointestinal tract, organs, and body fluids after oral application of ^{13}C - ΔAN2 , high- M_w ^{13}C -HA (1600 kDa) and low- M_w ^{13}C -HA (15 kDa) to wild type or germ free mice ($n=3$).

time, h	stomach content	small int. content	cecal content	colon content	stomach	small int.	cecum	colon	plasma	urine	liver	kidneys	spleen	lungs	knee	cartil age	node
^{13}C-ΔAN2 (wild type)																	
0.5	2469 $\pm$ 475	613 $\pm$ 253	2.12 $\pm$ 0.17	0.00 $\pm$ 0	71.6 $\pm$ 25.2	60.8 $\pm$ 7.64	5.16 $\pm$ 5.4	19.3 $\pm$ 30.3	0.05 $\pm$ 0	47.1 $\pm$ 13.6	1.31 $\pm$ 0.47	2.09 $\pm$ 1.06	0.05 $\pm$ 0	1.4 $\pm$ 1	-	-	-
1	1738 $\pm$ 364	1630 $\pm$ 530	627 $\pm$ 1061	1.28 $\pm$ 3.08	33.0 $\pm$ 16.2	79.9 $\pm$ 44.0	91.9 $\pm$ 146	3.88 $\pm$ 2.69	0.03 $\pm$ 0	84.9 $\pm$ 40.4	0.29 $\pm$ 0.18	1.49 $\pm$ 0.7	0 $\pm$ 0	0 $\pm$ 0	-	-	-
2	341.0 $\pm$ 163.3	854 $\pm$ 214	37.4 $\pm$ 32.5	2.13 $\pm$ 3.01	9.62 $\pm$ 1.38	36.3 $\pm$ 20.3	14.4 $\pm$ 15.2	14.5 $\pm$ 20.9	0.01 $\pm$ 0	17.2 $\pm$ 6.1	0.04 $\pm$ 0.04	0.38 $\pm$ 0.11	0 $\pm$ 0	0 $\pm$ 0	-	-	-
4	108.3 $\pm$ 35.6	155.9 $\pm$ 109.8	14 $\pm$ 11.12	2.55 $\pm$ 3.13	5.49 $\pm$ 4.27	11.33 $\pm$ 4.9	6.07 $\pm$ 3.22	5.3 $\pm$ 5.29	0.01 $\pm$ 0	53.4 $\pm$ 28.9	0.12 $\pm$ 0.02	0.54 $\pm$ 0.15	0.02 $\pm$ 0	0 $\pm$ 0	-	-	-
8	19.4 $\pm$ 15.1	41.8 $\pm$ 65.8	4.39 $\pm$ 5.61	0.29 $\pm$ 0.27	0.59 $\pm$ 0.35	5.62 $\pm$ 8.39	2.31 $\pm$ 2.79	0.4 $\pm$ 0.67	0 $\pm$ 0	41.1 $\pm$ 37.5	0.09 $\pm$ 0.06	0.15 $\pm$ 0.15	0 $\pm$ 0	0 $\pm$ 0	-	-	-
12	0.84 $\pm$ 1.04	2.13 $\pm$ 0.45	0.08 $\pm$ 0.05	0.02 $\pm$ 0	0.1 $\pm$ 0.18	0.15 $\pm$ 0.06	0.04 $\pm$ 0	0.02 $\pm$ 0.02	0 $\pm$ 0	0.23 $\pm$ 0.13	0.02 $\pm$ 0	0.03 $\pm$ 0.03	0 $\pm$ 0	0 $\pm$ 0	-	-	-
24	0.24 $\pm$ 0	0.15 $\pm$ 0.03	0.05 $\pm$ 0	0.03 $\pm$ 0.01	0.19 $\pm$ 0.17	0.06 $\pm$ 0.06	0.04 $\pm$ 0	0.01 $\pm$ 0	0 $\pm$ 0	0.08 $\pm$ 0.02	0.02 $\pm$ 0	0.02 $\pm$ 0.02	0 $\pm$ 0	0 $\pm$ 0	-	-	-
48	0.82 $\pm$ 0.58	0.11 $\pm$ 0	0.05 $\pm$ 0	0.02 $\pm$ 0	0.1 $\pm$ 0.17	0.04 $\pm$ 0.05	0.04 $\pm$ 0	0.01 $\pm$ 0	0 $\pm$ 0	0.03 $\pm$ 0	0.02 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	-	-
72	0.54 $\pm$ 0.43	0.12 $\pm$ 0.01	0.05 $\pm$ 0	0.02 $\pm$ 0	0.12 $\pm$ 0.17	0.01 $\pm$ 0	0.04 $\pm$ 0	0.01 $\pm$ 0	0 $\pm$ 0	0.03 $\pm$ 0	0.01 $\pm$ 0	0.03 $\pm$ 0.04	0 $\pm$ 0	0 $\pm$ 0	-	-	-
1600 kDa ^{13}C-HA (wild type)																	
0.5	1452 $\pm$ 807	816 $\pm$ 546	12.78 $\pm$ 9.51	14.5 $\pm$ 16.2	3636 $\pm$ 2726	85.8 $\pm$ 16.9	18.8 $\pm$ 15	35.9 $\pm$ 33.9	0 $\pm$ 0	0.02 $\pm$ 0.03	1.86 $\pm$ 3.22	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
1	2508 $\pm$ 1283	1076 $\pm$ 226	77.8 $\pm$ 86.5	3.82 $\pm$ 2.69	2508 $\pm$ 1283	261 $\pm$ 201	19.7 $\pm$ 13.3	17.8 $\pm$ 16.7	0 $\pm$ 0	0.03 $\pm$ 0.05	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
2	3635 $\pm$ 2726	1826 $\pm$ 909	298 $\pm$ 271	22.2 $\pm$ 6.9	1452 $\pm$ 807	142.2 $\pm$ 60.6	66.7 $\pm$ 54.4	6.23 $\pm$ 2.81	0 $\pm$ 0	0.76 $\pm$ 0.95	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
4	28.5 $\pm$ 28.27	849 $\pm$ 1138	397 $\pm$ 490	153 $\pm$ 213	20.11 $\pm$ 17.5	73.4 $\pm$ 83.9	164 $\pm$ 160	38.7 $\pm$ 39.0	0 $\pm$ 0	3.55 $\pm$ 5.87	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
8	9.2 $\pm$ 15.8	27.4 $\pm$ 41.3	1.34 $\pm$ 1.79	0.56 $\pm$ 0.57	1.75 $\pm$ 3.03	6.63 $\pm$ 8.81	0.54 $\pm$ 0.5	0 $\pm$ 0	0 $\pm$ 0	5.99 $\pm$ 5.9	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
12	1.09 $\pm$ 1.37	2.51 $\pm$ 4.16	0 $\pm$ 0	0 $\pm$ 0	1.1 $\pm$ 1.9	2.05 $\pm$ 2.24	0 $\pm$ 0	0 $\pm$ 0	0.1 $\pm$ 0	1.33 $\pm$ 1.88	0.53 $\pm$ 0.91	-	1.4 $\pm$ 2.5	-	0 $\pm$ 0	-	-
24	0.27 $\pm$ 0.25	0.0 $\pm$ 0.0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.00 $\pm$ 0.43	0 $\pm$ 0	0.06 $\pm$ 0.10	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
48	0 $\pm$ 0	0.71 $\pm$ 1.06	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.26 $\pm$ 0.43	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
72	0.16 $\pm$ 0.22	0.0 $\pm$ 0.0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	1.13 $\pm$ 0.53	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	-	0 $\pm$ 0	-	-
^{13}C-ΔAN2 (germ free)																	
0.5	2663 $\pm$ 1181	163.7 $\pm$ 14.02	0.2 $\pm$ 0	0.02 $\pm$ 0	51.3 $\pm$ 22.2	91.7 $\pm$ 35.7	0.34 $\pm$ 0.72	0.54 $\pm$ 0.94	0.2 $\pm$ 0	26.15 $\pm$ 12.7	0.23 $\pm$ 0.06	1.28 $\pm$ 0.64	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	2.1 $\pm$ 1.2
2	788 $\pm$ 338	198.72 $\pm$ 13.01	0.2 $\pm$ 0	0.24 $\pm$ 0.38	16.32 $\pm$ 4.02	30.12 $\pm$ 10.3	0.02 $\pm$ 0.1	0.00 $\pm$ 0	0.03 $\pm$ 0	28.6 $\pm$ 15.9	0.22 $\pm$ 0.13	0.53 $\pm$ 0.14	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0.4 $\pm$ 0.3
4	235.4 $\pm$ 88.2	366 $\pm$ 205	0.72 $\pm$ 0.9	0.19 $\pm$ 0.23	13.9 $\pm$ 20.3	46.1 $\pm$ 20.4	4.23 $\pm$ 6.38	2.47 $\pm$ 3.05	0.03 $\pm$ 0	85.1 $\pm$ 64.97	0.19 $\pm$ 0.02	0.96 $\pm$ 0.86	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	1.1 $\pm$ 0.9
8	64.7 $\pm$ 40.9	39.63 $\pm$ 18.46	69.9 $\pm$ 18.8	0.23 $\pm$ 0.17	11.3 $\pm$ 8.14	4.33 $\pm$ 2.45	47.3 $\pm$ 23.9	6.77 $\pm$ 3.34	0.01 $\pm$ 0	34.04 $\pm$ 27.4	0.1 $\pm$ 0.2	0.1 $\pm$ 0.14	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0.2 $\pm$ 0.1
24	10.61 $\pm$ 13.15	1.87 $\pm$ 1.9	34.38 $\pm$ 4.64	0.48 $\pm$ 0.58	0 $\pm$ 0	0.77 $\pm$ 0.43	16.69 $\pm$ 3.76	1.27 $\pm$ 1.02	0 $\pm$ 0	1.5 $\pm$ 0.97	-0.01 $\pm$ 0.01	0.02 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	-	0 $\pm$ 0	0.03 $\pm$ 0
1600 kDa ^{13}C-HA (germ free)																	
0.5	1471 $\pm$ 359	151.3 $\pm$ 51.9	0 $\pm$ 0	1.52 $\pm$ 1.34	17.7 $\pm$ 12.1	94.9 $\pm$ 17.2	0.52 $\pm$ 0.37	4.69 $\pm$ 5.39	0 $\pm$ 0	-	-	-	-	-	-	-	-
2	563 $\pm$ 372	1037 $\pm$ 421	0 $\pm$ 0	0.67 $\pm$ 0	34.1 $\pm$ 51.5	94.7 $\pm$ 15.7	0.13 $\pm$ 0	0.43 $\pm$ 0.05	0 $\pm$ 0	-	-	-	-	-	-	-	-
4	494 $\pm$ 495	922.8 $\pm$ 192.6	0 $\pm$ 0	0.9 $\pm$ 0.4	4.59 $\pm$ 3.72	58.39 $\pm$ 2.63	0.13 $\pm$ 0	12.7 $\pm$ 21.1	0 $\pm$ 0	-	-	-	-	-	-	-	-
8	18.71 $\pm$ 20.48	96.93 $\pm$ 82.74	225.6 $\pm$ 71.1	899 $\pm$ 217	0.53 $\pm$ 0.39	5.45 $\pm$ 4.24	112.3 $\pm$ 27.1	39 $\pm$ 11.5	0 $\pm$ 0	-	-	-	-	-	-	-	-
24	8.71 $\pm$ 4.57	2.29 $\pm$ 3.96	149.7 $\pm$ 13.8	103 $\pm$ 20	0.33 $\pm$ 0.18	0.92 $\pm$ 0.37	40.5 $\pm$ 7.11	8.9 $\pm$ 1.5	0 $\pm$ 0	-	-	-	-	-	-	-	-
15 kDa ^{13}C-HA (wild type)																	
1	1000.8 $\pm$ 482.8	1433.7 $\pm$ 541	150 $\pm$ 225	42.8 $\pm$ 69.6	-	-	-	-	0.04 $\pm$ 0	-	-	-	-	-	-	-	-
2	390.6 $\pm$ 335	1012.4 $\pm$ 840.7	507 $\pm$ 149	331 $\pm$ 301	-	-	-	-	0.05 $\pm$ 0	-	-	-	-	-	-	-	-
4	220.7 $\pm$ 133.5	79.3 $\pm$ 37.7	247 $\pm$ 289	178 $\pm$ 126	-	-	-	-	0.03 $\pm$ 0	-	-	-	-	-	-	-	-
6	220.1 $\pm$ 97.3	129.5 $\pm$ 77.5	17.5 $\pm$ 8.6	146 $\pm$ 167	-	-	-	-	0.03 $\pm$ 0	-	-	-	-	-	-	-	-
24	1.8 $\pm$ 0.0	3.9 $\pm$ 0.4	3.5 $\pm$ 0.0	1.8 $\pm$ 0.0	-	-	-	-	0.00 $\pm$ 0	-	-	-	-	-	-	-	-

S.Tab. 4 Concentrations ($\mu\text{g g}^{-1}$) of unsaturated HA oligosaccharides in various parts of gastrointestinal tract after oral administration of high- M_w ^{13}C -HA (1600 kDa) and low- M_w ^{13}C -HA (15 kDa) ($n=3$).

time. h	high- M_w ^{13}C -HA (1600 kDa)					
	ΔAN2	ΔAN8	ΔAN10	ΔAN12	ΔAN14	ΔAN16
	stomach					
1	0.20 ± 0.23	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00
2	7.45 ± 10.8	0.09 ± 0.02	0.08 ± 0.08	0.19 ± 0.22	0.3 ± 0.34	0.16 ± 0.22
4	2.83 ± 4.31	0.11 ± 0.12	0.11 ± 0.06	0.32 ± 0.55	0.24 ± 0.24	0.36 ± 0.63
6	27.3 ± 20.0	0.35 ± 0.12	0.09 ± 0.07	0.24 ± 0.23	0.15 ± 0.11	0.06 ± 0.06
24	0.00 ± 0.00	0.00 ± 0.16	0.00 ± 0.17	0.00 ± 0.00	0.04 ± 0.02	0.00 ± 0.00
	small intestine					
1	0.05 ± 0.03	0.00 ± 0.00	0.00 ± 0.02	-	0.06 ± 0.11	0.00 ± 0.01
2	0.45 ± 0.34	0.02 ± 0.02	0.01 ± 0.05	-	0.02 ± 0.04	0.00 ± 0.00
4	0.46 ± 0.28	0.02 ± 0.02	0.00 ± 0.01	-	0.00 ± 0.01	0.03 ± 0.05
6	4.57 ± 3.93	0.08 ± 0.06	0.04 ± 0.06	-	0.05 ± 0.10	0.12 ± 0.21
24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	-	0.01 ± 0.04	0.00 ± 0.00
	cecum					
1	1.82 ± 2.82	0.03 ± 0.05	0.06 ± 0.05	0.01 ± 0.03	0.04 ± 0.06	0.15 ± 0.21
2	19.1 ± 16.5	0.29 ± 0.36	0.06 ± 0.06	0.92 ± 1.18	0.72 ± 0.64	0.17 ± 0.21
4	27.4 ± 15.2	0.36 ± 0.32	0.11 ± 0.06	0.72 ± 0.99	0.22 ± 0.39	0.37 ± 0.51
6	9.78 ± 8.96	0.14 ± 0.31	0.05 ± 0.07	0.06 ± 0.1	0.00 ± 0.00	0.02 ± 0.04
24	0.06 ± 0.00	0.00 ± 0.26	0.01 ± 0.16	0.00 ± 0.00	0.07 ± 0.11	0.02 ± 0.03
	colon					
1	2.45 ± 4.42	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.04	0.00 ± 0.01	0.03 ± 0.06
2	15.21 ± 12.0	0.10 ± 0.11	0.01 ± 0.02	1.93 ± 1.68	0.28 ± 0.25	0.19 ± 0.27
4	272.0 ± 350.3	0.69 ± 0.39	0.31 ± 0.14	2.46 ± 0.10	1.24 ± 0.42	1.60 ± 1.14
6	45.4 ± 39.7	0.22 ± 0.20	0.02 ± 0.03	0.98 ± 0.97	0.52 ± 0.48	0.52 ± 0.67
24	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.36 ± 1.61	0.13 ± 0.13	0.00 ± 0.00
time, h	low- M_w ^{13}C -HA (15 kDa)					
	ΔAN2	ΔAN8	ΔAN10	ΔAN12	ΔAN14	ΔAN16
	stomach					
1	1.82 ± 3.1	0.03 ± 0.04	0.03 ± 0.05	0.19 ± 0.24	0.14 ± 0.09	0.06 ± 0.08
2	2.87 ± 2.59	0.06 ± 0.06	0.03 ± 0.03	0.28 ± 0.25	0.24 ± 0.17	0.11 ± 0.1
4	18.6 ± 27.7	0.21 ± 0.25	0.11 ± 0.20	0.37 ± 0.29	0.36 ± 0.21	0.21 ± 0.22
6	9.68 ± 4.81	0.30 ± 0.23	0.30 ± 0.26	0.89 ± 0.57	0.84 ± 0.48	0.52 ± 0.44
24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00
	small intestine					
1	0.11 ± 0.07	0.01 ± 0.01	-0.02 ± 0.01	-	0.04 ± 0.03	0.03 ± 0.04
2	0.50 ± 0.42	0.03 ± 0.03	0.04 ± 0.07	-	0.12 ± 0.13	0.03 ± 0.03
4	3.63 ± 4.98	0.12 ± 0.16	0.04 ± 0.13	-	0.10 ± 0.13	0.04 ± 0.07
6	4.13 ± 1.27	0.46 ± 0.29	0.58 ± 0.43	-	0.42 ± 0.26	0.73 ± 0.58
24	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.08	-	0.14 ± 0.13	0.01 ± 0.03
	cecum					
1	1.45 ± 1.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.09 ± 0.11	0.01 ± 0.02
2	15.1 ± 10.1	0.50 ± 0.39	0.32 ± 0.13	2.43 ± 1.57	2.00 ± 1.49	1.48 ± 1.17
4	21.4 ± 22.7	0.30 ± 0.36	0.08 ± 0.13	1.06 ± 1.73	0.59 ± 1.03	1.19 ± 2.05
6	2.88 ± 1.76	0.04 ± 0.34	0.01 ± 0.38	0.00 ± 0.00	0.03 ± 0.05	0.00 ± 0.00
24	0.06 ± 0.01	0.00 ± 0.36	0.02 ± 0.38	0.00 ± 0.00	0.10 ± 0.09	0.00 ± 0.00
	colon					
1	0.43 ± 0.54	0.00 ± 0.00	0.00 ± 0.00	0.24 ± 0.2	0.00 ± 0.00	0.00 ± 0.00
2	55.9 ± 49.7	0.16 ± 0.16	0.11 ± 0.09	1.79 ± 0.77	0.54 ± 0.55	0.56 ± 0.49
4	172.7 ± 101.7	0.46 ± 0.29	0.12 ± 0.21	1.85 ± 2.24	0.64 ± 1.05	0.72 ± 1.16
6	35.8 ± 37.5	0.42 ± 0.66	0.20 ± 0.35	1.84 ± 2.69	1.03 ± 1.63	1.47 ± 2.54
24	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.85 ± 0.74	0.02 ± 0.03	0.00 ± 0.00

3. SUPPLEMENTARY METHODS

S.Met. 1 Histochemistry localization of biotin-labelled HA in the jejunal loop

Mouse jejunal loops were fixed in a solution composed of ethanol, formalin, and glacial acetic acid (EFG; 70%, 10%, 5% v/v) (Rowley et al., 2020) for at least 24 hours. The fixation was followed by a standard procedure of dehydration and paraffin wax infiltration (TP1020, Leica Biosystems). A Leica embedded station (EG1150 C) was used for the formation of paraffin blocks, from which 5 μm slices were prepared (RM2255, Leica Biosystems). After deparaffinization, the slices were washed in Tris buffered saline (TBS; 20 mmol l^{-1} Tris, 137 mmol l^{-1} NaCl) and incubated for 1 h at room temperature with blocking solution (0.3 mol l^{-1} glycine, 10% FBS, 0.1 % Tween20). Afterwards, the slices, diluted 1:100 in blocking solution, were incubated with streptavidin-Alexa Fluor 647 (Thermo Fisher Scientific, S21374) for 1 h at room temperature. To verify signal specificity, a section of jejunal loop tissue from a mouse without HA-biotin treatment was incubated with streptavidin-Alexa Fluor 647. Samples were washed 5 times in demineralized water and mounted in ROTI Mount FluorCare DAPI (HP20.1, Carl Roth). Images were acquired using a Leica TCS SP8 X confocal microscope with a 20x water immersion Leica objective (HC PL APO CS2, 0.75 NA) and the 405 nm laser line for DAPI excitation and the 650 nm white light laser line for Alexa Fluor 647 nm were used. For samples with biotin labelled HA, the white light laser was set to 40% power. Final images were acquired in z-stacks that covered the whole thickness of the section, with 2 μm steps applied at a resolution 1024 $\times$ 1024 pixels with 1.0 zoom. Images were post processed using Leica LasX software.

S.Met. 2 MALDI-MS imaging of ^{13}C -HA oligosaccharides

Frozen tissue sections (15 μm) were mounted on ITO glass slides and placed in a desiccator for at least 2 h at room temperature. 9-aminoacridone in ethanol/water (70/30, v/v) was deposited on the slides by means of 8 passes using an HTX Sprayer, (HTX Technologies) to form a homogeneous layer (0.22 mg cm^{-2}). The spray parameters were as follows: flow rate (0.1 ml min^{-1}), pressure (10 psi), track spacing (2 mm), velocity (1200 mm min^{-1}), nozzle temperature (100 $^{\circ}\text{C}$), and dry time (0.5 min). MALDI-MS imaging data were acquired on a scimaX magnetic resonance mass spectrometer (Bruker Daltonics) equipped with a MALDI ion source. With respect to the MALDI-MS parameter settings: the intensity of laser light was 30% with a frequency of 300 Hz; the spatial resolution was 35 μm , and the m/z range 150-2000. The data were processed using Fleximaging software (Bruker, version 5.0) and SCiLS Lab software (Bruker, version 5.12.2).

S.Met. 3 Cultivation of oMM¹² strains with ^{13}C -HA

oMM¹² strains were obtained from the DSMZ- German Collection of Microorganisms and Cell cultures GmbH. The bacterial strains were grown in an anaerobic chamber (Concept 400, Ruskinn Technology LTD) filled with a gas mixture (7% H_2 , 10% CO_2 , 83% N_2) (Linde). All materials, including anaerobic medium, solutions and bottles, were pre-reduced in the anaerobic chamber for at least two days prior to use. Strains were grown in basal medium (Derrien et al., 2004). For the cultivation of each strain, 0.1 ml of cryostock was inoculated in 10 ml of reduced medium and the culture was incubated for 24-48 hours at 37 $^{\circ}\text{C}$ until bacterial growth was observed. Then, the culture was diluted 1:10 with fresh AAM medium and grown for 24 hours. Lastly, the grown culture was diluted in the ratio 1:20 with AAM medium to achieve a final volume of 40 ml. When the optical density reached 0.4, a sample of the culture medium was collected and ^{13}C -HA (3 mg ml^{-1}) solution was added to achieve a final concentration of 0.5 mg ml^{-1} . Samples were collected 8 h and 24 h after the addition of ^{13}C -HA. Collected culture samples were centrifuged (7,000 g, 10 min), cells were harvested, and the supernatant was stored at -80 $^{\circ}\text{C}$. Cells were washed 3 times with sterile pre-reduced PBS and then stored at -80 $^{\circ}\text{C}$.

S.Met. 4 DNA extraction and gut microbiome analysis

DNA from cecal contents was extracted using PureLink™ Microbiome DNA Purification Kit (Invitrogen) following the manufacturer's instructions. The concentration of DNA was measured by a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and the quality was checked by running the samples on 1% agarose gel. DNA was amplified in the V3-V4 region of the 16S rRNA gene using a two-step PCR protocol on an Illumina MiSeq instrument with the 2×300bp paired-end read module (INVIEW Microbiome profiling 3.0 service; Eurofins Genomics). Raw sequencing data were processed using Mothur 1.46.1 software and its MiSeq standard operating procedure (Kozich et al., 2013). Sequencing errors and duplicates were removed from the dataset. Subsequently, reads were aligned to SILVA-based bacterial reference alignment and chimeric sequences were removed. Sequences were assigned to the same Operational taxonomy units (OTUs) with a cutoff of 0.03. The Inverse Simpson Index representing alpha diversity was calculated on the normalized OTU-based dataset. Beta diversity was analyzed by means of statistical analysis of the molecular variance and by analysis of the homogeneity of the molecular variance. The method implemented in Mothur (get.communitytype) based on Dirichlet Multinomial Mixtures (Holmes et al., 2012) was used to predict the number of clusters of metacommunities. The difference in bacterial community between groups was calculated using the *metastats* and *LEfSe* methods. Processed data were visualized in MEGAN6 (Huson et al., 2016) and STAMP (Parks et al., 2014) software.

S.Met. 5 Histochemistry staining and confocal microscopy of mouse intestine

Mouse intestine was cut according to its morphological and functional parts as follows: ileum, cecum, and colon; these parts were then fixed in 4% paraformaldehyde for at least 24 hours and processed according to the procedure described in (S.Met. 1). Afterwards, slices were incubated with appropriate primary antibody overnight at room temperature; the antibody was diluted in blocking solution. Primary antibodies with the following dilutions were used for the detection of HA-related proteins: CD44 (ab157107, Abcam, 1:500), HAS1 (bs-2946R, Bioss, 1:100), HAS2 (PA525593, Invitrogen, 1:100), HAS3 (NBP-86328, Novus biologicals, 1:100), HYAL1 (bs-1235R, Bioss, 1:75), HYAL2 (bs-5888R, Bioss, 1:100), KIAA1199 (21129-1-AP, Proteintech, 1:100), Layilin (bs-4629R, Bioss, 1:400), LYVE-1 (NB100-725, Novus biologicals, 1:100) and TMEM2 (PA5-85901, Invitrogen, 1:100). Afterwards, glass slides were washed 5 times with water, and a drop of TBS was applied as drying prevention. The TBS was dried just before secondary antibody was applied. The secondary antibody used was goat anti-rabbit IgG H&L Alexa Fluor 555 (ab150078, Abcam, 1:500) and slices were incubated with it for 1h at room temperature in a dark place. After incubation, glasses were washed 5 times in water and mounted in ROTI Mount FluorCare DAPI (HP20.1, Carl Roth). Images were acquired and processed on the same instrument as described in (S.Met. 1). The 550 nm white light laser line for Alexa Fluor 555 nm was used for appropriate protein detection.

S.Met. 6 GC-MS analysis of short chain fatty acids

First, samples (100 µl) were acidified by HCl (10 µl, 5 mol l⁻¹) and diethylether (300 µl) was added. Subsequently, samples were stored at 4 °C for 15 min and then centrifugated (15,000 g, 4 °C, 10 min), and 200 µl of organic phase was transferred to a new vial and 10 µl of internal standard solution (100 µg ml⁻¹, undecanoic acid) was added. The analysis of extracts was performed on a Shimadzu GCMS-TQ8050 NX instrument. Samples were injected into an HP-FFAP capillary column (30 m, 0.25 mm, 0.25 µm) (Agilent) with He as the carrier gas. The injector temperature was 240 °C, the split ratio 1:10, the injected volume 1 µl, and the detector temperature 240 °C. The initial temperature in the oven was 80 °C (held for 2 min), then the temperature was gradually increased at a rate of 20 °C min⁻¹ to 230 °C and held for 7 min. Data were acquired in scan mode (*m/z* 30-300); the analysis of the isotope pattern was performed manually.

References

- Blundell, C. D., & Almond, A. (2006). Enzymatic and chemical methods for the generation of pure hyaluronan oligosaccharides with both odd and even numbers of monosaccharide units. *Analytical Biochemistry*, 353(2), 236–247. <https://doi.org/10.1016/j.ab.2006.03.013>
- Derrien, M., Vaughan, E. E., Plugge, C. M., & de Vos, W. M. (2004). *Akkermansia muciniphila* gen. Nov., sp. Nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*, 54(5), 1469–1476.
- Holmes, I., Harris, K., & Quince, C. (2012). Dirichlet Multinomial Mixtures: Generative Models for Microbial Metagenomics. *PLOS ONE*, 7(2), e30126. <https://doi.org/10.1371/journal.pone.0030126>
- Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.-J., & Tappu, R. (2016). MEGAN Community Edition—Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLOS Computational Biology*, 12(6), e1004957. <https://doi.org/10.1371/journal.pcbi.1004957>
- Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*, 79(17), 5112–5120. <https://doi.org/10.1128/AEM.01043-13>
- Parks, D. H., Tyson, G. W., Hugenholtz, P., & Beiko, R. G. (2014). STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics*, 30(21), 3123–3124. <https://doi.org/10.1093/bioinformatics/btu494>
- Rowley, J. E., Rubenstein, G. E., Manuel, S. L., Johnson, N. L., Surgnier, J., Kapitsinou, P. P., Duncan, F. E., & Pritchard, M. T. (2020). Tissue-specific Fixation Methods Are Required for Optimal In Situ Visualization of Hyaluronan in the Ovary, Kidney, and Liver. *Journal of Histochemistry & Cytochemistry*, 68(1), 75–91. <https://doi.org/10.1369/0022155419884879>

Article III

Excluded for confidentiality (unpublished)

Article IV

RESEARCH

Open Access



Extracellular vesicles of the probiotic bacteria *E. coli* O83 activate innate immunity and prevent allergy in mice

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Abstract

Background *E. coli* O83 (Colinfant Newborn) is a Gram-negative (G-) probiotic bacterium used in the clinic. When administered orally, it reduces allergic sensitisation but not allergic asthma. Intranasal administration offers a non-invasive and convenient delivery method. This route bypasses the gastrointestinal tract and provides direct access to the airways, which are the target of asthma prevention. G- bacteria such as *E. coli* O83 release outer membrane vesicles (OMVs) to communicate with the environment. Here we investigate whether intranasally administered *E. coli* O83 OMVs (EcO83-OMVs) can reduce allergic airway inflammation in mice.

Methods EcO83-OMVs were isolated by ultracentrifugation and characterised their number, morphology (shape and size), composition (proteins and lipopolysaccharide; LPS), recognition by innate receptors (using transfected HEK293 cells) and immunomodulatory potential (in naïve splenocytes and bone marrow-derived dendritic cells; BMDCs). Their allergy-preventive effect was investigated in a mouse model of ovalbumin-induced allergic airway inflammation.

Results EcO83-OMVs are spherical nanoparticles with a size of about 110 nm. They contain LPS and protein cargo. We identified a total of 1120 proteins, 136 of which were enriched in OMVs compared to parent bacteria. Proteins from the flagellum dominated. OMVs activated the pattern recognition receptors TLR2/4/5 as well as NOD1 and NOD2. EcO83-OMVs induced the production of pro- and anti-inflammatory cytokines in splenocytes and BMDCs. Intranasal administration of EcO83-OMVs inhibited airway hyperresponsiveness, and decreased airway eosinophilia, Th2 cytokine production and mucus secretion.

Conclusions We demonstrate for the first time that intranasally administered OMVs from probiotic G- bacteria have an anti-allergic effect. Our study highlights the advantages of OMVs as a safe platform for the prophylactic treatment of allergy.

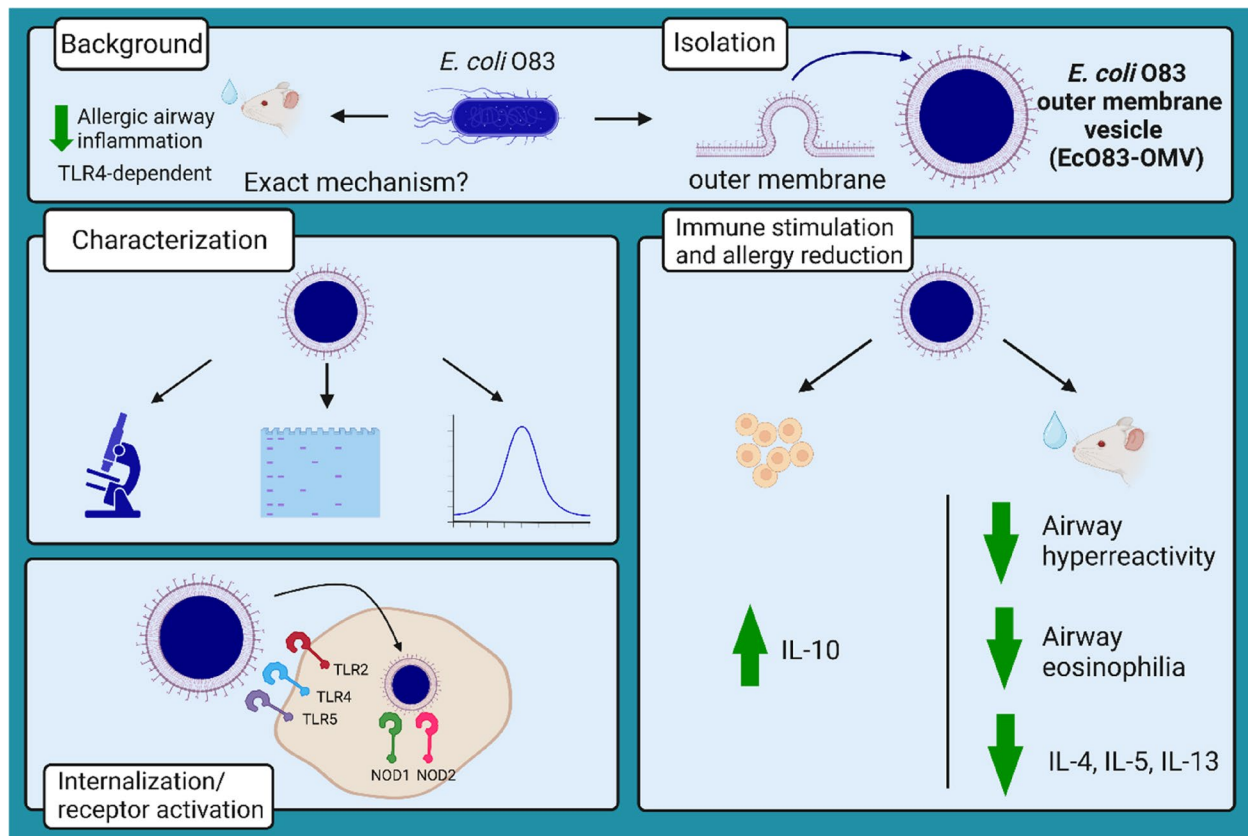
Keywords Allergic airway inflammation, Extracellular vesicles, Outer membrane vesicles, Microbiota and innate immunity, Nasal route of administration

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Graphical Abstract



Introduction

Allergies are one of the most common chronic inflammatory diseases, the prevalence of which has increased in industrialised countries in recent decades, resulting in a significant socioeconomic health burden [1]. To date, the only disease-modifying treatment available is allergen-specific immunotherapy. Although highly effective, it has several disadvantages such as regular administration over a long period of time and the occurrence of adverse reactions [2].

The exact mechanisms responsible for the development of allergies are largely unknown, but both genetic and environmental factors play a role [3]. The increasing prevalence of allergic diseases has been linked to changes in the microbial load in the environment due to profound changes in lifestyle and hygiene [4, 5]. Therefore, restoring a balanced microbiota at mucosal surfaces, such as the lung and gut, with probiotics is a promising strategy to prevent or treat allergies. Several clinical trials have tested the effect of oral administration

of live probiotic bacterial strains in the treatment of allergic diseases [6]. Although some beneficial effects of probiotic bacteria in reducing allergic sensitisation have been reported [7, 8], the efficacy in preventing or treating allergic asthma appears inconsistent [9–11].

Studies in mice have shown that intranasal administration of *Lactobacillus paracasei* NCC246 during aerosol challenge reduced the recruitment of inflammatory cells and the concentrations of IL-5 in the lungs of ovalbumin (OVA)-sensitised and challenged mice [12]. Along these lines, intranasal administration of *Lactobacillus rhamnosus* GG, but not the *L. rhamnosus* strain GR-1, prevented allergic asthma in a mouse model [13]. We have shown that *Bifidobacterium longum* NCC 3001, but not *L. paracasei*, administered intranasally prior to sensitisation and challenge, suppressed allergen-specific immune responses in mice [14].

The Gram-negative (G-) probiotic bacterial strain *E. coli* A0 34/86 (*E. coli* O83; serotype O83:K24:H31) is a commercially available live oral vaccine (Colinfant

Newborn). Although oral administration of *E. coli* O83 to high-risk infants reduced allergic sensitisation (skin disease), this bacterium did not provide protection against the development of respiratory allergies [15, 16]. We recently demonstrated that the route of delivery of probiotics might be important since *E. coli* O83 administered intranasally reduced allergic lung inflammation in mice. Mechanistically, the TLR4 signalling pathway was required for this beneficial effect [17].

Probiotic bacteria are generally well tolerated and considered safe. However, probiotic therapy in clinics may be associated with some risks in vulnerable target groups such as pregnant women, young children, and immunocompromised individuals [18]. Although rare, intentional supplementation of probiotics was linked with side effects such as systemic infections, toxic metabolic activities, or gene transfer [19]. Therefore, the discovery of non-living bacterial products that modulate immune function and can be conveniently used in daily life will open up new possibilities for the prevention or treatment of allergies.

G- bacteria produce extracellular vesicles called outer membrane vesicles (OMVs). They are produced during normal bacterial growth by detachment of the outer membrane and consist largely of outer membrane components such as lipopolysaccharide (LPS), lipids and proteins, but may also contain material from the periplasmic space or from within the bacterial cell, such as nucleic acids [20]. Several studies have investigated OMVs of pathogenic [21–24] or non-pathogenic [25–27] *E. coli* strains, but OMVs of *E. coli* O83 have not yet been investigated. OMVs contain microbe-associated molecular patterns that give them immunostimulatory properties. They can interact with different pattern recognition receptors in and on host cells to stimulate innate and adaptive immune responses [28]. As natural and non-replicative particles, OMVs are ideal candidates for use as safe vaccine platforms due to their nanosize (20–200 nm), low toxicity, cargo protection, ability to cross biological barriers, and the simplicity of their manufacturing and purification processes [29–31]. Although some OMVs-based vaccines against infectious diseases are already on the market [32], the potential of OMVs to treat non-communicable diseases, such as allergies, has not yet been satisfactorily investigated.

In this study, we hypothesised that non-proliferating *E. coli* O83-derived vesicles have immunomodulatory potential and can recapitulate the allergy-preventive effect of their parent strain in a mouse model of experimental asthma, providing a mucosal vaccine candidate with fewer risks associated with their use in humans.

Materials and methods

Bacteria

Escherichia coli strain A0 34/86 (*E. coli* O83; serotype O83:K24:H31) was grown in 10 ml Luria–Bertani (LB) medium (Sigma Aldrich, St. Louis, MO) as described before [17]. The culture was inoculated with a sample from a frozen glycerol stock and incubated for 3 h at 37 °C and 200 rpm. The colony-forming units (CFU) were calculated using a growth curve established in house. For experiments in cell culture, the bacteria were inactivated with 1% paraformaldehyde (PFA; SAV Liquid production, Flintsbach, Germany) for 3 h at room temperature (RT), washed twice in phosphate-buffered saline (PBS; Gibco, Thermo Scientific, Waltham, MA) and stored at -20 °C until further use. For the preparation of the lysate, 10 ml overnight culture of *E. coli* O83 in brain heart infusion broth (BHI; Sigma Aldrich, St. Louis, MO) was centrifuged (10 min at 4 °C and 3 000×g) and the pellet was washed twice with PBS. The washed pellet was then dissolved in 1 ml of lysis buffer (20 mM Tris–HCl, 0.14 M NaCl, 10% glycerol; pH=7) containing protease inhibitor cocktail (Roche, Mannheim, Germany) and then disrupted in a Precellys tissue homogenizer using microorganism disruption beads (both from Bertin Technologies, Montigny-le-Bretonneux, France) (30 s at RT and 6 500 rpm). The supernatant was kept at -20 °C until further use.

Isolation of *E. coli* O83-derived outer membrane vesicles (EcO83-OMVs)

Vesicles were isolated according to published protocol [33]. Briefly, *E. coli* O83 was grown in BHI in an overnight culture at 37 °C and 180 rpm. The main culture was inoculated 1:100 from the overnight culture and grown for 8 h at the same conditions. The culture was centrifuged for 15 min at 4 °C and 10 000×g. The supernatant was filtered through a 0.22 µm-pore-size filter (Merck Millipore, Burlington, MA). OMVs were isolated by ultracentrifugation (4 h at 4 °C and 150 000×g; Beckman Coulter 45Ti rotor). The pellet was dissolved in 500 µl of sterile 0.9% NaCl (B. Braun, Maria Enzersdorf, Austria). OMVs were stored at 4 °C for immediate use or at -20 °C for long-term storage.

Protein and endotoxin analysis

Protein concentration was determined by Bradford protein assay (BioRad, Hercules, CA) and the endotoxin content was analysed using the PyroGene™ Recombinant Factor C Endpoint Fluorescent Assay (Lonza, Basel, Switzerland) according to manufacturer's instructions. The absorbance and fluorescence were measured with TECAN Spark 10 M plate reader (Tecan Trading AG,

Switzerland). The protein profile of OMV samples was analysed by Agilent Bioanalyzer 2100 with the Protein 230 kit (Agilent Technologies, CA, USA) according to manufacturer's instructions.

Transmission electron microscopy (TEM)

The bacterial culture sample taken before filtration and centrifugation and the isolated EcO83-OMVs were imaged with TEM. The bacterial culture was centrifuged at 5 000×g and the pellet was washed twice with PBS before microscopy. EcO83-OMVs were dissolved in NaCl. Ultrastructural visualization was carried out by single-droplet negative stain. Briefly, 5 µl of each OMV preparation were left to adhere on formvar- and carbon-coated grids for 20 s. Simultaneous addition of 5–10 µl 1% uranylacetate and absorption of liquid was repeated twice to contrast the samples. Dried grids were imaged in an FEI Tecnai20 electron microscope (FEI, Eindhoven, NL) equipped with a 4 K Eagle-CCD camera.

Liquid chromatography/mass spectrometry (LC-MS) analyses of proteins

The label-free proteomics analyses were performed as previously described [34]. Briefly, 20 µg of protein was dissolved in SDS-PAGE loading buffer, subjected to SDS-PAGE followed by reduction, blocking of reduced cysteine residues and in-gel trypsin digestion. Resulting peptides were purified with PepClean™ C18 Spin Columns and dried on a miVac Duo Concentrator (Genevac, Ipswich, UK). Samples were stored at -20 °C until LC-MS/MS analyses. Peptide samples were analysed by nano LC (Easy-nLC 1200, Thermo Scientific, Waltham, MA)-tandem MS (Q-Exactive HF-X Hybrid Quadrupole Orbitrap, Thermo Scientific, Waltham, MA). Proteins were identified and quantified using MaxQuant [35] (version 1.5.3.30) with its Andromeda algorithm against the *E. coli* (strain K12) sequence database (*E. coli* proteome with 4448 reviewed sequences downloaded from UniProt database, <https://www.uniprot.org/>). The “match between runs” was applied and LFQ data were used for quantification using Perseus v1.6 [36]. Values below detection limit were assumed to be zero. A fold change of two or higher was considered for enrichment. The Venn diagram was created using DeepVenn (arXiv:2210.04597) and the volcano plot was created using VolcanoR [37]. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [38] with the dataset identifier PXD040963.

Culture and stimulation of human embryonic kidney (HEK) 293 cells

Recognition of EcO83-OMVs by innate pattern recognition receptors was tested on HEK 293 cells transfected

with human receptors hNOD1, hNOD2, hTLR2, hTLR5 and hTLR4/CD14/MD2. Cells were cultured according to published protocols [39] using the appropriate culture media (Table S1). Briefly, cells were stored in liquid nitrogen in freezing medium containing 10% DMSO (Merck, Darmstadt, Germany). The cells were thawed and transferred to the required growth medium (Table S1). Cells were grown at 37 °C and 5% CO₂. Generally, the growth medium was changed every 2–3 days and cultures were split when they reached ~80% confluence. HEK-293/hTLR4/CD14/MD2 cells were grown in cell culture flasks for challenging adherent cells (Sarstedt, Nürnbrecht, Germany). HEK-293 cells were seeded at the density of 4×10⁵ cells/ml, 100 µl/well, in sterile 96-well flat bottom plates and incubated at 37 °C in the respective growth media (Table S1) overnight before the stimulation. The cells were stimulated with EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml, and 1000 ng/ml), PFA-fixed *E. coli* O83 (10⁷ CFU/ml) and the respective positive control Pam3CysSerLys4 (Pam3CSK4; 1 µg/ml) for TLR2, ultrapure LPS (1 µg/ml) for TLR4, recombinant flagellin protein from *Salmonella typhimurium* (recFLA-ST; 100 ng/ml) for TLR5, L-Ala-γ-D-Glu-mDAP (Tri-DAP; 5 µg/ml) for NOD1 and muramyl dipeptide (MDP; 5 µg/ml) for NOD2 (all from Invivogen, Toulouse, France) at 37 °C and 5% CO₂ for 24 h. Untreated cells were used as negative controls. The activation of a specific receptor was confirmed by measuring IL-8 in the cell culture supernatants.

Mice

Wild type BALB/c mice (female, aged 6–8 weeks) were purchased from Charles River (Sulzfeld, Germany). Animals were kept in conventional housing at the Institute for Pathophysiology and Allergy Research of the Medical University of Vienna with free access to food and water. Experiments were approved by the Animal Experimentation Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Culture (BMBWF-66.009/0277-V/3b/2019).

Isolation and stimulation of splenocytes and bone marrow-derived dendritic cells (BMDCs) derived from naïve mice

Spleens from naïve mice were isolated, cultured, stimulated and supernatants were analysed as previously described [40]. Briefly, they were collected in RPMI 1640 media containing 100 µg/ml gentamycin, pushed through a metal net and the cell suspension was then pipetted through a 70 µm cell strainer. Samples were incubated in ACK Lysing Buffer (Lonza, Basel, Switzerland) for 1.5 min to lyse erythrocytes and cells were resuspended in complete RPMI medium (Table S1). 5×10⁵ cells/well

were plated in 96-well plates and stimulated with EcO83-OMVs (1 ng/ml, 10 ng/ml, and 100 ng/ml), PFA fixed *E. coli* O83 (10^6 , 10^7 , and 10^8 CFU /ml) and LPS (1 µg/ml;) at 37 °C and 5% CO₂ for 72 h. Cell culture supernatants were collected and cytokines were analysed. The isolation and differentiation of the bone marrow cells into BMDCs was performed as described previously [41]. In short, femur and tibia were extracted from BALB/c mice. The bones were cleaned from any remaining tissue and washed in 70% ethanol by shaking for one min followed by rinsing twice in PBS. Both ends of the bones were cut off and the bone marrow was flushed out with 2.5 mL BMDC medium (Table S1) from each side. The cell suspension was then pipetted through a 70 µm cell strainer, red blood cells were lysed using ACK lysing buffer and cells were re-suspended in BMDC medium containing 20 ng/mL recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF; Peprotech, London, UK). Cells were seeded in a Petri dish (2×10^5 cells/ dish in 10 ml medium) and maintained as follows: on day 3, 10 ml of DC-medium with rGM-CSF were added to each dish; on day 6, 10 ml were taken out of each dish and replaced by fresh DC-medium with rGM-CSF; on day 9, cells were harvested by centrifugation, and seeded at 1×10^7 cells/ml in 48-well flat bottom plates. The cells were stimulated for 24 h with the stimuli indicated above for the splenocytes and the cytokines were measured in the cell culture supernatants.

Allergic sensitisation, allergic challenge, and intranasal treatment with EcO83-OMVs

Airway inflammation was induced in female 6–8 weeks old BALB/c mice with ovalbumin (OVA; grade V; Sigma-Aldrich, St. Louis, MO) as previously described [17]. Briefly, mice were intraperitoneally (i.p) sensitized with 10 µg OVA and 65 µg aluminiumhydroxide (alum; SERVA Electrophoresis, Heidelberg, Germany) or PBS/alum in 150 µl on days 0 and 14. On days 21–24, mice were anesthetized with 5% isoflurane (CP-pharma, Burgdorf, Germany) and treated with 100 µg OVA in 30 µl PBS intranasally (i.n.) or 30 µl PBS alone. Thirty minutes prior to each OVA application, mice received i.n. 0.1 µg (group OMVs 0.1 µg/OVA), 1 µg of EcO83-OMVs (group OMVs 1 µg/OVA), or 0.9% NaCl (groups Sham/PBS and Sham/OVA). On day 25, airway hyperresponsiveness was tested using unrestrained whole-body plethysmography (Buxco® Small Animal Whole-body Plethysmography, Data Sciences International; St Paul, MN). Mice were subjected to increasing doses (0–50 mg/mL) of nebulized methacholine (Sigma-Aldrich, St. Louis, MO) and the enhanced pause was measured as an index for airway obstruction. Mice were terminally anesthetized on day

26, spleens and lungs were harvested and bronchoalveolar lavage (BAL) and blood were taken.

Characterisation of cell populations in the BAL

Lungs were lavaged twice with 500 µl of ice-cold PBS, and cells were collected by centrifugation. The pellet was resuspended in 200 µL PBS and 4×10^4 cells were spun onto a microscopic slide (Shandon Cytospin; Shandon Southern Instruments, Waltham, MA), air-dried and stained with haematoxylin and eosin (Hemacolor; Merck, Darmstadt, Germany). Slides were scanned using the Tissue FAXSi Plus system and cells were counted in Tissue FAXS viewer (TissueGnostics, Vienna, Austria).

Lung histology and isolation and restimulation of lung cells ex vivo

Histological evaluation of lungs and isolation, stimulation, and analysis of pulmonary single cell suspensions were performed according to published protocol [40]. Briefly, lungs were fixed in 7.5% formaldehyde (SAV Liquid production, Flintsbach, Germany) and the tissue was embedded in paraffin (Carl Roth, Karlsruhe, Germany), cut into three µm sections and stained using haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS, Sigma-Aldrich). Pathology score was evaluated based on a scoring system adapted from Zaiss et al. [42] by analysing 5 independent regions on each sample (H&E: inflammatory cells in peribronchial sites: 0=no inflammation; 1=few inflammatory cells; 2=layer of 1–4 inflammatory cells; 3=layer of 4–10 inflammatory cells; 4=layer of more than 10 inflammatory cells; PAS: mucus secretion and presence of goblet cells: 0=no goblet cells; 1=mild mucus secretion, 1/3 of epithelium occupied by goblet cells; 2=moderate mucus secretion, 1/3–2/3 of epithelium occupied by goblet cells; 3=high mucus secretion and >2/3 of epithelium occupied by goblet cells).

Isolation and restimulation of lung cells ex vivo

Lungs were minced and incubated in 6 ml RPMI 1640 (Biowest, Nuaillé, France) containing 100 µg/ml gentamycin (Carl Roth, Karlsruhe, Germany), 10 µg/mL liberase TL (Roche, Mannheim, Germany) and 0.5 mg/mL DNase (Sigma-Aldrich, St. Louis, MO) at 37 °C and 5% CO₂ for 45 min. The lung pieces were then pushed through a 70 µm cell strainer and red blood cells were lysed as described above. Cells were resuspended in complete RPMI (Table S1) and cells were plated at a concentration of 5×10^6 cells/ml in 96-well plates. Cells were stimulated with endotoxin-free OVA (100 µg/ml; Endo-Grade; Hyglos, Bernried am Starnberger See, Germany) at 37 °C and 5% CO₂ for 72 h. Cell culture supernatants were collected and cytokines were analysed.

ELISA

For detection of cytokines, eBioscience Ready-SET-Go! ELISA kits (eBioscience, San Diego, CA) were used according to manufacturer's instructions. Briefly, a 96-well-plate (Nunc MaxiSorp™, Thermo Scientific, Waltham, MA) was coated with the provided capture antibody and incubated at 4 °C overnight. The plate was washed 3X with PBS containing 0.05% Tween 20 (Carl Roth, Karlsruhe, Germany) (PBST). The plate was blocked with 200 µl of the kit's ELISA/ELISPOT diluent at RT for 1 h. Samples and standards were diluted in the same diluent, 50 µl were added to the plate and incubated at 4 °C overnight. Technical triplicates of the samples were used in all assays. This and all subsequent steps were followed by a washing step as described above. Biotinylated detection antibody was incubated at RT for 1 h. Then, horseradish-peroxidase conjugated avidin was added and incubated at RT for 30 min, and for detection, the substrate TMB was used. The reaction was stopped using 0.18 M H₂SO₄ (Carl Roth, Karlsruhe, Germany) and the absorbance at 450/570 nm was measured in a TECAN Spark™ 10 M plate reader.

Statistics

Statistical analysis was performed in GraphPad Prism Software 9 (GraphPad Software Inc.) using One-Way ANOVA or Two-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. Data are shown as mean ± SD. Significant differences are marked as **p* < 0.05; ***p* < 0.05; ****p* < 0.01, *****p* < 0.001.

Results

Probiotic *E. coli* O83 produces outer membrane vesicles

EcO83-OMVs were isolated from cell-free culture supernatants by ultracentrifugation (Fig. 1A). In parallel, we processed the vesicle-free supernatant and mock control (Supplementary Material and Methods). To exclude the possibility of contamination of purified vesicles with live bacteria, EcO83-OMVs and both control samples were plated on agar plates and examined for the presence of bacterial colonies. Even after 96 h of culture, no bacterial colonies were detectable on the plates with neither of the sample (Figure S1). TEM visualization showed that EcO83-OMVs are spherical structures with a diameter of

approximately 100 to 200 nm (Fig. 1B and C). Figure 1B shows a TEM image of budding vesicles on the surface of the parent bacteria and their release into the surrounding medium. This sample was taken before the purification process. Dynamic light scattering (DLS) measurement revealed a high concentration of vesicles with a mean size around 110 nm (Fig. 1D). In total, we isolated 7×10^{12} particles from a two litre culture of *E. coli* O83 (O.D. = 2) (Table S2). To compare the protein profile of the vesicles with that of the parental bacteria, we performed automated electrophoresis with the Bioanalyzer. The lysate of the whole bacteria and the OMVs had different protein profiles (Fig. 1E and S2). Several prominent bands of about 19, 34, 49 and 64 kDa, can be identified in the OMV sample (Fig. 1E; Table S3). The most dominant protein had a size of 64 kDa and accounted for 27.5% of the total protein content (Table S3). We performed a LC-MS-based label-free protein quantification to compare the protein expression profile in *E. coli* O83 bacteria and EcO83-OMVs in more detail. While most of the identified proteins (545) are shared by the OMVs and the parent bacteria, there is a group of 136 proteins (Fig. 1F), including flagellar proteins FlgL 1 and 3, FlgE, FlgG, and FlhC, detected exclusively in the vesicles (Fig. 1G and Table S4). The molecular mass of flagellin ranges from 37 to 67 kDa depending on the *E. coli* strain [43]. Our preliminary sequencing data confirm that the band of 64 kDa is *E. coli* O83 flagellin (data not shown). LPS is the major microbe-associated molecular pattern of the outer membrane of Gram- bacteria and we have shown that LPS content in OMVs is approximately 5 times higher than the protein content (Table S2).

EcO83-OMVs activate the surface receptors TLR4, TLR2, and TLR5 and the intracellular receptors NOD1 and NOD2

OMVs derived from G- bacteria have previously been shown to interact with the host via their cargoes such as LPS, lipoproteins and flagellin [23, 44, 45] leading to modulation of the innate immune response. We have previously shown that *E. coli* O83 prevents the development of allergies in mice and that this effect depends on its interaction with TLR4 [17]. Here we show that EcO83-OMVs, similar to the parent bacteria, target TLR4, confirming the presence of the TLR4 ligands on

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Fig. 1 Isolation and characterisation of EcO83-OMVs. **A** OMVs were isolated from the culture supernatant of *E. coli* O83 by ultracentrifugation. **B** Visualisation of the budding process of EcO83-OMVs in the sample taken before filtration and centrifugation by TEM. **C** Visualisation of the isolated EcO83-OMVs by TEM. Scale bar = 200 nm. **D** The size of the isolated EcO83-OMVs were determined by Dynamic Light Scattering. **E** Protein profiles of *E. coli* O83 lysate and EcO83-OMVs analysed by Bioanalyzer. The unaltered original picture is shown in Fig. S1. **F** Intersection of proteins associated with *E. coli* O83 lysate and EcO83-OMVs depicted by Venn diagram. **G** Volcano plot visualization of enriched proteins in EcO83-OMVs compared to *E. coli* O83 lysate with annotated flagella proteins

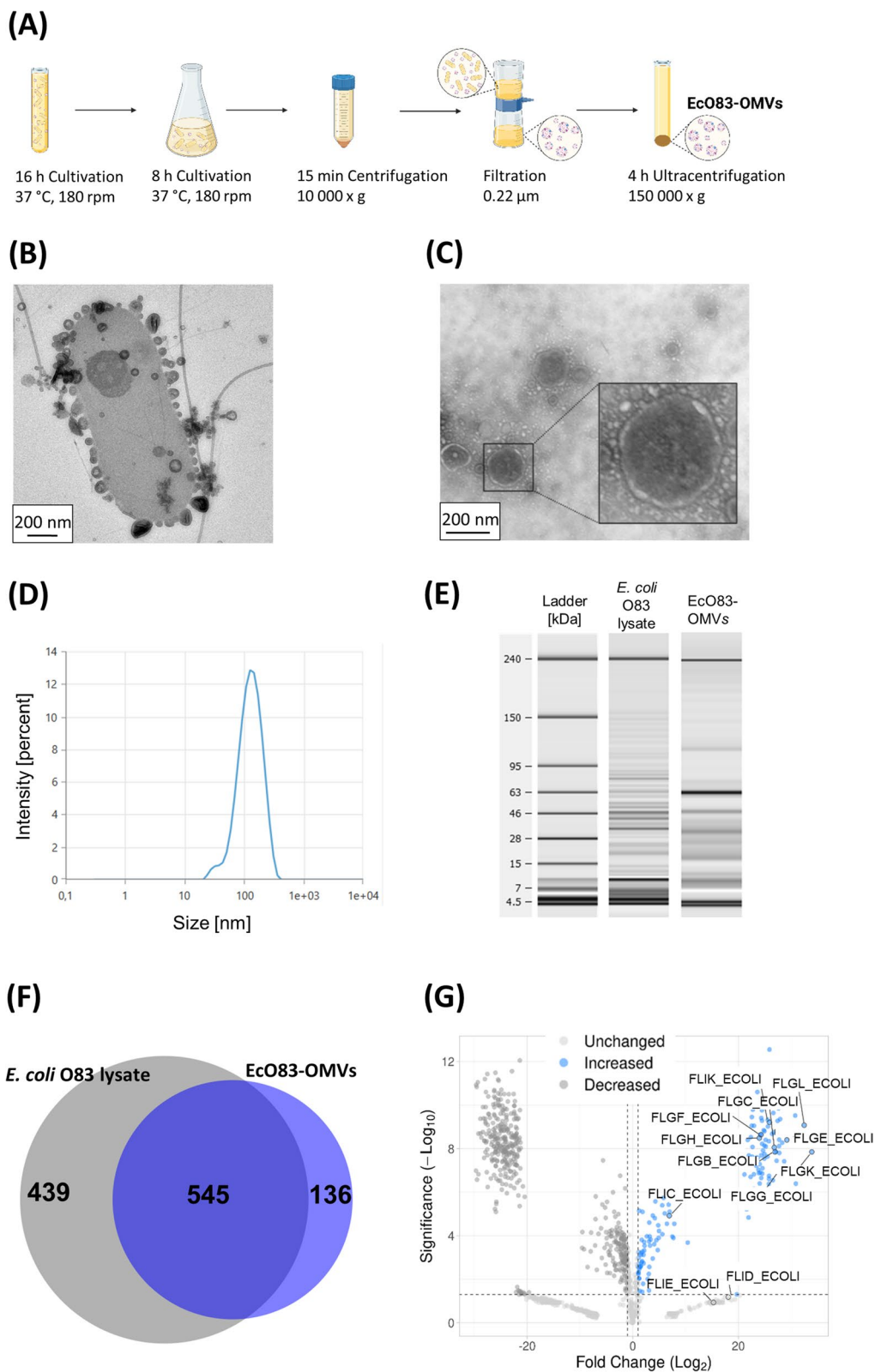


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the bacteria and also on their vesicles (Fig. 2A). Similarly, we observed the involvement of TLR2 (lipoprotein receptor) and TLR5 (flagellin receptor) in the interaction with EcO83-OMVs (Fig. 2A). TLR2, but not TLR5, was activated by the parent bacteria, confirming our observation from proteomic analysis showing that flagellum-associated proteins are more abundant in OMVs than in the parent bacteria.

Unlike TLR2/4/5, which recognise microbial ligands on the cell surface, NOD1 and NOD2 sense bacterial products such as peptidoglycan in the cytosol [46, 47] and bacterial OMVs have been shown to deliver peptidoglycan into the cytoplasm [47]. NOD1 and NOD2 recognised EcO83-OMVs and whole bacteria lysate, and EcO83-OMVs interact with these receptors in a dose-dependent manner (Fig. 2B).

HEK-293 cells transfected with TLR2, TLR4, and TLR5 produce IL-8 when incubated with EcO83-OMVs, as detected by ELISA. IL-8 is a chemokine produced in response to TLR agonists in an NF- κ B/AP-1-dependent manner [48, 49]. Since NF- κ B is a crucial transcription factor for the induction of a plethora of inflammatory genes [50], we investigated whether EcO83-OMVs can induce the expression of other NF- κ B target genes. RAW264.7 cells were treated with medium, LPS (1 μ g/ml), *E. coli* O83 (10⁷ CFU/ml) and EcO83-OMVs (10 ng/ml and 100 ng/ml) for 6 h. The results show that EcO83-OMVs induced mRNA expression of IL-6, TNF- α and IL-1 β in a dose-dependent manner, further confirming the involvement of the NF- κ B pathway (Figure S3).

EcO83-OMVs have an immunomodulatory effect in vitro

EcO83-OMVs induced high levels of IL-10, IL-17 and IFN- γ in naïve splenocytes in a dose-dependent manner (Fig. 3A). The pro-inflammatory cytokine TNF- α was induced to a much lesser extent and IL-6 was not induced by OMVs at any of the concentrations used (Fig. 3A). EcO83-OMVs are potent inducers of IL-10, a cytokine with regulatory potential, because 1 ng of vesicles induced amounts comparable to the response elicited by 10⁷ CFU/ml fixed *E. coli* O83 bacteria (Fig. 3A). Dendritic cells (DCs) are the most potent antigen-presenting cells and are directly associated with the

instruction and regulation of the adaptive immune response [51]. BMDCs stimulated by vesicles produced significant amounts of IL-6, IL-23 and IL-1 β (Fig. 3B). The levels of IL-12p70 were not significantly different from those triggered by medium alone (Fig. 3B). To demonstrate that the observed functional effects were associated with EcO83-OMVs and not soluble non-vesicular components, the immunostimulatory effect of EcO83-OMVs was compared with a vesicle-free supernatant and the mock control (Supplementary Material and Methods). Lung cells treated with LPS, *E. coli* O83 and EcO83-OMVs produced IL-6 and TNF- α . No production of these cytokines could be measured in the cultures with vesicle-free supernatant and mock control (Figure S4), ruling out the possibility of a contributing effect of non-vesicular material. We have shown that EcO83-OMVs contain LPS (Table S2) and that they are recognised by TLR4, the key receptor involved in LPS recognition and signal initiation (Fig. 2A). To investigate the role of EcO83-OMVs LPS in immunomodulation, EcO83-OMVs were treated with Polymyxin B or Polymyxin B mock (Supplementary Material and Methods) and then these vesicles were used to treat lung cell cultures. The data show that treatment of EcO83-OMVs with Polymyxin B reduced their ability to trigger the production of IL-6 and TNF- α compared to vesicles treated with Polymyxin mock (Figure S5), suggesting that LPS may be important for the immunostimulatory effect of EcO83-OMVs. Finally, EcO83-OMVs were heat-inactivated at 95 °C for 15 min and added to lung cells (Supplementary Material and Methods) to test whether heat-labile surface molecules are involved in their immunostimulatory properties. The data show that heat inactivation did not reduce the levels of IL-6 and TNF- α in the cell cultures compared to untreated vesicles (Figure S6).

Preventive effect of intranasally administered EcO83-OMVs on experimental asthma

In our previous study, intranasal administration of live *E. coli* O83 reduced OVA-induced allergic asthma in a mouse model [17]. Here we tested whether EcO83-OMVs can recapitulate the beneficial effects of live bacteria (Fig. 4A). In this model, repeated exposure of mice

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Fig. 2 Receptor activation by EcO83-OMVs. HEK-293 cells expressing (A) hTLR2, hTLR4 and hTLR5, (B) hNOD1 and hNOD2 were treated with the respective positive control (Pam3CSK 1 μ g/ml, LPS 1 μ g/ml, recFLA-ST 100 ng/ml, Tri-DAP 5 μ g/ml, MDP 5 μ g/ml), fixed *E. coli* O83 (10⁷ CFU/ml), EcO83-OMVs (OMVs; 1 ng/ml, 10 ng/ml, and 100 ng/ml for TLR4; and 10 ng/ml, 100 ng/ml and 1000 ng/ml for TLR2, TLR5, NOD1 and NOD2) or medium only. Receptor activation was detected as an increase in IL-8 production. Data were analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; + = significant difference between positive and negative control. Data are representative of three independent experiments. Mean $\pm$ SD is shown. Significant differences between EcO83-OMVs and the negative controls are indicated n.d. = not detected

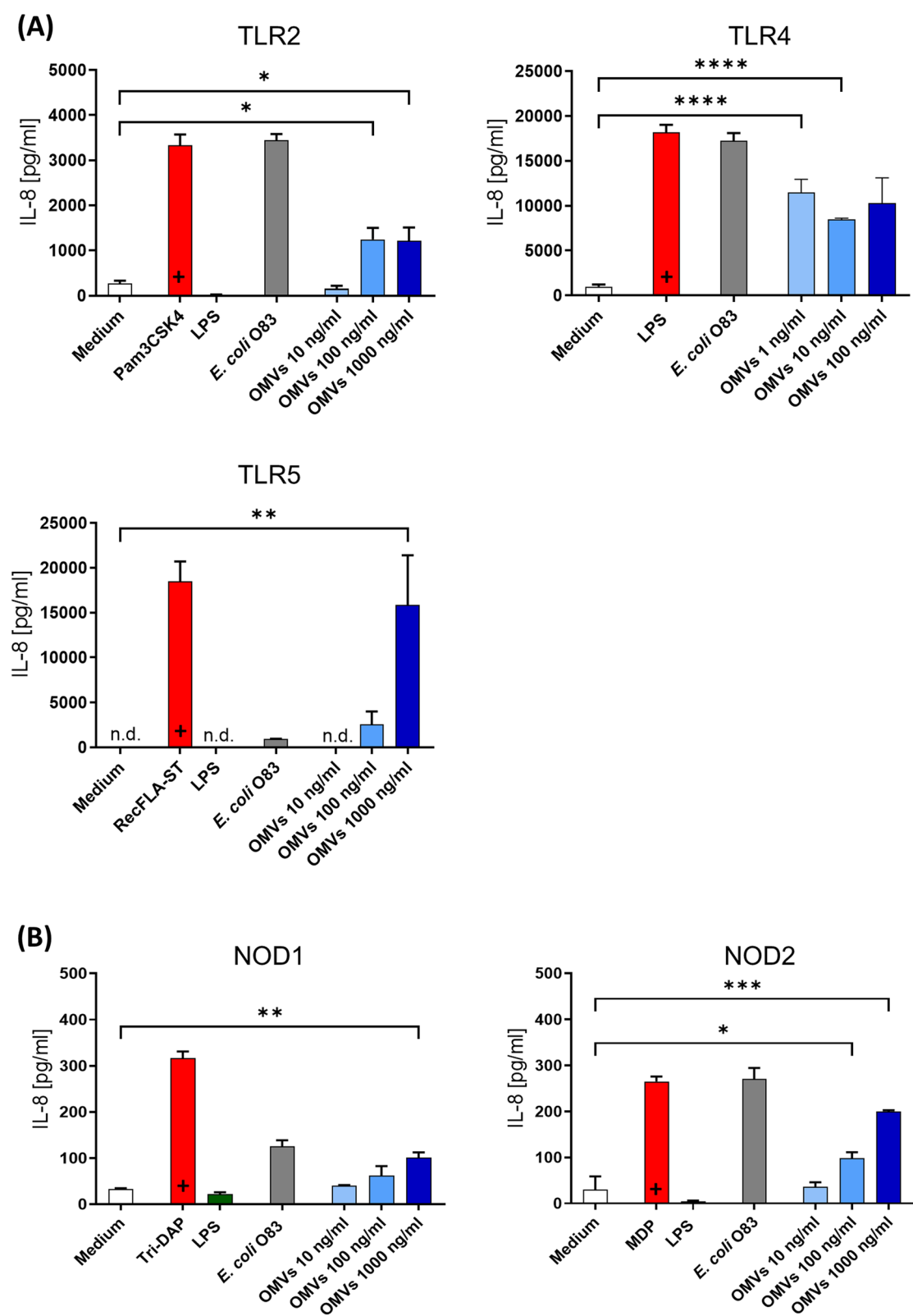


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to OVA leads to airway hyperresponsiveness, the main feature of asthma (Fig. 4B). Intranasal pretreatment with 1 µg EcO83-OMVs significantly decreased enhanced pause levels after exposure to methacholine compared to sham-treated OVA-sensitised mice, demonstrating the protective effect of vesicles (Fig. 4B).

Intranasal treatment of mice with EcO83-OMVs reduced the total number of cells in the lung (Fig. 4C) and the number of eosinophils in the BAL compared to the sham-treated OVA group (Fig. 4D). The number of macrophages, neutrophils and lymphocytes in the BAL did not change in the EcO83-OMVs group compared to the allergic control group (Fig. 4D). Decreased levels of allergen-specific IgA and IgE in BAL fluid were observed in the OMV-treated mice compared to controls (Figure S7).

Histological analysis of lung sections confirmed reduced eosinophil infiltration (Fig. 4E) and a decrease in mucus-producing cells (Fig. 4F) in mice treated with 1 µg EcO83-OMVs. This dose also decreased the disease score based on evaluation of H&E-stained samples (Fig. 4G) and mucus production (Fig. 4H). The lower dose of 0.1 µg reduced the disease score based on evaluation of H&E-stained samples (Fig. 4G), but not based on mucus production (Fig. 4H). Single-cell lung suspensions were stimulated with OVA and the levels of IL-4, IL-5, IL-13, IL-10 and IFN-γ were measured in the supernatant. The group treated with 1 µg EcO83-OMVs exhibited significantly lower levels of IL-4, IL-5, IL-13 and IL-10 compared to sham-treated controls (Fig. 5). Treatment with 0.1 µg EcO83-OMVs reduced the levels of IL-5, IL-13 but not IL-4 and IL-10 (Fig. 5) compared to allergic controls. IFN-γ levels were increased in both treatment groups compared to the allergic control, but the effect was not significant (Fig. 5).

The systemic response was assessed by measuring the level of specific antibodies in serum and by the production of cytokines in OVA-restimulated spleen cell cultures. Treatment with EcO83-OMVs had no effect on the levels of OVA-specific serum antibodies (Figure S8A) and OVA-specific IFN-γ in stimulated splenocytes (Figure S8B). We observed a decrease in the levels of IL-5 in OVA-stimulated splenocytes (Figure S8B) after treatment with 1 µg EcO83-OMVs and IL-4, IL-5 and IL-10 after

treatment with 0.1 µg EcO83-OMVs compared to sham-treated OVA-sensitised controls.

Discussion

This is the first study demonstrating a protective effect of OMVs from a probiotic *G-* bacterium in a mouse model of allergic asthma. Here we describe the isolation, identification, biophysical characterisation, interaction with innate receptors and anti-allergic properties of *E. coli* O83 OMVs.

Although numerous epidemiological studies propose that continuous high exposure to environmental bacteria and endotoxins has a protective effect against allergic sensitisation and asthma, the exact mechanisms are not yet clear [52, 53]. Preclinical studies confirmed the beneficial effect of intranasal administration of farm dust [53, 54] or farm dust microbes [55–57] and suggested that LPS is at least largely responsible for the beneficial effect [53, 54, 58]. We have shown in our previous study that intranasally administered *E. coli* O83 reduced allergy in a TLR4-dependent manner, suggesting the role of LPS [17]. Here we show that EcO83-OMVs, like their parent bacteria contain endotoxin and are recognised by TLR4.

Furthermore, we have shown that treatment of EcO83-OMVs with Polymyxin B (a cyclic cationic polypeptide antibiotic that inhibits LPS-induced TLR4 activation) reduces their ability to trigger the production of IL-6 and TNF-α in lung cells, suggesting that LPS may be important for the immunostimulatory effect of EcO83-OMVs. As the role of LPS displayed on the surface of EcO83-OMVs in the prevention of allergy is tantalising, we are planning a follow-up study using TLR4-deficient mice in an in vivo model of allergic airway inflammation.

The effects of intranasal administration of LPS on pulmonary allergy are complex and to some extent controversial, with some studies showing a worsening, while others show a reduction in allergy [59, 60]. It is now understood that variables such as timing, dosage, chemical structure, and the resulting biological activity of the used LPS can affect the type of immune response that occurs [61]. Therefore, based on its safe and unique properties, EcO83-OMVs have clear advantages over LPS and offer significant potential for a range of clinical applications.

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Fig. 3 Stimulation of naïve mouse splenocytes and BMDCs with EcO83-OMVs. **A** Mouse splenocytes and **B** BMDCs were stimulated with LPS (1 µg/ml), fixed *E. coli* O83 (10^7 CFU/ml), EcO83-OMVs (OMVs; 1 ng/ml, 10 ng/ml, and 100 ng/ml) for 72 and 48 h, respectively. Unstimulated cells served as controls (Medium). Cytokine levels were measured in the cell culture supernatants by ELISA. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.05$; *** $p < 0.01$. **A** Data are representative of four and **B** two independent experiments, respectively. Mean $\pm$ SD is shown. Significant differences between EcO83-OMVs and the negative controls are indicated. BMDC = bone marrow-derived dendritic cell; n.d. = not detected

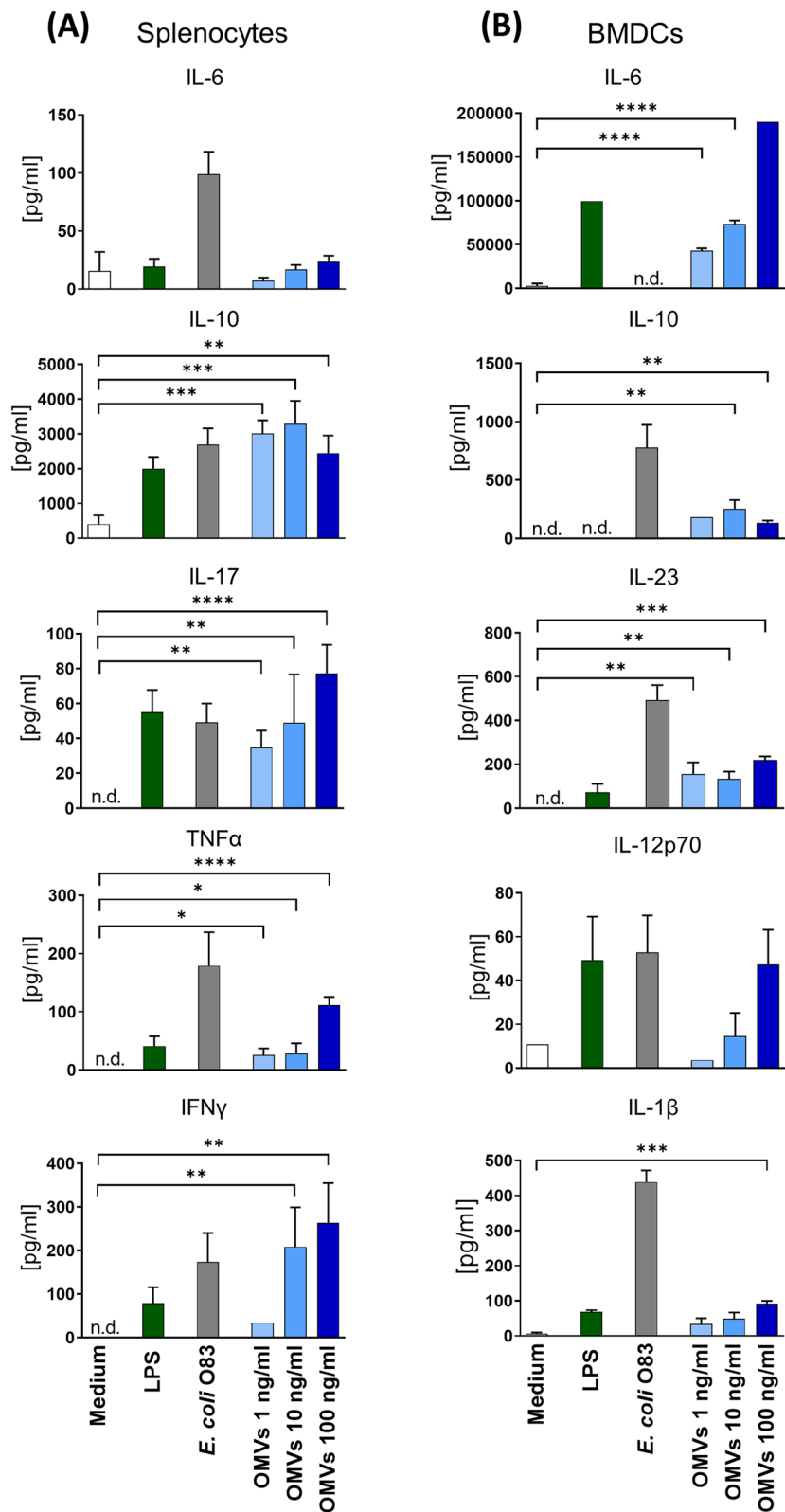


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In terms of timing, EcO83-OMVs were administered concomitantly with the allergen, at the time of allergic sensitization and challenge, using the same experimental protocol as we used for the application of live bacteria in our previous study [17]. In this sense, Tulic et al. reported that *Salmonella typhimurium* LPS reduced pulmonary allergy when administered prior or up to 4 days after OVA sensitization, but worsened it when administered more than 6 days after sensitization [62]. Furthermore, Bickert et al. have shown that LPS from *E. coli* 026:B6 reduced eosinophilia only when applied at the time of OVA challenge but not when administered before or after sensitization [63]. Whether prophylactic administration of vesicles prior to sensitization and challenge has the potential to reduce allergy or whether the beneficial effect is long-lasting remains to be investigated.

It has been demonstrated that the dose of LPS delivered intranasally also determines the direction in which the immune system reacts. A high dose (100 µg) of LPS from a bacterial strain other than *E. coli* O83 reduced allergy to inhaled allergens in mice, but a low dose (0.1 µg) had the reverse effect [64]. Here we show that the higher dose (1 µg) of EcO83-OMVs reduced allergy, whereas a lower dose (0.1 µg) did not worsen the disease severity but improved allergic inflammation in several parameters. Although LPS is a dominant OMV antigen, our results cannot be directly compared with those of Eisenbarth et al. also because they used LPS from other bacteria and it is known that LPS can vary greatly between different bacterial strains.

Flagella synthesis has been shown to play a key role in the budding of OMVs from *E. coli* [65]. Here we show that OMVs from *E. coli* O83 contain flagellin (FliC), a major structural protein of bacterial flagellum, and proteins required for anchoring the flagella in the membrane (flagellar hook-associated proteins FlgK, FlgL; hook protein FlgE; basal body rod proteins FlgB, FlgC, FlgE, FlgF; and L-ring protein FlgH). These proteins

showed a significant fold enrichment in vesicles compared to the whole bacteria. The cost of constructing flagella is high in bacteria such as *E. coli*, and the reason behind the release of these energy intensive molecules into the environment has yet to be investigated.

Antigen-presenting cells such as DCs recognise flagellin on their apical surface through TLR5, which activates NF-κB and MAPK, leading to the production of pro-inflammatory cytokines [66]. In our studies, EcO83-OMVs, containing high levels of flagellin, induced the expression of pro-inflammatory cytokines such as IL-6 and TNF-α in BMDCs, and the involvement of TLR5 in their recognition was confirmed in TLR5-transfected HEK293 cells. Vesicle-stimulated BMDCs also produce caspase-1-dependent IL-1β, suggesting activation of the NLR neuronal apoptosis inhibitory protein 5 or 6 (NAIP5/6), the sensor for intracellular flagellin, followed by assembly of the NLRP4 inflammasome [67]. However, vesicle-induced IL-1β levels were several 100-fold lower compared to levels induced by whole bacteria, suggesting that vesicles can be viewed as potent immunomodulators that lack some of the negative properties of the parent bacteria. On the other hand, vesicles but not whole bacteria were recognized by TLR5, implying that this receptor is important for vesicles recognition but less important for whole bacteria recognition.

In vivo, we have shown that intranasal treatment with vesicles reduces the number of eosinophils in the lungs compared to allergic controls. A similar effect was observed with intranasal application of recombinant flagellin [68, 69]. A recent study suggests that the binding of flagellin to surface receptors on eosinophils such as TLR5 may prevent eosinophil sensitization [70]. According to Luo et al., flagellin can reduce oxidative stress in eosinophils [69]. We are currently conducting studies to elucidate the effect of EcO83-OMVs on oxidative stress in immune cells.

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Fig. 4 Effect of intranasal application of EcO83-OMVs on the development of allergic airway inflammation. **A** Experimental setup for intranasal application of EcO83-OMVs and induction of experimental allergic airway inflammation to OVA. BALB/c mice were sensitised (i.p.) with either OVA/alum (groups Sham/OVA, OMVs 0.1 µg/OVA and OMVs 1 µg/OVA) or PBS/alum (group Sham/PBS) on days 0 and 14 and challenged (i.n.) with either OVA or PBS on days 21–24 with. Mice were treated i.n. with 30 µl of 0.1 µg EcO83-OMVs (group OMVs 0.1 µg/OVA), 1 µg EcO83-OMVs (group OMVs 1 µg/OVA) or 0.9% NaCl (groups Sham/PBS and Sham/OVA). **B** Airway hyperresponsiveness was measured in response to increasing doses of metacholine by whole body plethysmography. Penh = enhanced pause. **C–D** Differential cell counts in bronchoalveolar lavage (BAL): **(C)** Total cell count and **(D)** total numbers of macrophages, eosinophils, neutrophils and lymphocytes. **E–F** Representative H&E and PAS-stained lung tissue sections showing recruited inflammatory cells and mucus-producing goblet cells, respectively. **G–H** Quantification of histological experiments (Disease score 0–3 for PAS and 1–4 for H&E). Scale bar = 50 µm. **B, C, E** and **F** Data were analysed using a One-Way ANOVA or **(D)** Two-Way ANOVA followed by post-hoc Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. $n = 5$ /group. Data are representative of three independent experiments. Mean $\pm$ SD is shown. Significant differences between Sham/OVA and EcO83-OMVs treatment groups (OMVs 0.1 µg/OVA or OMVs 1 µg/OVA) are indicated. alum = aluminiumhydroxide; i.n. = intranasal; i.p. = intraperitoneal; OVA = ovalbumin; OMVs = EcO83-OMVs; H&E = haematoxylin and eosin; PAS = periodic acid-Schiff

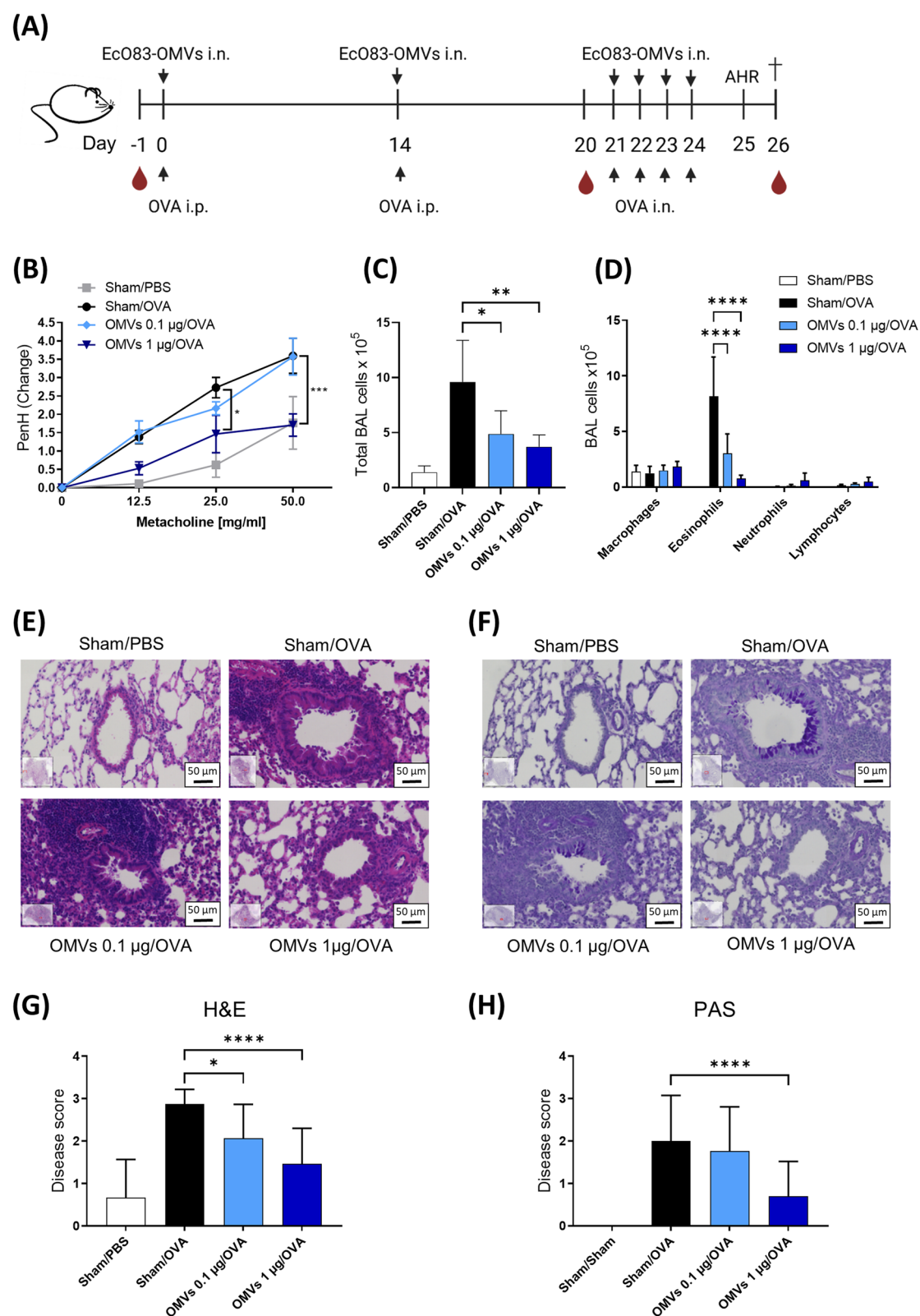


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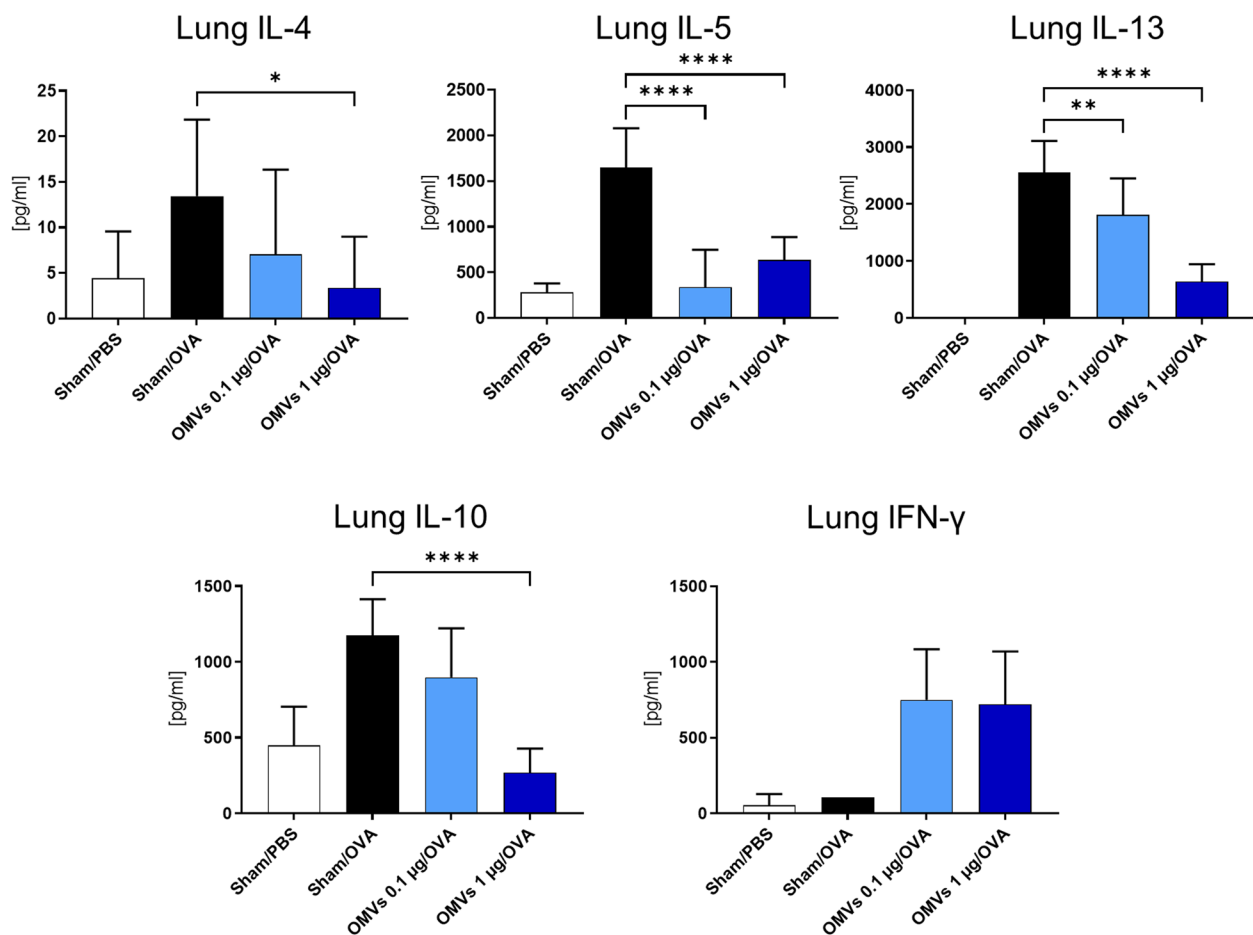


Fig. 5 Effect of intranasal application of EcO83-OMVs on the cellular recall response in the lung. Mice were treated as shown in the Fig. 4A. Single cell lung suspensions were stimulated with OVA for 72 h. Cytokines were measured in the supernatant by ELISA. Data were analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$, $n = 5$ /group. Data are representative of three independent experiments. Significant differences between sham-treated allergic mice (group Sham/OVA) and EcO83-OMVs treatment groups (OMVs 0.1 μg/OVA or OMVs 1 μg/OVA) are indicated. OVA = ovalbumin; OMVs = EcO83-OMVs

Recent studies have demonstrated the feasibility of using OMVs to deliver heterogeneous antigens. Eastwood et al. used an innovative expression system in *E. coli* based on a simple peptide tag that results in a high yield of functional proteins packaged into the vesicles [71]. Another study used the “plug-and-play” approach to decorate *S. typhimurium* OMVs with the spike receptor-binding domain [72]. This technology makes it possible to decorate the surface of OMVs with a variety of antigens or even multiple antigens. This could be of interest for the production of engineered OMVs decorated with allergens/peptides for specific immunotherapy. In our previous study, we constructed recombinant *E. coli* Nissle 1917 expressing the chimera with major birch and grass pollen allergenic protein/peptides and showed that its intranasal application prevented polysensitization in mice [73]. Therefore, future studies in our laboratory will focus on developing a recombinant

E. coli O83 to produce OMVs decorated with fluorescent markers or mono- and poly-allergens for specific immunotherapy.

Since there are no studies in which OMVs from different G- bacteria have been tested against allergies, we cannot answer the question of whether it is necessary to use OMVs from probiotic bacteria for allergy prevention or whether OMVs from non-probiotic Gram- bacteria would serve the same purpose. *Neisseria meningitidis*, for example, is a G- pathogenic bacterium and vaccines based on its OMVs have been used in clinics against meningitis [32]. These OMVs contain an extremely potent hexa-acylated LPS that leads to adverse effects. Therefore, several attempts have been made to reduce toxicity by removing the LPS [74]. In summary, we believe that the type of OMVs used depends on the context and purpose of the treatment and that further studies are needed to clarify the effect of OMVs in allergy.

In summary, we show that the probiotic *E. coli* O83 produces OMVs that exhibit potent immunomodulatory and anti-allergic properties. The ease of manufacture and the availability of technologies for genetically engineered vesicles, decorated with heterologous antigens in the lumen or on the surface, provide an extraordinarily versatile platform with great potential for therapeutic applications of bacterial vesicles in allergy research and clinic.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12964-023-01329-4>.

Additional file 1: Figure S1. Examination of EcO83-OMVs, EcO83-OMVs-depleted supernatant and mock control for bacterial contamination. The isolated EcO83-OMVs, EcO83-OMVs-depleted supernatant and mock control were examined for the presence of live bacteria. Samples were placed on LB agar plates and incubated at 37 °C for 96 h. The plates were examined for visible bacterial colonies. **Figure S2.** Unprocessed image of Bioanalyzer protein analysis of EcO83-OMVs and *E. coli* O83 lysate (modified image in Fig. 1E). Samples 1 and 2: EcO83-OMVs in duplicates. Samples 9 and 10: *E. coli* O83 lysate in duplicates. Samples 3–8: Material not relevant to this publication. **Figure S3.** Activation of the NF-κB pathway by EcO83-OMVs. The macrophage-like mouse cell line RAW264.7 was stimulated with medium, LPS (1 µg/ml), *E. coli* O83 (10⁷ CFU/ml) and EcO83-OMVs (10 ng/ml and 100 ng/ml) at 37 °C and 5 % CO₂ for 6 h. The expression of IL-6, TNF-α and IL-1β mRNA was measured with RT-PCR and is presented as the fold change to the housekeeping gene GAPDH. OMVs = EcO83-OMVs. **Figure S4.** Immunostimulatory potential of EcO83-OMVs compared to EcO83-OMVs-depleted supernatant and mock control. Cells isolated from the lungs of naive mice (*n* = 5) were treated with medium, LPS (1 µg/ml), *E. coli* O83 (10⁸ CFU/ml), EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml), EcO83-OMVs-depleted supernatant and mock control. The supernatant and mock control were added in the amount equal to the volume of vesicles used in cultures with 100 ng/ml EcO83-OMVs and incubated at 37 °C and 5 % CO₂ for 48 h. IL-6 and TNF-α were measured in the cell culture supernatant by ELISA. The mean ± SD is shown. Data were analysed using a One-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. ***p* < 0.05; *****p* < 0.001. OMVs = EcO83-OMVs. **Figure S5.** Immunostimulatory potential of EcO83-OMVs compared to Polymyxin B-treated EcO83-OMVs. Cells isolated from the lungs of naive mice (*n* = 5) were treated with medium, LPS (1 µg/ml), *E. coli* O83 (10⁸ CFU/ml), EcO83-OMVs treated with Polymyxin B (1 ng/ml, 10 ng/ml, 100 ng/ml), EcO83-OMVs treated with Polymyxin B mock (1 ng/ml, 10 ng/ml, 100 ng/ml) and stimulated with Polymyxin B alone at 37 °C/ 5 % CO₂ for 48 h. IL-6 and TNF-α were measured in the supernatants by ELISA. The mean ± SD is shown. Data were analysed using a One-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. ****p* < 0.01; *****p* < 0.001. OMVs = EcO83-OMVs; PmB = Polymyxin B. **Figure S6.** Immunostimulatory potential of EcO83-OMVs compared to heat-treated EcO83-OMVs. Cells isolated from the lungs of naive mice (*n* = 5) were treated with medium, LPS (1 µg/ml), *E. coli* O83 (10⁸ CFU/ml), EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml) and heat-treated EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml) at 37 °C/ 5 % CO₂ for 48 h. IL-6 and TNF-α were measured in the supernatants by ELISA. The mean ± SD is shown. Data were analysed using a One-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. *****p* < 0.001. OMVs = EcO83-OMVs. **Figure S7.** Local allergen-specific responses: Levels of OVA-specific antibodies in BAL. BAL was collected on sacrifice day by flushing the lungs with PBS and OVA-specific antibodies were measured by ELISA. Data were analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. **p* < 0.5; ***p* < 0.05. *n* = 5/group. Data are representative of three independent experiments. Mean ± SD is shown. Significant differences between Sham/OVA and EcO83-OMVs treatment groups (OMVs 0.1 µg/OVA or OMVs 1 µg/OVA) are indicated. BAL = bronchoalveolar lavage; OVA = ovalbumin; OMVs = EcO83-OMVs. **Figure S8.** Systemic allergen-specific responses: Levels

of OVA-specific antibodies in serum and cytokines produced by OVA-stimulated splenocytes *ex vivo*. (A) Blood was sampled on days -1, 20 and 26 (Experimental setup see Figure 5 A) and centrifuged to obtain serum. OVA-specific antibodies in serum were measured by ELISA. (B) Single cell suspensions of excised spleens of Sham or EcO83-OMVs-treated allergic mice were stimulated with OVA for 72 h and cytokine levels were measured by ELISA. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. **p* < 0.5; ***p* < 0.05; ****p* < 0.001. (A) Data are representative of three independent experiments for samples collected on day -1 and a pool of two experiments for samples collected on days 20 and 26; *n* = 5–10/group. (B) Data are representative of three independent experiments, *n* = 5/group. Mean ± SD is shown. Significant differences between Sham/OVA and EcO83-OMVs treatment groups (OMVs 0.1 µg/OVA or OMVs 1 µg/OVA) are indicated. OVA = ovalbumin; OMVs = EcO83-OMVs. **Table S1.** Cell culture media. **Table S2.** Size distribution and concentration of vesicles and amount of protein, and endotoxin corresponding to material collected from a two litre culture. Average ± SD is shown from three independent measurements. **Table S3.** Protein content in EcO83-OMVs measured with Bioanalyzer. Marked in grey are dominant visible bands. **Table S4.** List of proteins that were found to be upregulated in EcO83-OMVs compared to *E. coli* O83 lysate and met the false discovery rate (FDR) criteria. (P-value < 0.05). n.d. = not determined.

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Authors' contributions

I.S., A.I.K. and U.W. conceived the study and designed the experiments. I.S. and A.R. supervised the experiments and interpreted the data. S.S. assisted in the design of the experiments. P.K. and S.S. were involved in establishing methods and protocols for the isolation and characterisation of EcO83-OMVs. A.S., A.R., M.W., P.K., E.K. and M.T. isolated and characterised EcO83-OMVs and bacterial lysate. P.N., J.P. and B.W. planned and performed the proteomics analysis and interpreted the data. M.S. assisted in the analysis and interpretation of the proteomic data. K.S. performed transmission electron microscopy. A.S. performed and analysed in vivo and in vitro experiments. D.K., M.H. and G.B. performed and analysed in vitro experiments. N.G. assisted in the animal experiments and performed the histological analysis. T.W. isolated and characterised EcO83-OMVs and performed the experiment on stimulation RAW264.7 cells. A.S., A.R. and I.S. wrote the manuscript and drafted the figures. The final version of the manuscript was approved by all authors after they had reviewed it.

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Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (<http://www.ebi.ac.uk/pride>) with the dataset identifier PXD040963.

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Animal Experimentation Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Culture (BMBWF-66.009/0277-V/3b/2019).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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References

- CA Akdis 2021 Does the epithelial barrier hypothesis explain the increase in allergy, autoimmunity and other chronic conditions? *Nat Rev Immunol* 21 739–51
- Y Dorofeeva I Shilovskiy I Tulaeva M Focke-Tejkl S Flicker D Kudlay 2021 Past, present, and future of allergen immunotherapy vaccines *Allergy: Europ J Allergy Clinical Immunol* 76 131–49
- DE Campbell RJ Boyle CA Thornton SL Prescott 2015 Mechanisms of allergic disease - environmental and genetic determinants for the development of allergy *Clinical Experiment Allergy* 45 844–58
- M Sokolowska R Frei N Lunjani CA Akdis L O'Mahony 2018 Microbiome and asthma. *Asthma Res Pract* 4 1
- G Jatzlauk S Bartel H Heine M Schlöter S Krauss-Etschmann 2017 Influences of environmental bacteria and their metabolites on allergies, asthma, and host microbiota *Allergy: Europ J Allergy Clinical Immunol* 72 1859–67
- A Lopez-Santamarina EG Gonzalez A Lamas AC Mondragon del P Regal JM Miranda 2021 Probiotics as a possible strategy for the prevention and treatment of allergies A narrative review *Foods* 10 1–21
- L Cao L Wang L Yang S Tao R Xia W Fan 2015 Long-term effect of early-life supplementation with probiotics on preventing atopic dermatitis: A meta-analysis *J Dermatolog Treatment* 26 537–40
- M Panduru NM Panduru CM Sălavăstru GS Tiplica 2015 Probiotics and primary prevention of atopic dermatitis: A meta-analysis of randomized controlled studies *J Europ Academ Dermatology Venereol* 29 232–42
- TR Abrahamsson T Jakobsson B Björkstén G Oldaeus MC Jenmalm 2013 No effect of probiotics on respiratory allergies: A seven-year follow-up of a randomized controlled trial in infancy *Pediatric Allergy Immunol* 24 556–61
- MB Azad GJ Coneys AL Kozyrskyj CJ Field CD Ramsey AB Becker 2013 Probiotic supplementation during pregnancy or infancy for the prevention of asthma and wheeze Systematic review and meta-Analysis *BMJ (Online)*. 347 1–15
- X Wei P Jiang J Liu R Sun L Zhu 2020 Association between probiotic supplementation and asthma incidence in infants: a meta-analysis of randomized controlled trials *J Asthma* 57 167–78
- C Pellaton S Nutten A-C Thierry C Boudousquié N Barbier C Blanchard 2012 Intragastric and intranasal administration of lactobacillus paracasei NCC2461 modulates allergic airway inflammation in mice *Int J Inflam.* 2012 1–8
- I Spacova MI Petrova A Fremau L Pollaris J Vanoirbeek JL Ceuppens 2019 Intranasal administration of probiotic *Lactobacillus Rhamnosus* GG prevents birch pollen-induced allergic asthma in a murine model *Allergy: Europ J Allergy Clinical Immunol* 74 100–10
- I Schabussova K Hufnagl C Wild S Nutten AW Zuercher A Mercenier 2011 Distinctive anti-allergy properties of two probiotic bacterial strains in a mouse model of allergic poly-sensitization *Vaccine* 29 1981–90
- R Lodinova-Zadnikova L Prokešová I Kocourková J Hrdý J Žižka 2010 Prevention of allergy in infants of allergic mothers by probiotic *Escherichia coli* *Int Arch Allergy Immunol.* 153 201–6
- R Lodinova-Zadnikova B Cukrowska H Tlaskalova-Hogenova 2003 Oral administration of probiotic *Escherichia coli* after birth reduces frequency of allergies and repeated infections later in life (after 10 and 20 years) *Int Arch Allergy Immunol.* 131 209–11
- C Zwicker P Sarate M Drinić K Ambroz E Korb U Smole 2018 Prophylactic and therapeutic inhibition of allergic airway inflammation by probiotic *Escherichia coli* O83 *J Allergy Clinical Immunol* 142 1987–1990.e7
- D Pradhan RH Mallappa S Grover 2020 Comprehensive approaches for assessing the safety of probiotic bacteria *Food Control* 108 106872
- D Kothari S Patel SK Kim 2019 Probiotic supplements might not be universally-effective and safe: a review *Biomedic Pharmacother* 111 537–47
- C Schwechheimer MJ Kuehn 2015 Outer-membrane vesicles from Gram-negative bacteria: Biogenesis and functions *Nat Rev Microbiol* 13 605–19
- R Hu J Li Y Zhao H Lin L Liang M Wang 2020 Exploiting bacterial outer membrane vesicles as a cross-protective vaccine candidate against avian pathogenic *Escherichia coli* (APEC) *Microb Cell Fact* 19 119
- L David P Bordinon E Meunier E Oswald 2021 Outer membrane vesicles produced by pathogenic strains of *Escherichia coli* block autophagic flux and exacerbate inflammasome activation *Autophagy* 18 12 2913–2925
- M Bielaszewska M Marejková A Bauwens L Kunsmann-Prokscha A Mellmann H Karch 2018 Enterohemorrhagic *Escherichia coli* O157 outer membrane vesicles induce interleukin 8 production in human intestinal epithelial cells by signaling via Toll-like receptors TLR4 and TLR5 and activation of the nuclear factor NF- κ B *Int J Medical Microbiol* 308 882–9
- Bielaszewska M, Rüter C, Bauwens A, Greune L, Jarosch K-A, Steil D, et al. Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: Intracellular delivery, trafficking and mechanisms of cell injury. *PLoS Pathog.* 2017;13(2):e1006159.
- R Hu H Lin J Li Y Zhao M Wang X Sun 2020 Probiotic *Escherichia coli* Nissle 1917-derived outer membrane vesicles enhance immunomodulation and antimicrobial activity in RAW264.7 macrophages *BMC Microbiol* 20 1–13
- MA Cañas R Giménez MJ Fábrega L Toloza L Baldomà J Badia 2016 Outer membrane vesicles from the probiotic *Escherichia coli* Nissle 1917 and the commensal ECOR12 enter intestinal epithelial cells via clathrin-dependent endocytosis and elicit differential effects on DNA damage *PLoS One.* 11 1–22
- MJ Fábrega A Rodríguez-Nogales J Garrido-Mesa F Algieri J Badia R Giménez 2017 Intestinal anti-inflammatory effects of outer membrane vesicles from *Escherichia coli* Nissle 1917 in DSS-experimental colitis in mice *Front Microbiol* 8 1–13
- Diaz-Garrido N, Badia J, Baldoma L. Microbiota-derived extracellular vesicles in interkingdom communication in the gut. *J Extracell Vesicles.* 2021;10(13):e12161.
- M Kaparakis-Liaskos RL Ferrero 2015 Immune modulation by bacterial outer membrane vesicles *Nat Rev Immunol* 15 375–87
- NJ Bitto M Kaparakis-Liaskos 2017 The therapeutic benefit of bacterial membrane vesicles *Int J Mol Sci.* 18 1–15
- WJ Gilmore EL Johnston L Zavan NJ Bitto M Kaparakis-Liaskos 2021 Immunomodulatory roles and novel applications of bacterial membrane vesicles *Mol Immunol.* 134 72–85
- SN Ladhani M Ramsay R Borrow A Riordan JM Watson AJ Pollard 2016 Enter B and W: Two new meningococcal vaccine programmes launched *Arch Dis Child.* 101 91–5
- Kohl P, Zingl FG, Eichmann TO, Schild S. Isolation of outer membrane vesicles including their quantitative and qualitative analyses. *Methods Mol Biol.* 2018;1839:117–34.

34. AV Edhager JA Povlsen B Løfgren HE Bøtker J Palmfeldt 2018 Proteomics of the Rat Myocardium during Development of Type 2 Diabetes Mellitus Reveals Progressive Alterations in Major Metabolic Pathways *J Proteome Res.* 17 2521 32
35. J Cox M Mann 2008 MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification *Nat Biotechnol* 26 1367 72 <https://doi.org/10.1038/nbt.1511>
36. S Tyanova T Temu P Sinitcyn A Carlson MY Hein T Geiger 2016 The Perseus computational platform for comprehensive analysis of (prote)omics data *Nat Methods.* 13 731 40
37. J Goedhart MS Luijsterburg 2020 VolcanoR is a web app for creating, exploring, labeling and sharing volcano plots *Sci Rep.* 10 20560
38. Y Perez-Riverol J Bai C Bandla D García-Seisdedos S Hewapathirana S Kamatchinathan 2022 The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences *Nucleic Acids Res.* 50 D543 52
39. Srutkova D, Schwarzer M, Hudcovic T, Zakostelska Z, Drab V, Spanova A, et al. Bifidobacterium longum CCM 7952 Promotes Epithelial Barrier Function and Prevents Acute DSS- Induced Colitis in Strictly Strain-Specific Manner. *PLoS ONE.* 2015;10(7):e0134050.
40. E Korb M Drinić A Wagner N Geissler A Inic-Kanada R Peschke 2021 Reduction of allergic lung disease by mucosal application of toxoplasma Gondii-Derived molecules: possible role of carbohydrates *Front Immunol* 11 1 14
41. Drinic M, Wagner A, Sarate P, Zwicker C, Korb E, Loupal G, et al. Toxoplasma gondii tachyzoite-extract acts as a potent immunomodulator against allergic sensitization and airway inflammation. *Sci Rep.* 2017;7(1):15211.
42. MM Zaiss A Rapin L Lebon LK Dubey I Mosconi K Sarter 2015 The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation *Immunity* 43 998 1010
43. G Schoenhals C Whitfield 1993 Comparative analysis of flagellin sequences from Escherichia coli strains possessing serologically distinct flagellar filaments with a shared complex surface pattern *J Bacteriol* 175 5395 402
44. O Takeuchi K Hoshino T Kawai H Sanjo H Takada T Ogawa 1999 Differential roles of TLR2 and TLR4 in recognition of Gram-Negative and Gram-Positive bacterial cell wall components *Technol Japan Scie* 11 443 51
45. F Hayashi KD Smith A Ozinsky TR Hawn EC Yi DR Goodlett 2001 The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5 *Nature.* 410 1099 103
46. JD Cecil NM O'Brien-Simpson JC Lenzo JA Holden YY Chen W Singleton 2016 Differential responses of pattern recognition receptors to outer membrane vesicles of three periodontal pathogens *PLoS One.* 11 1 20
47. M Kaparakis L Turnbull L Carneiro S Firth HA Coleman HC Parkington 2010 Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells *Cell Microbiol* 12 372 85
48. Ou T, Lilly M, Jiang W. The Pathologic Role of Toll-Like Receptor 4 in Prostate Cancer. *Front Immunol.* 2018;9:1188.
49. Cochet F, Peri F. The Role of Carbohydrates in the Lipopolysaccharide (LPS)/Toll-Like Receptor 4 (TLR4) Signalling. *Int J Mol Sci.* 2017;18(11):2318.
50. T Liu L Zhang D Joo S-C Sun 2017 NF-κB signaling in inflammation *Signal Transduct Target Ther.* 2 17023
51. MJ Schuijs H Hammad BN Lambrecht 2019 Professional and 'Amateur' Antigen-Presenting Cells In Type 2 Immunity *Trends Immunol* 40 22 34
52. M Ege M Mayer AC Normand J Genueneit W Cookson Ch Braun-Fahrlander 2011 Exposure to environmental microorganisms and childhood asthma *N Engl J Med* 364 701 9
53. MM Stein CL Hrusch J Gozdz C Igartua V Pivniouk SE Murray 2016 Innate Immunity and Asthma Risk in Amish and Hutterite farm children *New England Medicine* 375 411 21
54. MJ Schuijs MA Willart K Vergote D Gras K Deswarte MJ Ege 1979 Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells *Science* 205 349 1106 10
55. J Debarry H Garn A Hanuszkiewicz N Dickgreber N Blumer E Mutius von 2007 Acinetobacter lwoffii and Lactococcus lactis strains isolated from farm cowsheds possess strong allergy-protective properties *J Allergy Clin Immunol.* 119 1514 21
56. S Hagner H Harb M Zhao K Stein O Holst MJ Ege 2013 Farm-derived Gram-positive bacterium Staphylococcus sciuri W620 prevents asthma phenotype in HDM- and OVA-exposed mice *Allergy: Europ J Allergy Clinical Immunol* 68 322 9
57. ML Conrad R Ferstl R Teich S Brand N Blumer AO Yildirim 2009 Maternal TLR signaling is required for prenatal asthma protection by the non-pathogenic microbe Acinetobacter lwoffii F78 *J Exp Med.* 206 2869 77
58. J Debarry A Hanuszkiewicz K Stein O Holst H Heine 2010 The allergy-protective properties of Acinetobacter lwoffii F78 are imparted by its lipopolysaccharide *Allergy: Europ J Allergy Clinical Immunol* 65 690 7
59. D Murakami H Yamada T Yajima A Masuda S Komune Y Yoshikai 2007 Lipopolysaccharide inhalation exacerbates allergic airway inflammation by activating mast cells and promoting Th2 responses *Clinical Experimental Allergy* 37 339 47
60. N Blumer U Herz M Wegmann H Renz N Blümer U Herz 2005 Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma *Clinical Experiment Allergy* 35 397 402
61. AH Liu 2002 Endotoxin exposure in allergy and asthma: Reconciling a paradox *J Allergy Clinical Immunol* 109 379 92
62. MK Tulić JL Wale PG Holt PD Sly 2000 Modification of the inflammatory response to allergen challenge after exposure to bacterial lipopolysaccharide *Am J Respir Cell Mol Biol.* 22 604 12
63. T Bickert CM Trujillo-Vargas M Duechs G Wohlleben T Polte G Hansen 2009 Probiotic Escherichia coli Nissle 1917 suppresses Allergen-Induced Th2 responses in the Airways *Int Arch Allergy Immunol* 149 219 30
64. SC Eisenbarth DA Piggott JW Huleatt I Visintin CA Herrick K Bottomly 2002 Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen *J Experimental Medicine* 196 1645 51
65. T Manabe M Kato T Ueno K Kawasaki 2013 Flagella proteins contribute to the production of outer membrane vesicles from Escherichia coli W3110 *Biochem Biophys Res Commun* 441 151 6
66. V Feuillet S Medjane I Mondor O Demaria PP Pagni JE Galán 2006 Involvement of toll-like receptor 5 in the recognition of flagellated bacteria *Proc Natl Acad Sci U S A.* 103 12487 92
67. M Zhong H Yan Y Li 2017 Flagellin: A unique microbe-associated molecular pattern and a multi-faceted immunomodulator *Cell Mol Immunol.* 14 862 4
68. DH Lee HK Park HR Lee H Sohn S Sim HJ Park 2022 Immunoregulatory effects of Lactococcus lactis-derived extracellular vesicles in allergic asthma *Clin Transl Allergy.* 12 1 11
69. XQ Luo J Liu LH Mo G Yang F Ma Y Ning 2021 Flagellin alleviates airway allergic response by stabilizing eosinophils through modulating oxidative stress *J Innate Immun* 13 333 44
70. X Lv Q Chang Q Wang QR Jin HZ Liu SB Yang 2022 Flagellin maintains eosinophils in the intestine *Cytokine.* 150 155769
71. TA Eastwood K Baker BR Streather JR Hiscock C Lennon DP Mulvihill 2023 High-yield vesicle-packaged recombinant protein production from E coli *Cell Reports Methods* 3 100396
72. Jiang L, Driedonks TAP, Jong WSP, Dhakal S, Bart van den Berg van Saparoea H, Sitaras I, et al. A bacterial extracellular vesicle-based intranasal vaccine against SARS-CoV-2 protects against disease and elicits neutralizing antibodies to wild-type and Delta variants. *J Extracell Vesicles.* 2022;11.
73. PJ Sarate S Heintz S Poiret M Drinić C Zwicker I Schabussova E. coli Nissle, 1917 is a safe mucosal delivery vector for a birch-grass pollen chimera to prevent allergic poly-sensitization *Mucosal Immunol.* 2019 12 132 44
74. Z Afshin B Joep W Bas de HH Jan E Bth R Elly 2016 Meningococcal Outer Membrane Vesicle Composition-Dependent Activation of the Innate Immune Response *Infect Immun* 84 3024 33

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SUPPLEMENTARY MATERIAL

Title: Extracellular vesicles of the probiotic bacteria *E. coli* O83 activate innate immunity and prevent allergy in mice

Running title: Extracellular vesicles of *E. coli* O83 reduce allergy

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SUPPLEMENTARY MATERIALS AND METHODS

Table S1: Cell culture media

Purpose of media	Media and additives	Supplier
HEK-293/hTLR2	Dulbecco's modified Eagle's medium (DMEM) Heat inactivated fetal calf serum (FCS) 10 % Penicillin/streptomycin (Pen/Strep) 50 U/ml/ 50 µg/ml Blasticidin 10 µg/ml	Gibco Gibco Biochrom Invivogen
HEK-293/hTLR4/CD14/MD2	DMEM FCS 10 % Pen/Strep 50 U/ml/ 50 µg/ml Blasticidin 10 µg/ml Hygromycin B Gold 55 µg/ml	Gibco Gibco Biochrom Invivogen Invivogen
HEK-293/hTLR5	DMEM FCS 10 % Pen/Strep 50 U/ml/ 50 µg/ml Normocin 100 µg/ml L-glutamine 2 mM Blasticidin 10 µg/ml	Gibco Gibco Biochrom Invivogen Gibco Invivogen
HEK-293/hNOD1 HEK-293/hNOD2	DMEM FCS 10 % Blasticidin 10 µg/ml Normocin 110 µg/ml	Gibco Gibco Invivogen Invivogen
Complete RPMI	RPMI 1640 FCS 10 % Mercaptoethanol 2 mM L-glutamine 2 mM Gentamycin 100 µg/ml	Biowest Gibco Sigma Aldrich Sigma Aldrich Carl Roth
Wash RPMI	RPMI 1640 Gentamycin 100 µg/ml	Biowest Carl Roth
Bone marrow-derived dendritic cells	RPMI 1640 FCS 10 % L-glutamine 2 mM Pen/Strep 50 U/ml/ 50 µg/ml	Biowest Gibco Sigma Aldrich Biochrom

Isolation and restimulation of spleen cells *ex vivo*

Spleens from EcO83-treated OVA-allergic mice were processed as described in the main text. Cells were seeded at a concentration of 5×10^6 cells/ml and stimulated with endotoxin-free OVA (100 µg/ml; EndoGrade; Hyglos, Bernried am Starnberger See, Germany) for 72 h at 37 °C and 5 % CO₂. Cell culture supernatants were collected and cytokines were analysed by ELISA.

ELISA

To measure OVA-specific antibodies, an in-house established ELISA protocol was used as described before [1].

Preparation of a mock control and recovery of the EcO83-OMVs-depleted supernatant

To demonstrate that the immunomodulatory effects were related to EcO83-OMVs and not soluble molecules, we tested two different controls. For the mock control, the BHI medium was incubated for 8 h at 37 °C and 200 rpm without the addition of bacteria. The medium was processed in parallel with the bacterial culture (described in Materials and Methods). To obtain the fraction depleted of EcO83-OMVs, the bacterial culture supernatant was centrifuged, filtered and ultracentrifuged (described in Materials and Methods). The vesicle-free supernatant was collected after the final ultracentrifugation. The mock control and the vesicle-free supernatant were stored under the same conditions as the isolated EcO83-OMVs until further use.

Testing EcO83-OMVs, mock control and EcO83-OMVs-depleted supernatant for bacterial contamination

50 µl EcO83-OMVs (diluted 1:5 with NaCl), a mock control (diluted 1:5 with NaCl) and the supernatant depleted in EcO83-OMVs (undiluted) were applied to LB agar plates (LB medium containing 1.5 % agarose; both Carl Roth) and incubated at 37 °C for 96 h. The plates were examined every 24 h for visible bacterial colonies.

Culture of lung cells with mock control, EcO83-OMVs-depleted supernatant, Polymyxin B- and heat-treated EcO83-OMVs

Lung cells were isolated as described in Materials and Methods and seeded at a concentration of 5×10^6 cells/ml. EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml), medium, LPS (1 μ g/ml), *E. coli* O83 (10^8 CFU/ml), the mock control and EcO83-OMVs-depleted supernatant were added to the cultures. The mock control and EcO83-OMVs-depleted supernatant were used in the amount equivalent to the amount of EcO83-OMVs used for the culture at 100 ng/ml. To block TLR4 signalling by LPS, EcO83-OMVs were incubated with 20 μ g/ml Polymyxin B (Sigma-Aldrich) at 37 °C for 30 minutes, as previously described [1]. As a mock control, EcO83-OMVs were incubated 30 min at 37 °C without Polymyxin B. Lung cells were stimulated with the Polymyxin B-treated EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml), medium, LPS (1 μ g/ml) and *E. coli* O83 (10^8 CFU/ml). In parallel, EcO83-OMVs were incubated at 95 °C for 15 min and added to lung cells as described above. The cells were incubated for 48 h at 37 °C and 5 % CO₂. The cell culture supernatants were collected and stored at -20 °C until further use. The levels of IL-6 and TNF- α in the cell cultures were measured by ELISA.

Cultivation and stimulation of the macrophage-like cell line

The macrophage-like cell line RAW264.7 (a gift from Prof. Sylvia Knapp) was cultured in DMEM medium with 10 % FCS at 37 °C/5 % CO₂. In general, the growth medium was changed every 2-3 days and the cultures were divided when they reached ~80 % confluence. For stimulation, cells were plated out in 96-well plates at a concentration of 2×10^5 cells /ml and allowed to adhere for 16 h. Cells were then stimulated for 6 h at 37 °C/5 % CO₂ with medium, LPS (1 μ g/ml) and EcO83-OMVs (10 ng/ml and 100 ng/ml). The supernatant was discarded and the cells were scraped from the surface with a pipette tip, collected and transferred to 100 μ l RNeasy lysis solution (Qiagen).

RNA isolation

RNA was isolated from stimulated RAW264.7 cells using the innuPREP RNA Mini Kit 2.0 (Analytik Jena) according to the manufacturer's protocol for eukaryotic cells. Subsequently, the purity and concentration of the extracted RNA were quantified using a NanoDrop 2000 spectrophotometer (PreqLab).

Reverse transcription and quantitative real-time PCR (qRT-PCR)

To initiate cDNA synthesis, 1 µg of total RNA was subjected to reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). The mixture was incubated in a thermal cycler (Bio-Rad T100™). Real-time qPCR was performed with the SsoAdvanced Universal SYBR® Green Supermix (Bio Rad) using the CFX Duet (Bio Rad). The thermal cycles consisted of denaturation 98 °C/3 min, 44 amplification cycles at 95 °C/10 s and 60 °C/60 s, followed by melting at 60-95 °C/5 s. The primer sequences for the tested genes (Eurofins Genomics) are listed below:

IL-1β: 5'-GAT CCA CAC TCT CCA GCT GCA-3' (forward); 5'-CAA CCA ACA AGT GAT ATT CTC CAT-3' (reverse)

IL-6:

5'-CAC TTC ACA AGT CGG AGG CT-3' (forward); 5'-CTG CAA GTG CAT CAT CGT TGT-3' (reverse)

TNF-α:

5'-TGC CTA TGT CTC AGC CTC TTC-3' (forward); 5'-TTG TGA GTG TGA GGG TCT GG-3' (reverse)

GAPDH:

5'-AAC TTT GGC ATT GTG GAA GG-3' (forward); 5'-ACA CAT TGG GGG TAG GAA CA-3' (reverse)

Samples were analysed in duplicates. Fold expression change was calculated by the comparative 2-ΔΔCt method using CFX Manager software version 2.3 (Bio Rad), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene for normalisation.

SUPPLEMENTARY RESULTS

Table S2: Size distribution and concentration of vesicles and amount of protein, and endotoxin corresponding to material collected from a two litre culture. Average $\pm$ SD is shown from three independent measurements.

Measured Parameter	Method	Values
Particle size	Dynamic Light Scattering	112.9 $\pm$ 1.99 nm
Particle concentration	Dynamic Light Scattering	1.4 x 10 ¹³ particles/ml (7 x 10 ¹² particles)
Protein content	Bradford	1.84 $\pm$ 0,12 mg/ml (~0.92 mg)
LPS content	rFC assay	5 mg LPS/mg protein (~4.6 mg)

Table S3: Protein content in EcO83-OMVs measured with Bioanalyzer. Marked in grey are dominant visible bands.

Size [kDa]	Relative Concentration [ng/ μ l]	% of Total	Comments
0	0	0	
0,3	0	0	
4,5	0	0	Lower Marker
10	0	0	System Peak
12,2	0	0	System Peak
19	172,6	19,5	
27	94,3	10,7	
34,3	207,4	23,5	
49	77,6	8,8	
64,6	242,9	27,5	
77,5	12,8	1,4	
115,6	29,2	3,3	
178,3	0,9	0,1	
193,7	5,9	0,7	
209,1	16,4	1,9	
224,6	24,2	2,6	
240	60	0	Upper Marker
394,3	0	0	

137 **Table S4: List of proteins that were found to be upregulated in EcO83-OMVs**
138 **compared to *E. coli* O83 lysate and met the false discovery rate (FDR) criteria.**
139 (P-value<0.05). n.d. = not determined.

UniProt accession	Entry name	Name	Localization	Log ₂ (Fold change)	- Log ₁₀ (p-value)
P33235	FLGK_ECOLI	Flagellar hook-associated protein 1	cell envelope	33,90	7,85
P29744	FLGL_ECOLI	Flagellar hook-associated protein 3	cell envelope	32,46	9,08
P0A927	TSX_ECOLI	Nucleoside-specific channel-forming protein Tsx	outer membrane	30,88	6,39
P13036	FECA_ECOLI	Fe(3+) dicitrate transport protein FecA	outer membrane	30,76	9,52
P75937	FLGE_ECOLI	Flagellar hook protein FlgE	cell envelope	29,18	8,40
P77717	YBAY_ECOLI	Uncharacterized lipoprotein YbaY	cell membrane	28,67	7,42
P37665	YIAD_ECOLI	Probable lipoprotein YiaD	inner, outer membrane	28,31	8,27
P10100	RLPA_ECOLI	Endolytic peptidoglycan transglycosylase RlpA	cell membrane	28,16	8,81
P0A921	PA1_ECOLI	Phospholipase A1	outer membrane	27,92	9,36
P0ABX5	FLGG_ECOLI	Flagellar basal body rod protein FlgG	cell envelope	27,78	7,02
P0A935	MLTA_ECOLI	Membrane-bound lytic murein transglycosylase A	outer membrane	27,75	7,80
P02943	LAMB_ECOLI	Maltoporin	outer membrane	27,71	8,41
P10384	FADL_ECOLI	Long-chain fatty acid transport protein	outer membrane	27,70	8,12
P45464	LPOA_ECOLI	Penicillin-binding protein activator LpoA	outer membrane	27,60	9,85
P76115	YNCD_ECOLI	Probable TonB-dependent receptor YncD	outer membrane	27,43	9,27
P77774	BAMB_ECOLI	Outer membrane protein assembly factor BamB	outer membrane	27,23	7,44
P0ADA7	OSMB_ECOLI	Osmotically-inducible lipoprotein B	cell membrane	27,13	7,86
P0ABW9	FLGB_ECOLI	Flagellar basal body rod protein FlgB	cell envelope	26,93	7,85

P0ABX2	FLGC_ECOLI	Flagellar basal body rod protein FlgC	cell envelope	26,79	8,05
P61320	LOLB_ECOLI	Outer-membrane lipoprotein LolB	outer membrane	26,66	8,75
P60785	LEPA_ECOLI	Elongation factor 4	inner membrane	26,62	9,64
P0C960	EMTA_ECOLI	Endo-type membrane-bound lytic murein transglycosylase A	outer membrane	26,53	6,55
P76206	YDIY_ECOLI	Uncharacterized protein YdiY	n.d.	26,39	8,68
P0AC02	BAMD_ECOLI	Outer membrane protein assembly factor BamD	outer membrane	25,89	12,55
P52614	FLIK_ECOLI	Flagellar hook-length control protein	cell envelope	25,87	9,21
Q46798	YGER_ECOLI	Uncharacterized lipoprotein YgeR	inner membrane	25,80	6,75
P08189	FIMF_ECOLI	Protein FimF	cell envelope/secreted	25,80	6,61
P0ADC1	LPTE_ECOLI	LPS-assembly lipoprotein LptE	outer membrane	25,78	8,34
P08190	FIMG_ECOLI	Protein FimG	cell envelope	25,77	6,38
P75780	FIU_ECOLI	Catecholate siderophore receptor Fiu	outer membrane	25,59	9,01
P0AA16	OMPR_ECOLI	DNA-binding dual transcriptional regulator OmpR	cytoplasm	25,56	9,32
P65292	YGDI_ECOLI	Uncharacterized lipoprotein YgdI	cell membrane	25,50	7,63
P76506	MLAA_ECOLI	Intermembrane phospholipid transport system lipoprotein MlaA	outer membrane	25,20	8,10
P11454	ENTF_ECOLI	Enterobactin synthase component F	cytoplasm	25,15	8,40
P0ACR9	MPRA_ECOLI	Transcriptional repressor MprA	cytosol	24,94	7,75
P77294	YDER_ECOLI	Uncharacterized fimbrial-like protein YdeR	cell envelope	24,93	8,80
P0A9E5	FNR_ECOLI	Fumarate and nitrate reduction regulatory protein	cytoplasm	24,92	8,14
P39264	FIMI_ECOLI	Fimbrin-like protein FimI	cell envelope	24,92	7,54

Q7DFV3	YMGG_ECOLI	UPF0757 protein YmgG	n.d.	24,79	8,84
P0A853	TNAA_ECOLI	Tryptophanase	cytoplasm	24,66	7,91
P0A6R3	FIS_ECOLI	DNA-binding protein Fis	cytoplasm	24,56	7,10
P77562	YAIW_ECOLI	Uncharacterized protein YaiW	outer membrane, cytosol	24,53	6,85
P0AAB6	GALF_ECOLI	UTP--glucose-1-phosphate uridylyltransferase	cytosol	24,49	8,22
P0ACJ0	LRP_ECOLI	Leucine-responsive regulatory protein	cytosol	24,46	9,29
P41052	MLTB_ECOLI	Membrane-bound lytic murein transglycosylase B	outer membrane	24,45	9,76
P0ADG4	SUHB_ECOLI	Nus factor SuhB	cytoplasm	24,33	9,41
P0ACP7	PURR_ECOLI	HTH-type transcriptional repressor PurR	cytosol	24,32	6,65
P0A9G6	ACEA_ECOLI	Isocitrate lyase	cytosol	24,32	7,70
P75938	FLGF_ECOLI	Flagellar basal-body rod protein FlgF	cell envelope	24,28	8,64
P46130	YBHC_ECOLI	Putative acyl-CoA thioester hydrolase YbhC	outer membrane	24,15	6,90
P0A6E9	BIOD2_ECOLI	ATP-dependent dethiobiotin synthetase BioD 2	cytoplasm	24,13	8,64
P0A7D1	PTH_ECOLI	Peptidyl-tRNA hydrolase	cytoplasm	24,05	7,29
P0A887	UBIE_ECOLI	Ubiquinone/menaquinone biosynthesis C-methyltransferase UbiE	cytoplasm	24,03	6,40
P0A6S0	FLGH_ECOLI	Flagellar L-ring protein	cell envelope	23,97	8,49
P0C058	IBPB_ECOLI	Small heat shock protein IbpB	cytoplasm	23,86	6,88
P76537	YFEY_ECOLI	Uncharacterized protein YfeY	n.d.	23,79	7,03
P0AA91	YEAY_ECOLI	Uncharacterized lipoprotein YeaY	cell membrane	23,68	7,56
P39180	AG43_ECOLI	Antigen 43	periplasm, secreted	23,60	5,72
P69924	RIR2_ECOLI	Ribonucleoside-diphosphate reductase 1 subunit beta	cytoplasm, cytosol	23,59	10,60

P77804	YDGA_ECOLI	Protein YdgA	inner membrane	23,45	7,59
P0ADN6	YIFL_ECOLI	Uncharacterized lipoprotein YifL	cell membrane	23,39	6,10
P0AEE5	DGAL_ECOLI	D-galactose/methyl-galactoside binding periplasmic protein MglB	periplasm	23,38	7,21
P63883	AMIC_ECOLI	N-acetylmuramoyl-L-alanine amidase AmiC	periplasm	22,96	9,05
P0A8I3	YAAA_ECOLI	Peroxide stress resistance protein YaaA	cytosol	22,96	6,15
P0CG19	RNPH_ECOLI	Truncated inactive ribonuclease PH	cytosol	22,93	6,82
P0C054	IBPA_ECOLI	Small heat shock protein IbpA	cytoplasm	22,73	9,79
P0ACP1	CRA_ECOLI	Catabolite repressor/activator	n.d.	22,68	8,08
P0A734	MINE_ECOLI	Cell division topological specificity factor	cytosol, plasma membrane	22,66	7,62
P21362	YCIF_ECOLI	Protein YciF	n.d.	22,56	9,45
P30958	MFD_ECOLI	Transcription-repair-coupling factor	cytoplasm	22,51	9,05
P46837	YHGF_ECOLI	Protein YhgF	cytosol	22,36	8,53
P64624	YHEO_ECOLI	Uncharacterized protein YheO	cytosol	22,31	6,70
P18196	MINC_ECOLI	Septum site-determining protein MinC	cell pole, cytosol	21,98	7,69
P09551	ARGT_ECOLI	Lysine/arginine/ornithine-binding periplasmic protein	periplasm	21,92	4,84
P27550	ACSA_ECOLI	Acetyl-coenzyme A synthetase	cytosol	21,77	8,40
P37650	BCSC_ECOLI	Cellulose synthase operon protein C	outer membrane	21,56	5,22
P66948	BEPA_ECOLI	Beta-barrel assembly-enhancing protease	periplasm	21,15	6,81
P31554	LPTD_ECOLI	LPS-assembly protein LptD	outer membrane	19,68	1,31
P09169	OMPT_ECOLI	Protease 7	outer membrane	10,40	3,68
P0A940	BAMA_ECOLI	Outer membrane protein assembly factor BamA	outer membrane	7,86	4,55

P0A908	MIPA_ECOLI	MltA-interacting protein	outer membrane	7,69	3,90
P06971	FHUA_ECOLI	Ferrichrome outer membrane transporter/phage receptor	outer membrane	7,50	3,96
P77330	BORD_ECOLI	Prophage lipoprotein Bor homolog	cell membrane	6,98	5,10
P04949	FLIC_ECOLI	Flagellin	cell envelope	6,91	4,92
P64596	YRAP_ECOLI	Outer membrane lipoprotein DolP	outer membrane	6,79	5,43
P0A912	PAL_ECOLI	Peptidoglycan-associated lipoprotein	outer mmebrane	6,46	4,55
P0A910	OMPA_ECOLI	Outer membrane protein A	outer membrane	6,06	5,02
P0A917	OMPX_ECOLI	Outer membrane protein X	outer membrane	5,83	5,74
P21420	NMPC_ECOLI	Putative outer membrane porin protein NmpC	outer membrane	5,60	4,07
P0A707	IF3_ECOLI	Translation initiation factor IF-3	cytoplasm	5,58	5,40
P69776	LPP_ECOLI	Major outer membrane lipoprotein Lpp	outer membrane, secreted, cell wall	4,96	4,22
P0ADB1	OSME_ECOLI	Osmotically-inducible putative lipoprotein OsmE	inner membrane	4,92	3,91
P06996	OMPC_ECOLI	Outer membrane porin C	outer membrane	4,82	4,48
P0ADA5	YAJG_ECOLI	Uncharacterized lipoprotein YajG	cell membrane	4,73	3,34
P0A915	OMPW_ECOLI	Outer membrane protein W	outer membrane	4,46	3,79
P76513	YFDQ_ECOLI	Uncharacterized protein YfdQ	n.d.	4,29	5,58
P0A905	SLYB_ECOLI	Outer membrane lipoprotein SlyB	outer membrane	4,18	4,80
P0A903	BAMC_ECOLI	Outer membrane protein assembly factor BamC	outer membrane	4,07	3,61
P0ACJ8	CRP_ECOLI	cAMP-activated global transcriptional regulator CRP	cytosol	3,79	4,93
P0A7G6	RECA_ECOLI	Protein RecA	cytoplasm	3,78	2,95
P0ADA3	NLPD_ECOLI	Murein hydrolase activator NlpD	inner membrane	3,39	3,83

P0A7M2	RL28_ECOLI	50S ribosomal protein L28	cytoplasm, cytosol	3,27	3,55
P0AAX8	YBIS_ECOLI	Probable L,D-transpeptidase YbiS	periplasm	3,20	3,97
P68688	GLRX1_ECOLI	Glutaredoxin 1	n.d.	3,14	2,57
P0A8F0	UPP_ECOLI	Uracil phosphoribosyltransferase	cytosol, cytoplasm	3,12	4,60
P60716	LIPA_ECOLI	Lipoyl synthase	cytoplasm	3,08	3,75
P0AB89	PUR8_ECOLI	Adenylosuccinate lyase	cytosol	3,03	2,24
P37194	SLP_ECOLI	Outer membrane protein Slp	outer membrane	2,67	3,97
P0A7G2	RBFA_ECOLI	30S ribosome-binding factor	cytoplasm	2,24	3,54
P0A9Q1	ARCA_ECOLI	Aerobic respiration control protein ArcA	cytoplasm	2,18	3,32
P0A7V3	RS3_ECOLI	30S ribosomal protein S3	cytoplasm, cytosol	2,16	3,71

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142 **SUPPLEMENTARY FIGURES**

143 Figure S1_Schmid *et al.*

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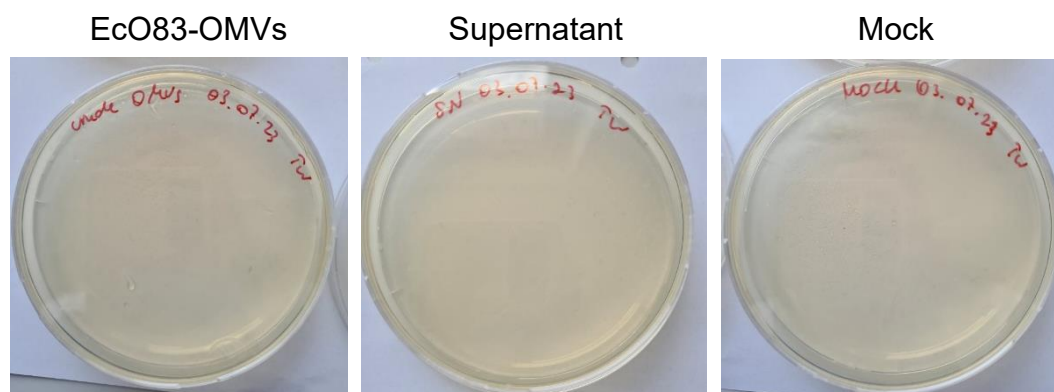
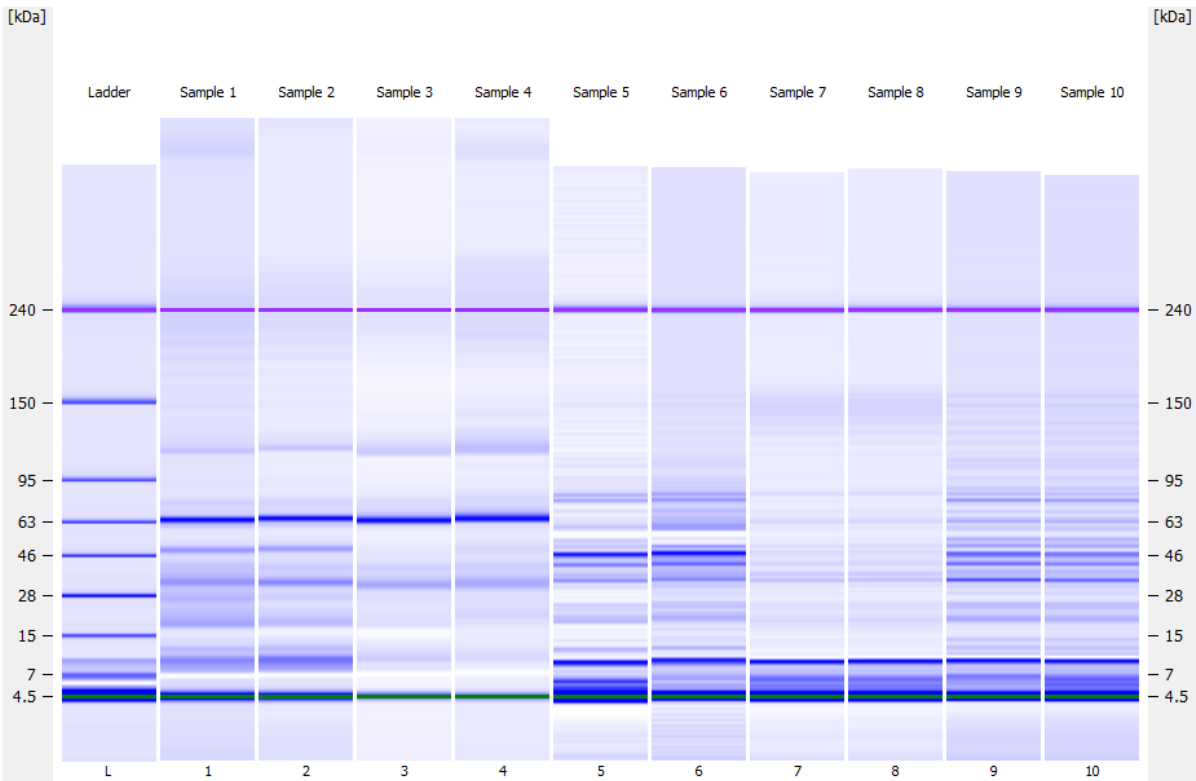
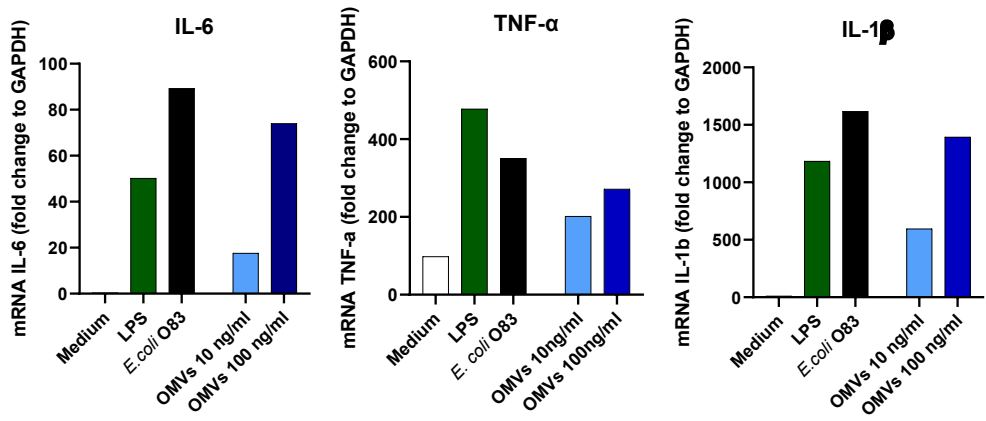
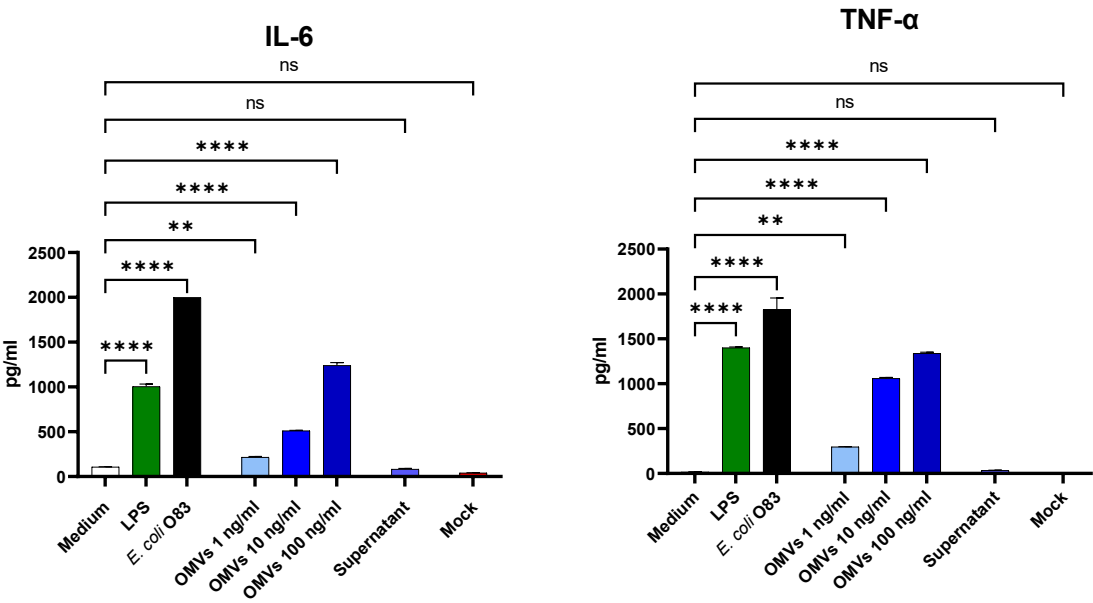
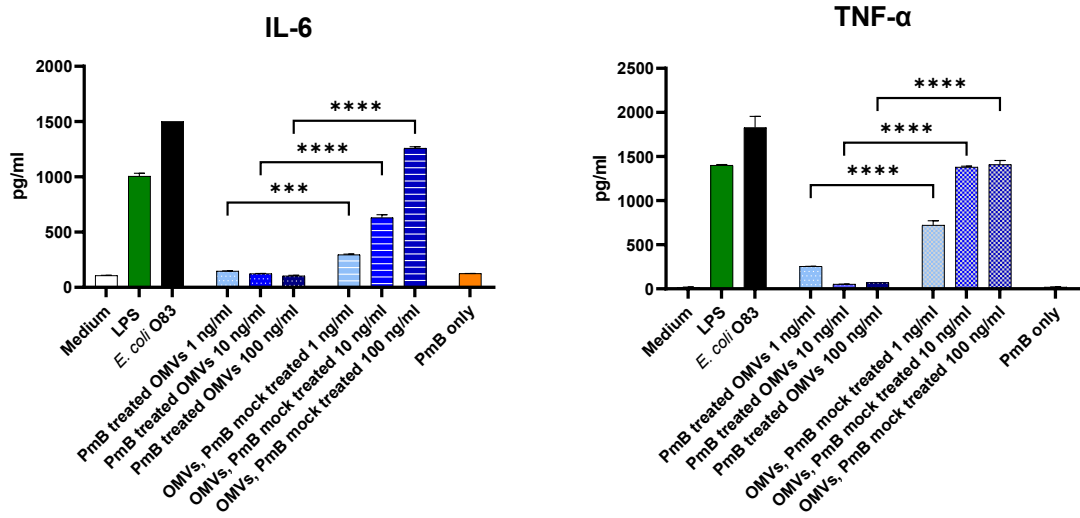


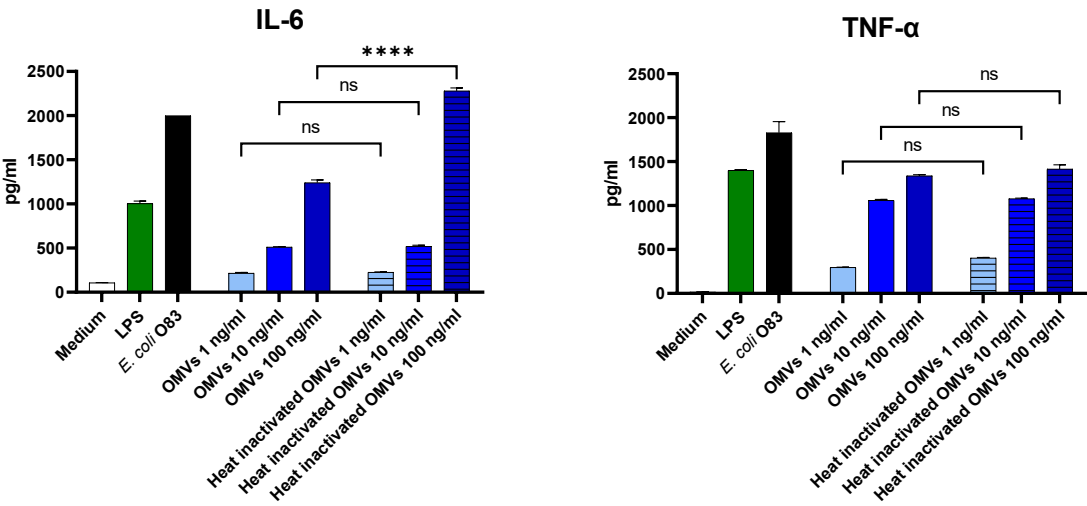
Figure S2_Schmid *et al.*











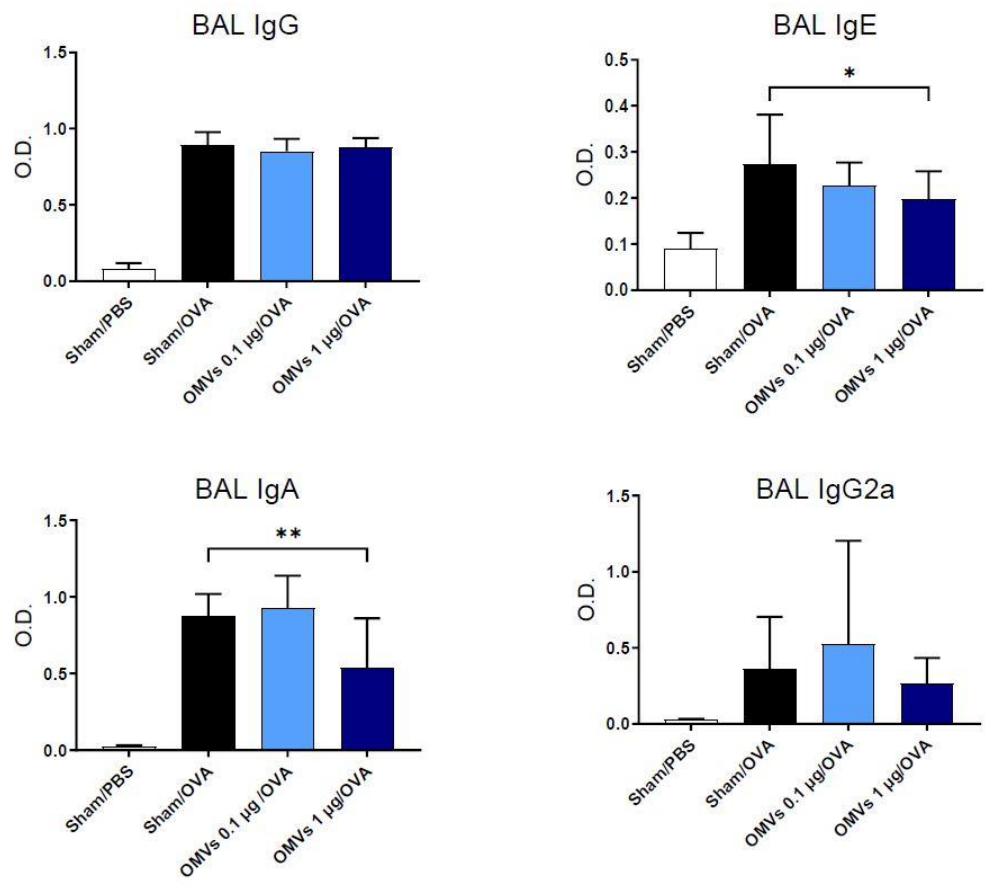
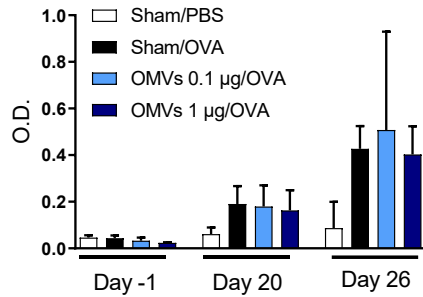


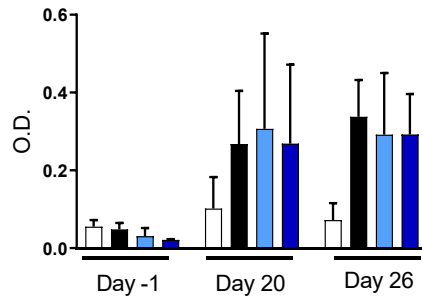
Figure S8_Schmid *et al.*

(A)

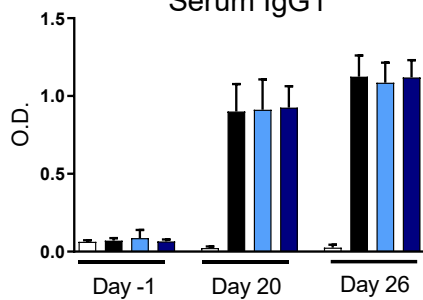
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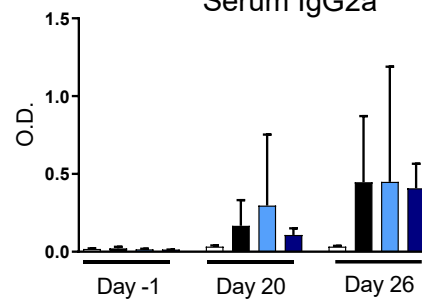
Serum IgE



Serum IgG1

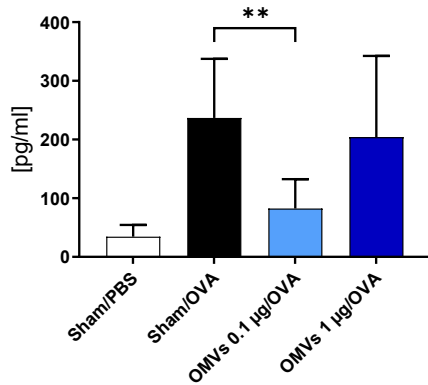


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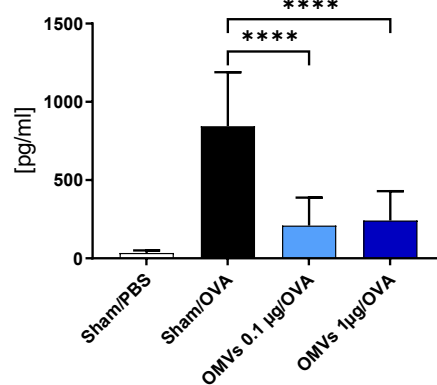


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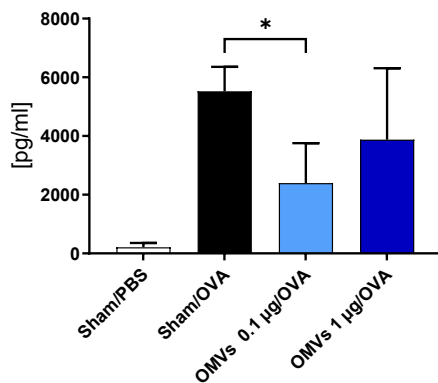
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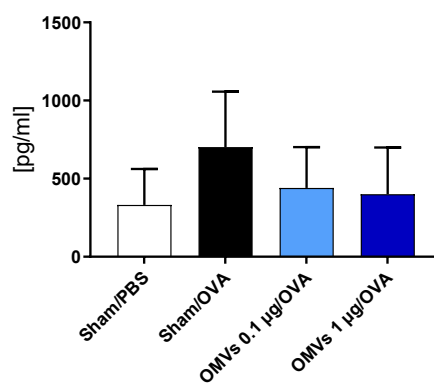
Spleen IL-5



Spleen IL-10



Spleen IFN-γ



SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Examination of EcO83-OMVs, EcO83-OMVs-depleted supernatant and mock control for bacterial contamination. The isolated EcO83-OMVs, EcO83-OMVs-depleted supernatant and mock control were examined for the presence of live bacteria. Samples were placed on LB agar plates and incubated at 37 °C for 96 h. The plates were examined for visible bacterial colonies.

Figure S2: Unprocessed image of Bioanalyzer protein analysis of EcO83-OMVs and *E. coli* O83 lysate (modified image in Fig. 1E). Samples 1 and 2: EcO83-OMVs in duplicates. Samples 9 and 10: *E. coli* O83 lysate in duplicates. Samples 3-8: Material not relevant to this publication.

Figure S3: Activation of the NF- κ B pathway by EcO83-OMVs. The macrophage-like mouse cell line RAW264.7 was stimulated with medium, LPS (1 μ g/ml), *E. coli* O83 (10^7 CFU/ml) and EcO83-OMVs (10 ng/ml and 100 ng/ml) at 37 °C and 5 % CO₂ for 6 h. The expression of IL-6, TNF- α and IL-1 β mRNA was measured with RT-PCR and is presented as the fold change to the housekeeping gene GAPDH. OMVs = EcO83-OMVs

Figure S4: Immunostimulatory potential of EcO83-OMVs compared to EcO83-OMVs-depleted supernatant and mock control. Cells isolated from the lungs of naive mice (n = 5) were treated with medium, LPS (1 μ g/ml), *E. coli* O83 (10^8 CFU/ml), EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml), EcO83-OMVs-depleted supernatant and mock control. The supernatant and mock control were added in the amount equal to the volume of vesicles used in cultures with 100 ng/ml EcO83-OMVs and incubated at 37 °C and 5 % CO₂ for 48 h. IL-6 and TNF- α were measured in the cell culture supernatant by ELISA. The mean $\pm$ SD is shown. Data were analysed using a One-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. **p<0.05; ****p<0.001. OMVs = EcO83-OMVs.

Figure S5: Immunostimulatory potential of EcO83-OMVs compared to Polymyxin B-treated EcO83-OMVs. Cells isolated from the lungs of naive mice (n =

5) were treated with medium, LPS (1 µg/ml), *E. coli* O83 (10⁸ CFU/ml), EcO83-OMVs treated with Polymyxin B (1 ng/ml, 10 ng/ml, 100 ng/ml), EcO83-OMVs treated with Polymyxin B mock (1 ng/ml, 10 ng/ml, 100 ng/ml) and stimulated with Polymyxin B alone at 37 °C/ 5% CO₂ for 48 h. IL-6 and TNF-α were measured in the supernatants by ELISA. The mean ± SD is shown. Data were analysed using a One-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. ***p < 0.01; ****p < 0.001. OMVs = EcO83-OMVs ; PmB = Polymyxin B

Fig. S6: Immunostimulatory potential of EcO83-OMVs compared to heat-treated EcO83-OMVs. Cells isolated from the lungs of naive mice (n = 5) were treated with medium, LPS (1 µg/ml), *E. coli* O83 (10⁸ CFU/ml), EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml) and heat-treated EcO83-OMVs (1 ng/ml, 10 ng/ml, 100 ng/ml) at 37 °C/ 5 % CO₂ for 48 h. IL-6 and TNF-α were measured in the supernatants by ELISA. The mean ± SD is shown. Data were analysed using a One-Way ANOVA followed by a post-hoc Tukey's multiple comparison test. ****p<0.001. OMVs = EcO83-OMVs

Figure S7: Local allergen-specific responses: Levels of OVA-specific antibodies in BAL. BAL was collected on sacrifice day by flushing the lungs with PBS and OVA-specific antibodies were measured by ELISA. Data were analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. *p<0.5; **p<0.05. n = 5/group. Data are representative of three independent experiments. Mean ± SD is shown. Significant differences between Sham/OVA and EcO83-OMVs treatment groups (OMVs 0.1 µg/OVA or OMVs 1 µg/OVA) are indicated. BAL = bronchoalveolar lavage; OVA = ovalbumin; OMVs = EcO83-OMVs.

Figure S8: Systemic allergen-specific responses: Levels of OVA-specific antibodies in serum and cytokines produced by OVA-stimulated splenocytes ex vivo. (A) Blood was sampled on days -1, 20 and 26 (Experimental setup see Figure 5 A) and centrifuged to obtain serum. OVA-specific antibodies in serum were measured by ELISA. **(B)** Single cell suspensions of excised spleens of Sham or EcO83-OMVs-treated allergic mice were stimulated with OVA for 72 h and cytokine levels were measured by ELISA. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. *p<0.5; **p<0.05; ***p<0.001. **(A)** Data are representative of three independent experiments for samples collected

260 on day -1 and a pool of two experiments for samples collected on days 20 and 26; n
261 = 5-10/group. **(B)** Data are representative of three independent experiments, n =
262 5/group. Mean $\pm$ SD is shown. Significant differences between Sham/OVA and
263 EcO83-OMVs treatment groups (OMVs 0.1 μ g/OVA or OMVs 1 μ g/OVA) are
264 indicated. OVA = ovalbumin; OMVs = EcO83-OMVs.

265 **SUPPLEMENTARY REFERENCES**

- 266 1. Korb, E. *et al.* Reduction of Allergic Lung Disease by Mucosal Application of
267 *Toxoplasma gondii*-Derived Molecules: Possible Role of Carbohydrates. *Front*
268 *Immunol* **11**, (2021).

Article V

RESEARCH ARTICLE

Growth phase matters: Boosting immunity via *Lactacaseibacillus*-derived membrane vesicles and their interactions with TLR2 pathways

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Abstract

Lipid bi-layered particles known as membrane vesicles (MVs), produced by Gram-positive bacteria are a communication tool throughout the entire bacterial growth. However, the MVs characteristics may vary across all stages of bacterial culture growth, leading to inconsistencies in MVs research. This, in turn, hinders their employment as nanocarriers, vaccines and other medical applications. In this study, we aimed to comprehensively characterize MVs derived from *Lactacaseibacillus rhamnosus* CCM7091 isolated at different growth stages: early exponential (6 h, MV6), late exponential (12 h, MV12) and late stationary phase (48 h, MV48). We observed significant differences in protein content between MV6 and MV48 (data are available via ProteomeXchange with identifier PXD041580), likely contributing to their different immunomodulatory capacities. *In vitro* analysis demonstrated that MV48 uptake rate by epithelial Caco-2 cells is significantly higher and they stimulate an immune response in murine macrophages RAW 264.7 (elevated production of TNF α , IL-6, IL-10, NO). This correlated with increased expression of lipoteichoic acid (LTA) and enhanced TLR2 signalling in MV48, suggesting that LTA contributes to the immunomodulation. In conclusion, we showed that *Lactacaseibacillus rhamnosus* CCM7091-derived MVs from the late stationary phase boost the immune response the most effectively, which pre-destines them for therapeutical application as nanocarriers.

KEYWORDS

growth curve, immunomodulation, *Lactacaseibacillus rhamnosus*, lipoteichoic acid, membrane vesicles, nanocarriers, TLR2

1 | INTRODUCTION

Extracellular vesicles (EVs) are bi-layered lipid membranous particles secreted by both eukaryotic and prokaryotic cells. Containing biological compounds such as nucleic acids, metabolites, lipids, polysaccharides and proteins (Gonzalez-Lozano et al., 2022), EVs are considered to be an essential tool in cell-to-cell interaction and intercellular communication (Nahui Palomino et al., 2021). When localized inside the vesicle, the bioactive molecules are protected from environmental conditions and can be

Miriam Sandanusova and Kristyna Turkova contributed equally to this study.

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transferred to near or distant sites to exert their functions. This has great potential in clinics, as the EVs can be used as carriers for drug delivery and vaccination.

Bacterial EVs come with great opportunities and many advantages in medical applications when compared with their maternal bacteria, such as higher safety, inability to replicate and possible fusion with the target cell when delivering the cargo, while keeping the capacity to boost the immune system. They were first shown to be released by Gram-negative bacteria more than 50 years ago (Chatterjee & Das, 1967) and were named outer membrane vesicles (OMVs). OMVs are already in use in clinics for vaccination (Micoli & MacLennan, 2020), however, their use is limited, because they can carry large quantities of lipopolysaccharide (LPS) causing too strong immune response (Park et al., 2021) and reduction of endotoxin activity is thus essential for the application (van der Ley & van den Dobbelsteen, 2011). The ability of Gram-positive bacteria to produce EVs was long questioned because the thick peptidoglycan layer in the bacterial cell wall was thought to inhibit EV formation. The first direct evidence of the existence of EVs derived from Gram-positive bacteria was provided by Lee et al. (2009). Since then, many genera of Gram-positive bacteria were confirmed as producers of EVs, including *Bacillus* (Rubio et al., 2020), *Streptococcus* (Mehanny et al., 2022; Yerneni et al., 2021), *Clostridium* (Ma et al., 2022), *Enterococcus* (Wagner et al., 2018), *Lactocaseibacillus* (Choi et al., 2020; Tong et al., 2021), *Lactiplantibacillus* (Kurata et al., 2022) and others. In Gram-positive bacteria, EVs are called membrane vesicles (MVs) and their size is approximately 20–500 nm in diameter (Champagne-Jorgensen et al., 2021; Zavan et al., 2019). In the medical context, the MVs derived from Gram-positive generally recognized as safe (GRAS) bacterial species such as *Lactocaseibacillus rhamnosus* emerge as even more tempting solution than the OMVs, due to their general safety and absence of endotoxins (Chen et al., 2022; Kim et al., 2017).

Lactocaseibacillus rhamnosus is a facultative anaerobic Gram-positive bacterium, isolated from a variety of habitats, including the human intestinal tract. Certain strains are known for their beneficial and immunomodulatory properties (Zheng et al., 2020). The effects of *L. rhamnosus*-derived MVs may be similarly useful, as they may reflect the contents of the maternal cells, which was already proven in many studies (Al-Nedawi et al., 2015; Behzadi et al., 2017; Champagne-Jorgensen et al., 2021; Gu et al., 2021; He et al., 2017; Keyhani et al., 2022; Mata Forsberg et al., 2019; Tong et al., 2021). Nevertheless, the proteome, transcriptome and functional properties of the culture of *L. rhamnosus* change depend on the cultivation conditions (Koskeniemi et al., 2009), growth phase (Laakso et al., 2011) or exposition to environmental stress (Aakko et al., 2014). Therefore, the information contained in the MVs can be altered accordingly (Kim et al., 2019; Mehanny et al., 2022; Tashiro et al., 2010; Wang et al., 2021). Up to date, to our best knowledge, no studies clarify how the different culture conditions influence the MVs uptake, interaction and recognition by the recipient cells (either epithelial or immune).

The lipoteichoic acid (LTA), Gram-positive bacteria membrane glycolipid that has a diacylated glycerol group attached to a sugar backbone and repeating units (Kang et al., 2009), is one of the pathogen-associated molecular patterns (PAMPs) recognized by the immune system of the host. LTA stimulates immune reaction through Toll-like receptor 2 (TLR2) (Knapp et al., 2008; Long et al., 2009; Nilsen et al., 2008), which in turn activates the NF- κ B pathway and pro-inflammatory mediators production (Kawai & Akira, 2007). LTA localization changes during the growth of several strains of *Lactocaseibacilli* (Shiraishi et al., 2018). As for their MVs, the presence of LTA in MVs was already confirmed (Champagne-Jorgensen et al., 2021; Uppu et al., 2023), although to our best knowledge, a study comparing LTA expression in MVs at different time points is lacking. Throughout the relatively short history of MV research, the MVs have often been isolated and characterized at only one selected time point of the growth phase (Choi et al., 2022; Han et al., 2023; Keyhani et al., 2022; Kurata et al., 2022). Only in the last 2 years have studies begun to compare MVs from at least two phases of maternal bacteria growth. For instance, da Luz et al. reported that the biochemical cargo of *Staphylococcus aureus*-derived MVs changed depending on the growth phase of the mother cells (da Luz et al., 2022). The time of the harvest influenced also *Streptococcus pneumoniae*-derived MVs' amount and differences were found in their interaction with primary macrophages (Mehanny et al., 2022).

Still, to the best of our knowledge, a comprehensive study covering the entire growth curve of the maternal bacteria and its influence on the composition and function of MVs is lacking. We believe that for using the entire potential of MVs in clinical applications (such as vaccine development and nanocarriers for drug delivery), it is of great importance to characterize and standardize the optimal time-point of the MVs isolation, with regards to their immunoboosting properties, maximal yields and absolute reproducibility. Here, we aimed to describe the impact of the growth phase of the microbial culture of *L. rhamnosus* CCM7091 on the biophysical characterization, uptake by the intestinal cells, immunomodulatory properties and content of its MVs. We hypothesized that the biophysical and biochemical composition of *Lactocaseibacillus rhamnosus*-derived MVs changes during the entire growth curve and thus the immunoboosting properties of these MVs may be different.

2 | MATERIALS AND METHODS

We have submitted all relevant data from our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230796) (EV-TRACK Consortium et al., 2017).

2.1 | Bacterial strain and growth conditions

The bacterial strain *Lactocaseibacillus rhamnosus* CCM 7091 (=ATCC 53103) was obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic) and cultivated aerobically in de Man, Rogosa and Sharpe broth (MRS) medium (VWR, Czech Republic) at 37°C for 48 h. The bacterial growth was determined by measuring the optical density at 600 nm in a 96-well plate (OD_{600nm}, Infinite M200 iControl, Tecan, Switzerland) and by plating on MRS agar medium prepared by adding 1.5% agar (VWR, Czech Republic). The colonies were counted after 48 h of incubation at 37°C (CFU mL⁻¹).

2.2 | Isolation and purification of MVs

Lactocaseibacillus rhamnosus CCM7091 was grown in 0.22- μ m-filtered MRS medium at 37°C for 24 h under aerobic conditions without shaking. The overnight culture (OD_{600nm} = 1.5) was sub-cultured at a ratio of 1:100 into the final volume of 500-mL MRS broth, followed by incubation at 37°C. Three cultivation end-time points of the growth curve were selected: the early exponential growth phase (6 h, MV6), the late exponential growth phase (12 h, MV12) and the late stationary phase (48 h, MV48). At the appropriate end-time point, the bacterial cell culture was centrifuged at 8800 $\times$ g for 30 min at 4°C to separate bacterial cells and unwanted debris (MR22i Refrigerated Centrifuge, Jouan, USA), and OD_{600nm} and CFU mL⁻¹ were determined. The supernatant was filtered using a 0.45- μ m pore membrane filter (PVDF, Whatman, Cyntiva, Germany) to remove any remaining bacterial cells. The filtrate was checked for sterility by plating 100 μ L of filtrate on MRS agar and cultivated for 48 h at 37°C. The filtrate was ultracentrifuged at 175,000 $\times$ g for 2 h (32,000 rpm for rotor SWTi32; Optima TM LE-80K Preparative Ultracentrifuge, Beckman Coulter, USA). After the ultracentrifugation, the supernatant was discarded, and the pellet was re-suspended in sterile 0.22- μ m-filtered phosphate-buffered saline (PBS; pH 7.5). Of note, in all the experiments when the PBS was needed, the sterile 0.22- μ m-filtered PBS (pH 7.5) was used. The crude fraction of MVs was then purified using a sucrose cushion as described by Pospichalova et al. (2015) with slight modifications. Briefly, the crude fraction was re-suspended in PBS, underlaid with 30% sucrose solution (30% sucrose in 20-mM Tris, pH 7.6 in D₂O, 0.22- μ m-filter sterilized) and ultracentrifuged at 175,000 $\times$ g for 2 h at 4°C (32,000 rpm for rotor SWTi32; slow deceleration; Optima TM LE-80K Preparative Ultracentrifuge, Beckman Coulter, USA). The interface was collected, refilled with PBS, ultracentrifuged at 175,000 $\times$ g for 2 h at 4°C (32,000 rpm for rotor SWTi32; Optima TM LE-80K Preparative Ultracentrifuge, Beckman Coulter, USA) and the pellet was re-suspended in $\pm$ 100 μ L of PBS to obtain the final purified MVs fraction (Figure S1). The MVs were stored at -80°C until use.

2.3 | Size and particle concentration analysis

The Multi-angle Dynamic Light Scattering (MADLS) technique was used for the size and particle concentration analysis. Measurements were performed using Zetasizer Ultra (Malvern Panalytical Ltd, UK) equipped with a He-Ne laser at a wavelength of 633 nm at a constant temperature of 25°C. The light scattering data were collected at three angles, 173°, 90° and 13°, and evaluated using ZS Xplorer software version 3.20 (Malvern Panalytical Ltd, UK). Results for the hydrodynamic diameter, polydispersity index (PDI) and particle concentration are reported as the mean value of four independent measurements $\pm$ standard deviation (SD). The difference in particle size between samples was determined using a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA).

2.4 | Visualization by electron microscopy

Four microlitres of the MVs sample were applied to freshly plasma-cleaned TEM grids (Quantifoil, Cu, 300mesh, R2/1) and vitrified in liquid ethane using Leica Automatic Plunge Freezer EM GP2 (4°C, 100% rel. humidity, 30-s waiting time, 4-s blotting time and using sensor blotting). The grids were subsequently mounted into the Autogrid cartridges and loaded into Talos Arctica (Thermo Fisher Scientific, USA) transmission electron microscope for imaging. The microscope was operated at 200 kV. The Cryo-TEM micrographs of MVs were collected on the K2 direct electron detection camera at a nominal magnification of 79,000 $\times$ with an average defocus of -2.2 μ m and an overall dose of $\sim$ 40 eÅ⁻².

2.5 | Fluorescent labelling of the MVs and their uptake by intestinal cells detection

The MVs were diluted to a concentration of 10¹² particles per mL in labelling buffer (50-mM Na₂CO₃, 100-mM NaCl, pH 9.2) and Octadecyl Rhodamine B Chloride (Biotium, USA) (R18) was used as the fluorescent probe. R18 was added to the MVs in

a final concentration of $1.5 \mu\text{g mL}^{-1}$ and incubated for 30 min at 25°C with shaking. Subsequently, 3.5 mL of PBS was added and the suspension was underlaid with 1 mL of 30% sucrose solution (30% sucrose in 20-mM Tris, pH 7.6 in D_2O , $0.22\text{-}\mu\text{m}$ -filter sterilized) and ultracentrifuged at $175,000 \times g$ for 2 h at 4°C (38,000 rpm for rotor SW55Ti; slow deceleration; Optima TM LE-80K Preparative Ultracentrifuge, Beckman Coulter; USA). The interface was collected, refilled with PBS to wash the free label and ultracentrifuged at $175,000 \times g$ for 2 h at 4°C (38,000 rpm for rotor SW55Ti; slow deceleration; Optima TM LE-80K Preparative Ultracentrifuge, Beckman Coulter; USA). The R18-labelled MVs were re-suspended in $100 \mu\text{L}$ of PBS and immediately used for Caco-2 cell treatment.

The human colorectal carcinoma cell line Caco-2 (American Type Culture Collection, USA) was routinely cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA), supplemented with 10% (vv^{-1}) foetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 100 U mL^{-1} of penicillin and $100 \mu\text{g mL}^{-1}$ of streptomycin (Gibco, USA) and 5-mM pyruvate. Cells were cultivated at 37°C in a humidified 5% CO_2 atmosphere. For the experiments, 2.0×10^4 cells per well were seeded to a 96-well plate and differentiated for 21 days. One day before the MV uptake experiment, the medium was replaced with a medium without antibiotics and FBS. To study the uptake mechanism, inhibitors of various MVs uptake pathways were employed: chlorpromazine ($30 \mu\text{M}$), genistein ($10 \mu\text{M}$), nystatin ($10 \mu\text{M}$) or wortmannin ($1 \mu\text{M}$) (all Sigma Aldrich, USA); 1 h prior to the MV48 addition. The R18-labelled MVs were added to the cells in a concentration of 10^5 particles per cell and the fluorescence was measured until it reached plateau (Infinite M200 iControl, Tecan, Switzerland) ($\lambda_{\text{Ex}} = 560 \text{ nm}$; $\lambda_{\text{Em}} = 590 \text{ nm}$). The time-course internalization was assessed as previously described (Gnopo & Putnam, 2020). Briefly, the fusion efficiency of the uptake was determined using Equation (1), where F_0 represents the fluorescence of the labelled MVs in the absence of cells, F_T is the increasing fluorescence over time, and F_I corresponds to the fluorescence observed at infinite dilution when 60-mM Triton X-100 (Sigma Aldrich, USA) was added (Triton X-100 being a detergent capable of dequenching all remaining R18, yielding a maximum fluorescence signal). Following these calculations, the resulting curve was fitted using Equation (2) to extract the kinetic parameters of the uptake. Specifically, Y represents the fusion efficiency at time t , Y_{max} corresponds to the maximum fusion efficiency and k represents the fusion rate constant. Fluorescence assays were repeated in four to six independent measurements, in technical triplicates. The statistical analysis was conducted using a t -test in R Studio.

$$\% = \frac{F_T - F_0}{F_I - F_0} \quad (1)$$

$$Y = Y_{\text{max}} (1 - e^{-kt}) \quad (2)$$

2.6 | Preparation of RAW 264.7 cell line and the treatment with MVs

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, USA) was routinely cultured in DMEM (Gibco, USA), supplemented with 10% (vv^{-1}) FBS (Thermo Fisher Scientific, USA), 100 U mL^{-1} of penicillin and $100 \mu\text{g mL}^{-1}$ of streptomycin (Gibco, USA). Cells were cultivated at 37°C in a humidified 5% CO_2 atmosphere. To analyse the production of cytokines and reactive nitrogen species (RNS), the cells were seeded in a 24-well plate at a density of 2.5×10^5 cells per well. Twenty-four hours after the seeding, the medium was replaced with a medium without antibiotics, and the cells were treated for 24 h with three concentrations of MVs: 1.0×10^7 , 1.0×10^5 and 1.0×10^3 MVs per cell. As a negative control, the vehicle alone (PBS) was used; whereas 100 ng mL^{-1} LPS (*Escherichia coli* O26:B6 LPS, cat. n. 297-473-0, Merck, Germany) was used as a positive control. The media were then collected, depleted of any remaining cells (by centrifugation, $250 \times g$ for 5 min at 4°C), and stored at -20°C for further cytokine and RNS detection. The statistical analysis was performed using ANOVA followed by Dunnett's multiple comparisons test in R Studio; three independent repetitions were performed.

2.7 | ELISA

Levels of the inflammatory cytokines $\text{TNF}\alpha$ and IL-6, the immunoregulatory cytokine IL-10 and IL-8 were detected in the cell culture medium collected after the treatment of RAW 264.7 cells or HEK293 with the MVs. Detection was performed by ELISA (cat. n. 88-7324-88, 88-7064-88, 88-7105-88, 88-8086-88, Thermo Fisher Scientific, USA) following the manufacturer's instructions with no modifications.

2.8 | Measurement of nitrite concentration by Griess reaction

Changes in RNS production were measured indirectly as the accumulation of nitrites, the end product of NO metabolism, in a medium using the Griess spectrophotometric assay, as previously described (Arias-Negrete et al., 2004). At the end of the

incubation period, the culture media were collected from the wells and centrifuged at $250 \times g$ at 4°C for 5 min. Then 80 μL of supernatant was mixed with an equal volume of Griess reagent (Sigma Aldrich, USA) in a 96-well plate and the mixture was incubated at room temperature and in the dark for 30 min. Absorbance was measured at 546 nm (Spectrophotometer Sunrise, Tecan, Switzerland). Sodium nitrite was used as a standard.

2.9 | Proteomic sample preparation and analysis by liquid chromatography–tandem mass spectrometry

Proteins extracted from MVs of *Lactocaseibacillus rhamnosus* CCM 7091 isolated at defined time points of the growth curve (6, 12 and 48 h) were subjected to filter-aided sample preparation as described elsewhere (Wisniewski et al., 2009). The resulting peptides were analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) performed using an UltiMate 3000 RSLCnano system (Thermo Fisher Scientific, USA) coupled on-line to a timsTOF Pro spectrometer (Bruker, USA). See the supplemental material section for full details of the analysis and data evaluation. The mass spectrometry proteomics data have been deposited at the proteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD041580.

2.10 | Bioinformatic processing of proteomic outputs

Proteins that did not meet the condition of identification by at least two unique peptides were filtered out. Further, all proteins that were found in at least one sample from triplicates of MV6, MV12 and MV48 were selected for processing. Protein cell localizations were predicted using PSORTb software (Yu et al., 2010). The annotation and classification of proteins into functional groups were carried out by KEGG Mapper (Kanehisa, 2019; Kanehisa & Goto, 2000; Kanehisa et al., 2023). To visualize the protein overlap, Venn diagrams were generated using the ggVennDiagram package in R. A heatmap was generated using $\log_2$ -transformed and imputed intensities with the ComplexHeatmap package in R, and the default clustering method, hierarchical clustering, was used to group similar data. Principal component analysis (PCA) was performed on $\log_2$ -transformed and imputed intensities using the ggplot2 package in R. Volcano plots were generated using the VolcanoR web application (Goedhart & Luijsterburg, 2020). LIMMA-calculated statistics and $\log_2$ -transformed fold changes were used to visualize the differential expression of proteins.

For the prediction of protein–protein interactions (PPI), the MV48 protein dataset was used, comprising proteins that were identified with at least two peptides in at least one replicate and exhibited at least a twofold increase in abundance compared to MV6 and MV12. The interactions were predicted with proteins from the mouse IL-6, IL-10 and TNF α signalling cascades and human TLR pathways, which were retrieved from the Reactome database (Milacic et al., 2024). The interolog prediction on the PredHPI Webserver (Loaiza & Kaundal, 2021) was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

2.11 | Western blotting and immunodetection of LTA

The LTA levels in MV6, MV12 and MV48 were detected by Western blot analysis with immunodetection, as previously described (Ambrozova et al., 2010). Briefly, equal volumes of MV pellets were mixed with Leammli buffer and heated to 100°C for 10 min, then loaded and subjected to SDS-polyacrylamide gel electrophoresis using 12.5% running gel. For reference, PageRuler Plus Prestained Protein Ladder (cat. n. 26619, Thermo Fisher Scientific, USA) was loaded. Anti-LTA monoclonal antibody (cat. n. MA1-7402, Thermo Fisher Scientific, USA) and anti-mouse IgG horseradish peroxidase-linked whole antibody (from the horse; Cell Signaling Technology, USA) were used. Immunoreactive bands were detected using an ECLTM detection reagent kit (Pierce, USA) by AmershamTM Imager 680 and quantified by scanning densitometry using the Image JTM programme. The individual band density value was expressed in arbitrary units. For statistical analysis, ANOVA followed by Dunnett's multiple comparison test in GraphPad Prism 6 (GraphPad Software, San Diego, California, USA) was used; three independent experiments were performed.

2.12 | Toll-like receptor (TLR) screening assay

HEK293 (ATCC, USA) transfectants TLR2/CD14, TLR6/2 and TLR1/2 were stably transfected with pDUO plasmids purchased from InvivoGen (USA) according to the manufacturer's instructions. pDUO-hCD14/TLR2, pDUO-hTLR1/TLR2 pDUO-hTLR6/TLR2 are expression vectors designed to co-express CD14 and TLR2 genes, TLR1 and TLR2 genes and TLR6 and TLR2 genes. Further, commercially available HEK293 cells stably transfected with the human TLR4a, MD2 and CD14 genes, NOD1

genes and NOD2 genes were used (293/hTLR4A-MD2-CD14, 293-hNOD1 and 293-hNOD2 cells) and HEK 293 control cell line (293/null cells) was tested as a scrambled control (InvivoGen, USA).

Transfected cells were grown in DMEM (Gibco, USA) supplemented with 10% (v/v⁻¹) FBS (Thermo Fisher Scientific, USA), 1% (v/v⁻¹) L-glutamine (Gibco, USA) and 100 U mL⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin (Gibco, USA) with selective antibiotic Blastcidin (10 µg mL⁻¹; InvivoGen, USA) applied every 5th passage. For the experiment, cells were seeded in a medium without antibiotics at 2.0×10^4 cells per well in 96-well plates and treated for 24 h with MVs in concentration 10^5 MVs per cell. Positive controls for each cell line were as follows: 10^8 HKLM mL⁻¹ for TLR2/CD14, 50 ng mL⁻¹ Pam3CSK4 for TLR1/2, 10 ng mL⁻¹ FSL1 for TLR6/TLR2, 10 mg mL⁻¹ LPS for TLR4A-MD2-CD14, 100 µg mL⁻¹ iE-DAP for NOD1, 10-ng mL⁻¹ L18-MDP for NOD2 (InvivoGen, USA) cells. Cell culture supernatants from four independent experiments were assayed for IL-8 ELISA according to the manufacturer's instructions. Differences between samples were verified using R Studio and statistically analysed by ANOVA. Data normalization was performed using a Z-score.

2.13 | Statistical analysis

Data are presented as mean $\pm$ SD. All measurements were performed at least in duplicate in n independent experiments. The statistical significance was assessed at $*p < 0.05$. Details regarding statistical analyses can be found in the description of the particular methods.

3 | RESULTS

3.1 | *Lactacaseibacillus rhamnosus*-derived MVs average size increases with a longer cultivation period

First, to detect changes in the growth of *Lactacaseibacillus rhamnosus* CCM7091 cells, the overnight culture was inoculated in MRS broth and incubated aerobically at 37°C for 48 h, and we determined OD_{600nm} and CFU mL⁻¹ throughout the 48-h incubation. According to the growth curve (Figure S2) and the CFU mL⁻¹ (Table S1), we selected the time points for MV isolation to represent different growth phases: the early exponential phase (6 h, MV6), the late exponential phase (12 h, MV12) and the late stationary phase (48 h, MV48).

After harvesting and purifying the MVs at three-time points of the growth curve, we performed biophysical characterization using MADLS. Generally, the *L. rhamnosus* CCM7091-derived MVs consisted of two main populations with peak maxima around 30 nm for the first population and around 100 nm in diameter for the second population, as shown in intensity distribution analysis (Figures 1a–c and S3). The third population with a peak maximum of around 500 nm (Figure 1a–c) represents potential impurities or aggregates in the sample, which were nevertheless in the minority as can be seen in the Number distribution analysis (Figure 1d). When comparing the particle size across the batches of MVs isolated at the three time points, the average particle diameter was 72 ± 14 , 95 ± 10 and 101 ± 7 nm for MV6, MV12 and MV48, respectively (Table 1), with the difference between MV6 and MV48 being statistically significant. This can be explained by a larger proportion of the smaller population around 30 nm in the MV6 sample compared to the MV48, where the population around 100 nm has a higher contribution to the total concentration of particles in the sample. The presence of multiple particle populations in all selected samples is also reflected in the PDI. Generally, high values (approximately 0.2 and higher) of the PDI index indicate a broad size distribution. Nevertheless, compared to other populations, MV48 shows a homogeneous population character with an average PDI of 0.263 ± 0.070 compared to MV6 with a PDI of 0.369 ± 0.054 . Next, we examined the morphology of *L. rhamnosus*-derived MVs using cryo-electron microscopy (cryo-EM). The representative image shows that the MV48 sample consisted of lipid bi-layer vesicles of varying sizes (Figure 1e).

3.2 | The uptake of late-stationary-phase *L. rhamnosus* MVs is the most efficient and the inhibitor of the caveolae-mediated pathway is the most effective in blocking their uptake by Caco-2 cells

Physiologically, the intestinal cells are the first cell type to encounter the gut microbiota and its EVs. For that reason, we have chosen the cell line Caco-2 (colorectal carcinoma cell line commonly used as a model of the intestinal monolayer) to monitor the uptake of MVs by the intestinal epithelium. We stained the MVs by lipophilic probe R18, which fluorescence is quenched at high R18 concentrations, but de-quenched when the probe is diluted. Then, we incubated the labelled MVs with Caco-2 cells, spectrophotometrically detected the fluorescence plateau and calculated the kinetic parameters.

Our results indicate that MVs were effectively internalized by the Caco-2 cell monolayer, as the fluorescence signal increased during the uptake (Figure 2a). The uptake of MV6 and MV12 reached a maximum of 19% by recipient cells, while the uptake of MV48 was significantly higher, reaching 25% (Figure 2a and Table 2). As a negative control, we subjected the vehicle alone (PBS)

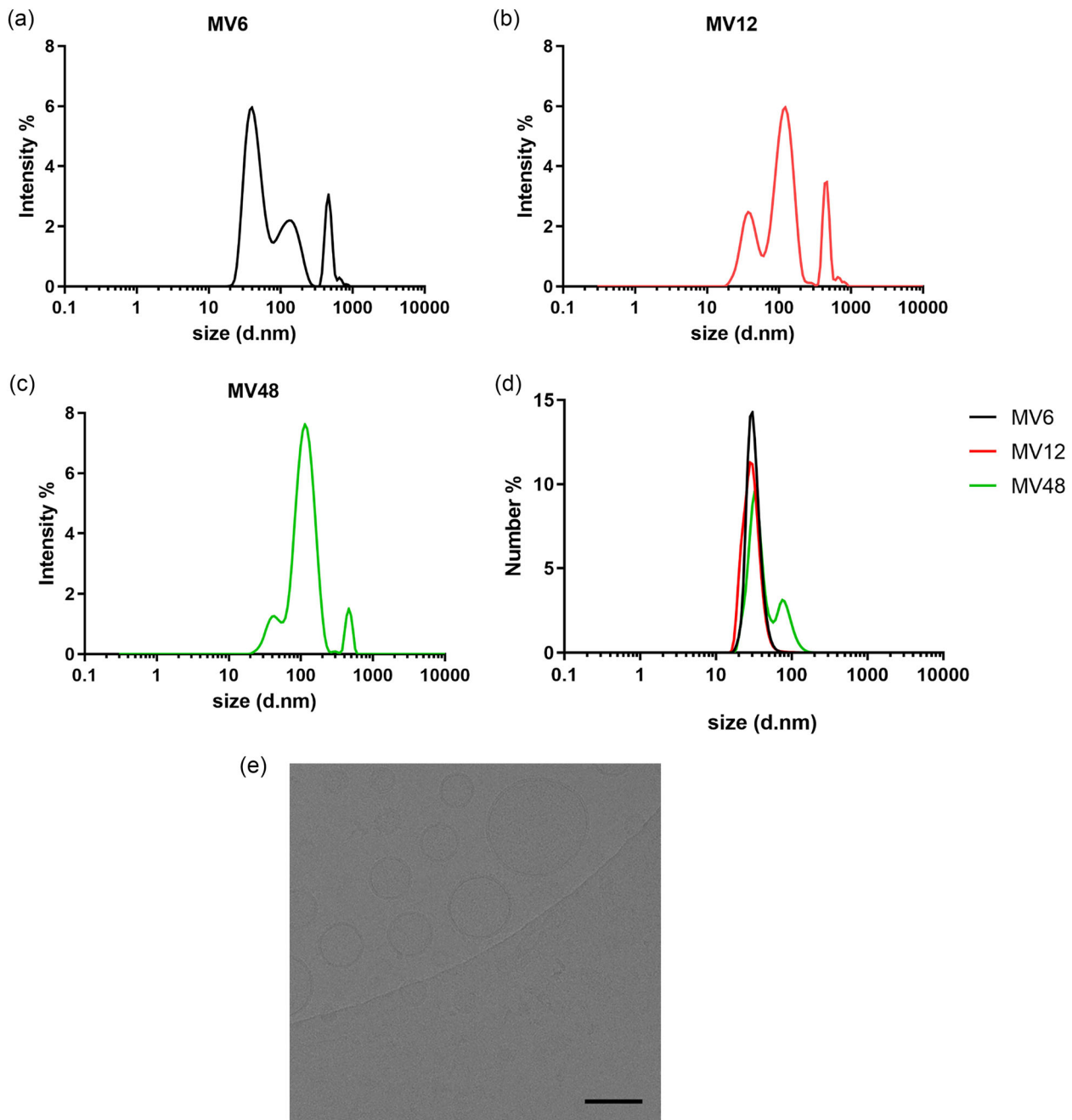


FIGURE 1 Biophysical characterization of *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs) isolated throughout bacterial growth (after 6, 12 and 48 h – MV6, MV12, MV48). (a)–(c) Size distribution by intensity (MV6, MV12 and MV48, respectively). (d) Size distribution by number. (e) Cryo-electron microscopy visualization of MV48 (bar length is 100 nm).

to the same labelling protocol as the MVs. Its internalization exhibited a different time-course of internalization compared to that of labelled MVs (Figure S4).

The uptake mechanism of the MVs may have several forms, of which the most probable for MVs uptake are endocytosis, micropinocytosis and lipid rafts-mediated internalization. To identify the prevailing uptake mechanism of *L. rhamnosus* MVs by Caco-2 cells, we tested the inhibitors of the chosen pathways: chlorpromazine, genistein, nystatin, wortmannin for clathrin- and caveolae-mediated endocytosis, lipid rafts-dependent and micropinocytosis, respectively (as nicely reviewed by O'Donoghue & Krachler (2016)). Treatment with all the inhibitors resulted in a statistically significant decrease in MV48 uptake. Genistein was the most effective in the uptake inhibition, reducing the saturation significantly compared with the rest of the inhibitors (Figure 2b and Table 3). Therefore, the MV uptake mechanism by Caco-2 cells is likely a combination of endocytosis, micropinocytosis and lipid rafts-dependent uptake, and the predominant pathway is the caveolae-mediated endocytosis.

TABLE 1 Size and concentration of *Lactacaseibacillus rhamnosus* CCM7091-derived membrane vesicles (MVs) after cultivation for 6, 12 and 48 h (MV6, MV12, MV48), analysed by MADLS.

Name	n	Z-average (nm)	Polydispersity index (PDI)	Concentration (particles mL ⁻¹)
MV6	1	80 ± 2	0.383 ± 0.015	1.80·10 ¹³
	2	91 ± 11	0.450 ± 0.062	7.40·10 ¹²
	3	56 ± 3	0.305 ± 0.006	1.40·10 ¹²
	4	60 ± 6	0.337 ± 0.026	3.80·10 ¹²
	Average	72 ± 14	0.369 ± 0.054	7.65 ± 6.35 ·10 ¹²
MV12	1	84 ± 2	0.408 ± 0.014	2.00·10 ¹²
	2	93 ± 3	0.447 ± 0.028	2.10·10 ¹²
	3	92 ± 0	0.245 ± 0.009	1.20·10 ¹⁴
	4	111 ± 2	0.616 ± 0.005	1.00·10 ¹³
	Average	95 ± 10	0.429 ± 0.132	3.35 ± 5.00 ·10 ¹³
MV48	1	103 ± 1	0.198 ± 0.010	5.00·10 ¹³
	2	88 ± 1	0.217 ± 0.005	5.30·10 ¹²
	3	106 ± 3	0.257 ± 0.017	9.93·10 ¹²
	4	105 ± 4	0.379 ± 0.006	1.40·10 ¹²
	Average	101 ± 7*	0.263 ± 0.070	1.67 ± 1.95 ·10 ¹³

Note: One-way ANOVA was followed by Tukey's honestly significant difference (HSD) test. $n = 4$.

* $p < 0.05$; statistical significance for MV48 Z-average versus MV6 Z-average.

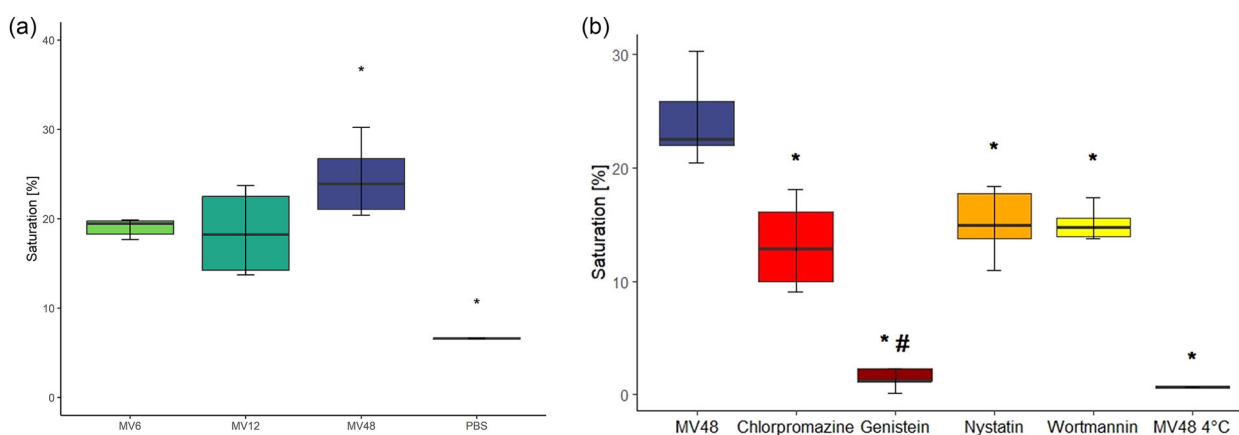


FIGURE 2 Uptake of *Lactacaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs) by Caco-2 cells. (a) The final saturation observed approximately after 10-h incubation of R-18 labelled MVs from different times of isolation: 6, 12 and 48 h (MV6, MV12, MV48) with Caco-2 cell monolayer. The increase in fluorescence was monitored until saturation. The vehicle alone, subjected to the same labelling protocol, was used as negative control (PBS). (b) Determination of MV48 uptake pathways. Selective inhibitors targeting different uptake pathways were employed 1 h before MV48 treatment: chlorpromazine (red) for clathrin-mediated endocytosis, genistein (dark red) for caveolin-mediated endocytosis, nystatin (orange) for lipid rafts and wortmannin (yellow) for micropinocytosis. The uptake of MV48 in the absence of inhibitors is represented as a control (blue). Additionally, MV uptake was assessed at 4°C to demonstrate the energy dependence of the process. The box plots show medians (lines), interquartile ranges (boxes) and minimum and maximum values (whiskers); statistical analysis was performed using the t -test and the significance was assessed at $p < 0.05$: * marks a statistically significant difference between MV48 without inhibitors versus Chlorpromazine, Genistein, Nystatin, Wortmannin, MV48 4°C; # marks a statistically significant difference between Genistein versus Chlorpromazine, Nystatin, Wortmannin; $n = 4-6$.

TABLE 2 Kinetic parameters of *Lactacaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs) uptake by Caco-2 cells.

	MV6	MV12	MV48	PBS
Plateau [%]	18.83	18.28	24.32	6.600
K [h ⁻¹]	0.293	0.259	0.280	0.273
Half-life [h]	2.364	2.677	2.478	2.537
Tau [h]	3.410	3.862	3.574	3.660

Note: Fluorescently labelled MVs isolated at different times of cultivation (MV6, MV12, MV48) were cultivated with Caco-2 cells until uptake saturation was reached. The parameters encompass the saturation plateau, kinetic rate constant (K), half-life (defined as the time required to attain half of the saturation level) and tau (signifying the time constant of saturation).

TABLE 3 Kinetic parameters of *Lactacaseibacillus rhamnosus* CCM7091-derived membrane vesicles (MVs) uptake by Caco-2 cells treated with selective inhibitors of uptake pathways.

	MV48	Chlorpromazine	Genistein	Nystatin	Wortmannin	MV48 4°C
Plateau [%]	24.32	14.76	1.47	15.59	15.49	0.70
K [h ⁻¹]	0.280	0.478	0.297	0.581	0.573	1.688
Half-life [h]	2.478	1.450	2.333	1.193	1.210	0.411
Tau [h]	3.574	2.092	3.366	1.722	1.745	0.592

Note: The MVs were obtained after 48-h bacterial cultivation (MV48). The Caco-2 cells were treated with the inhibitors (chlorpromazine, genistein, nystatin or wortmannin) for 1 h before the addition of MV48, and kinetic parameters were calculated. The parameters encompass the saturation plateau, kinetic rate constant, half-life (defined as the time required to attain half of the saturation level) and tau (signifying the time constant of saturation). As negative controls, the MV48 were added to Caco-2 cells without the inhibitors (MV48), and the same was measured also at 4°C without the presence of inhibitors (MV48 4°C).

To exclude the possibility that the MV uptake is a passive process, we conducted the experiment also at 4°C, when the transcription, translation and metabolic activity of the recipient cells are reduced (Hunt et al., 2005). The resulting fusion efficiency, which we calculated, reached values up to 0.8% for all MVs (Figure 2b and Table S2), thus we can confidently conclude that the uptake of MVs is indeed an active, energy-dependent process.

We further tested whether the MVs induce inflammation-related activation of the Caco-2 cells. We measured IL-8 levels by ELISA, however, the MVs did not have any significant effects on IL-8 production (data not shown).

3.3 | *L. rhamnosus* MVs from the later stages of the growth curve exert immunomodulatory properties

It is known that in the intestine, the bacterial EVs may pass across the epithelial monolayer also paracellularly (Jones et al., 2020; Krsek et al., 2023) and come in contact with immune cells of the host, so we proceeded to investigate their immunoregulatory effects on innate immune cells. We analysed the interaction of MV6, MV12 and MV48 with the murine macrophage cell line RAW 264.7, as a representative of the host's first defence against undesirable antigens. We measured the production of RNS, as well as the release of pro-inflammatory and immunoregulatory cytokines (TNF α , IL-6 and IL-10, respectively). To verify that the observed effects were caused by MVs, we used a series of dilutions of each MV pellet.

Stimulation of RAW 264.7 cells by MV48 resulted in an increased inflammatory reaction, in a concentration-dependent manner. The highest concentration of all the MVs (10^7 MVs per cell), and MV48 in particular, induced the most significant increase in the analysed inflammatory mediators (RNS, TNF α and IL-6) (Figure 3a–c). The middle concentration (10^5 MVs per cell) revealed the differences between the MVs isolated from different stages of the *L. rhamnosus* growth curve compared with vehicle-treated control – the production of pro-inflammatory mediators was elevated after the MV48 treatment, in the case of TNF α statistically significantly. To get a more comprehensive view of the macrophages' response to the MVs, we determined the production of the immunoregulatory cytokine IL-10. Treatment with MV48 induced an enhanced production of IL-10 (Figure 3d), corresponding with a heightened RNS and TNF α release at concentrations of 10^7 and 10^5 MVs per cell. MV6 caused a minor increase in the IL-10 production which did not reach statistical significance, not even at the concentration of 10^7 MVs per cell, compared to the vehicle-treated control.

In summary, functional tests of *L. rhamnosus*-derived MVs have shown that different-time-points MVs treatment of immune cells results in diverse responses. The MV48 elicits a pro-inflammatory response of murine macrophages, as well as the production of immunoregulatory cytokine IL-10, in contrast to MV6 and MV12.

3.4 | The protein profile of *L. rhamnosus* CCM7091-derived MVs changes with the growth phase of the maternal culture

To elucidate the cause of different immunomodulatory effects of the MVs isolated from different time points of the growth curve of *Lactacaseibacillus rhamnosus* CCM7091, we analysed their protein composition by LC-MS/MS (each time point MV sample in triplicate). A total of 879, 1081 and 882 proteins were associated with MV6, MV12 and MV48, respectively. Each protein was identified based on at least two unique peptides and was present in at least one replicate. Protein localization was similar across the different time points samples (Figure 4a). The majority of the proteins were classified as either cytoplasmic membrane (MV6 = 429, MV12 = 491, MV48 = 393) or cytoplasmic (MV6 = 285, MV12 = 364, MV48 = 302) proteins (Table S3), based on the predictions made using the PSORTb software. In contrast, extracellular proteins and cell wall-associated proteins were in the

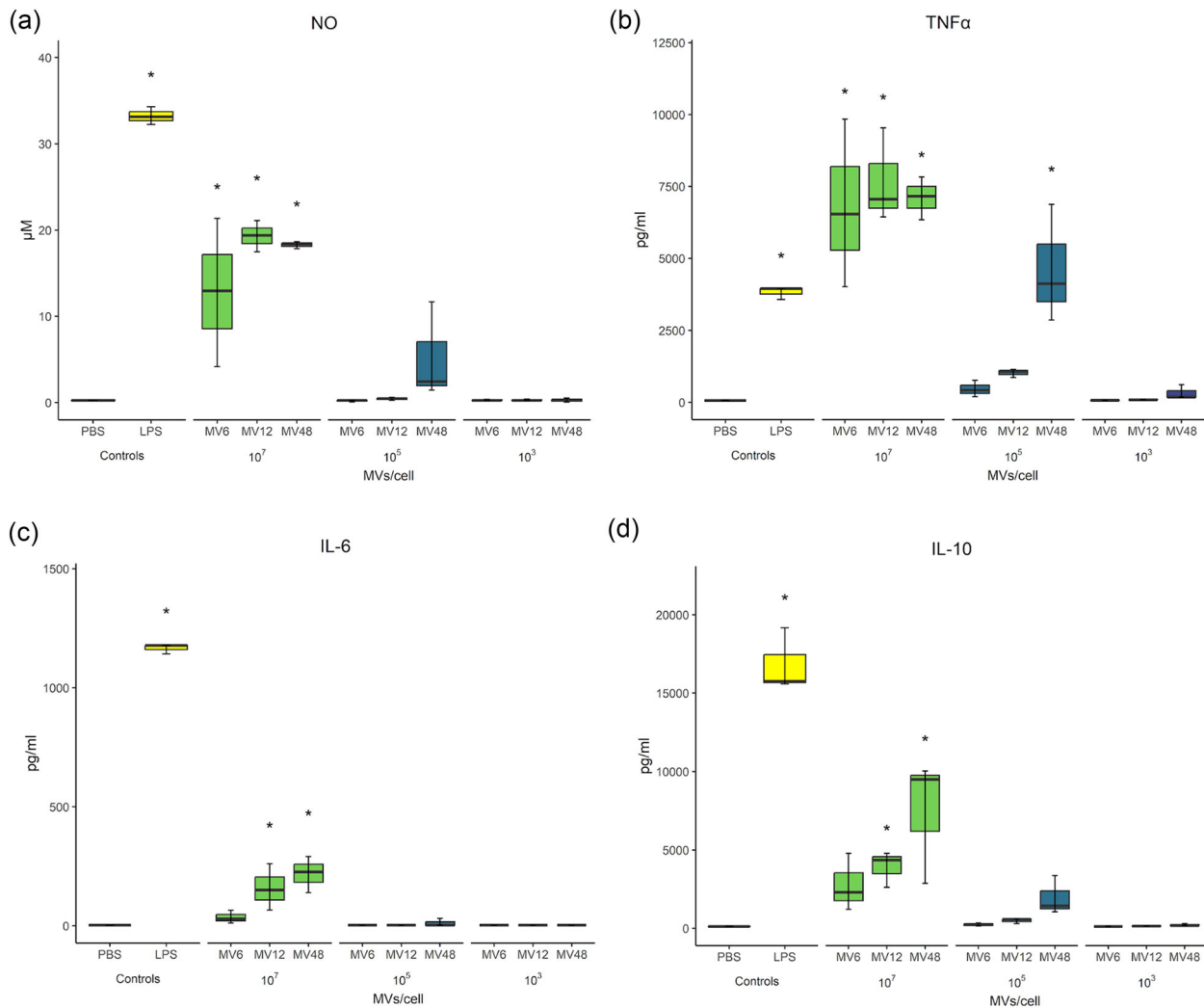


FIGURE 3 Pro-inflammatory (NO, TNFα, IL-6) and immunoregulatory (IL-10) mediators (a–d, respectively) produced by RAW 264.7 treated with *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs) in three concentrations (10⁷, 10⁵ and 10³ MVs/cell). MVs were isolated at different times of cultivation: 6, 12 and 48 h (MV6, MV12, MV48); PBS was used as negative control (Ctrl), and *Escherichia coli* lipopolysaccharide (LPS) was used as positive control. The box plots show medians (lines), inter-quartile ranges (boxes) and minimum and maximum values (whiskers); ANOVA was followed by Dunnett's multiple comparisons test versus negative control; statistical significance was assessed at * $p < 0.05$; $n = 3$.

minority. Part of the identified proteins were annotated only as predicted or inferred from homology, hence the origin of about 18% (MV6 = 140, MV12 = 196, MV48 = 158) proteins was unknown.

Then, we determined the functional classification of MV6, MV12 and MV48 proteomes using the KEGG Mapper (Figure 4b). Again, since a significant proportion of proteins in our dataset had not yet been reviewed in databases such as UniProt and were annotated as either predicted or inferred from homology, only approximately 65% of the total proteins were classified into functional categories (MV6 = 65.9%, MV12 = 65.4%, MV48 = 66%) (Table S4). The overall number of functional KEGG categories of the *L. rhamnosus* CCM7091 MV-associated proteins was 19. The majority of the proteins were assigned to Environmental information processing (MV6 = 10.6%, MV12 = 9.0%, MV48 = 9.4%), genetic information processing (MV6 = 11.1%, MV12 = 9.1%, MV48 = 10.4%), protein families: signalling and cellular processes (MV6 = 8.3%, MV12 = 7.9%, MV48 = 7.1%) and carbohydrate metabolism (MV6 = 6.9%, MV12 = 8.4%, MV48 = 8.3%). The distribution of MV proteins between the three time point samples according to their functional characteristics was similar with only subtle differences. For instance, MV12 and MV48 had a slightly higher percentage of proteins belonging to carbohydrate metabolism and amino acid metabolism compared to MV6. On the other hand, MV6 contained a slightly higher proportion of genetic information processing proteins (Table S4).

The core proteome, common to all MV samples, consisted of 708 proteins (Figure S5). Surprisingly, the MV6 proteome comprises 43 unique proteins, mainly (61.3%) belonging to genetic information processing proteins. The MV12 proteome consisted of 110 unique proteins, of which 27.9% belong to genetic information processing proteins and 20.1% belong to carbohydrate metabolism. Lastly, MV48 contained 17 unique proteins of which only six were annotated.

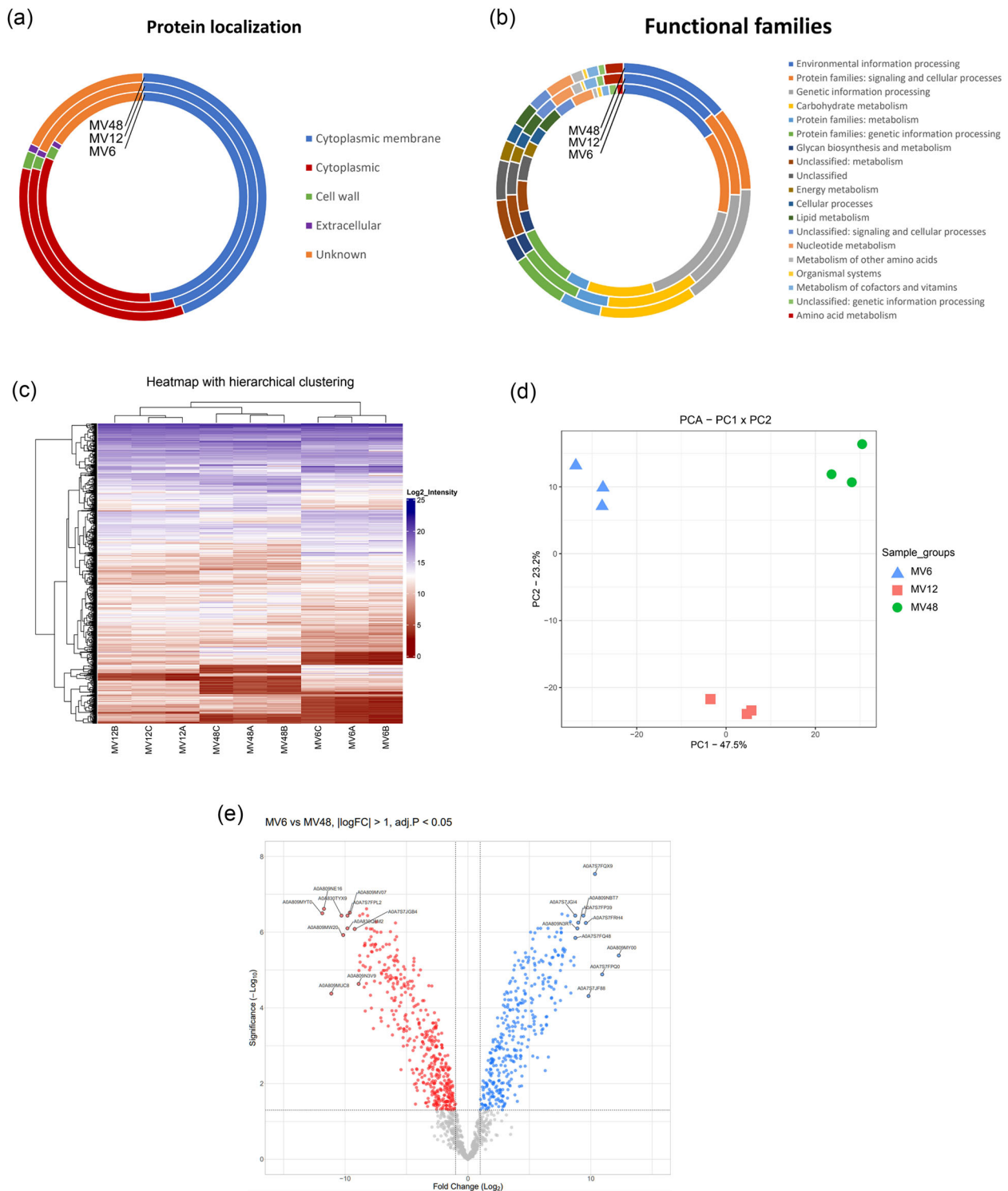


FIGURE 4 Proteomic analysis of *Lactacisibacillus rhamnosus* CCM7091 membrane vesicles (MVs) isolated at different times: 6, 12 and 48 h (MV6, MV12, MV48). (a) Pie chart showing the relative proportions of colocalized proteins in all MV samples. Predictions were performed using PSORTb software. (b) Pie chart showing the distribution of individual functional families in all MV samples. Predictions were performed using KEGG Mapper. (c) Heatmap representation of clustering according to the protein abundance in MV samples. Normalized and imputed values were used. Heatmap was created using ComplexHeatmap package in R. Hierarchical clustering method was used. (d) Principal component analysis of MV samples created using ggplot2 package in R. (e) Volcano plot showing proteomics data from MV6 versus MV48. The dots indicate different proteins that display both large magnitude fold changes (x -axis) and high statistical significance ($-\log_{10}$ of adjusted p -values, y -axis). The LIMMA package in R was utilized to calculate statistical significance. A log fold change >1 and an adjusted p -value < 0.05 were chosen as the thresholds to determine differential expression (grey – unchanged, red – decreased, blue – increased).

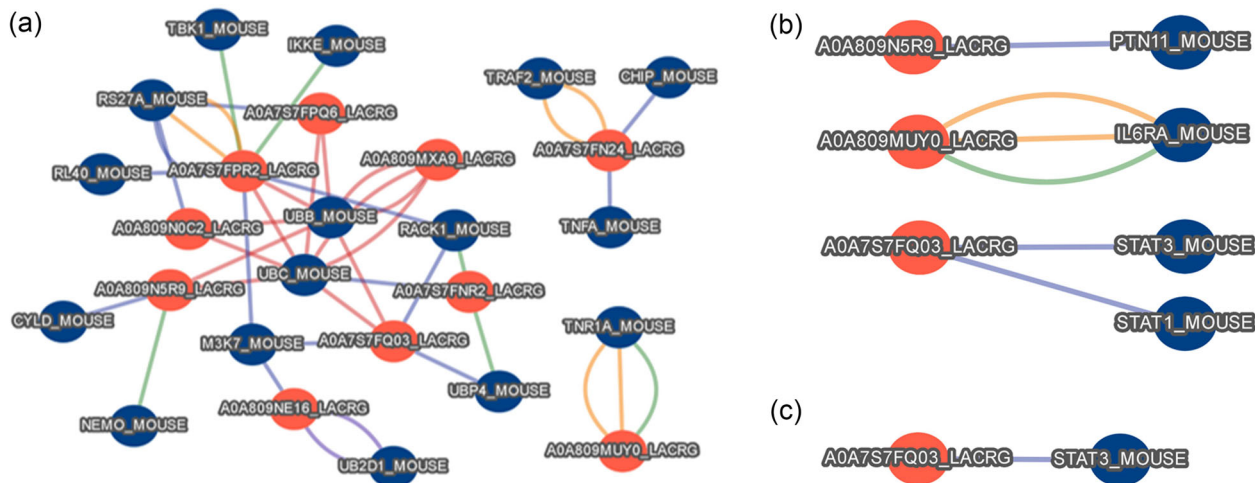


FIGURE 5 Predicted interaction network of up-regulated proteins from *Lacticaseibacillus rhamnosus* CCM7091 membrane vesicles isolated after 48 h (MV48) with murine (a) TNF α signalling cascade proteins, (b) IL-6 signalling cascade proteins and (c) IL-10 signalling cascade proteins. Red dots represent *L. rhamnosus* CCM7091 proteins; Uniprot ID is used as a protein identifier. Blue dots represent host proteins; Uniprot name is used as a protein identifier. The interactions were predicted with host proteins, which were retrieved from the Reactome database (Milacic et al. 2024). The interolog prediction on the PredHPI Webserver (Loaiza & Kaundal 2021) was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

We also quantitatively analysed the protein cargo and visualized it by heatmap clustering (Figure 4c). The biological triplicates clustered together, confirmed by PCA (Figure 4d) suggesting that protein loading into MV is a reproducible and selective process in *L. rhamnosus* CCM7091. Differences between samples based on protein intensities appeared to reflect the time-point of the isolation. Significant changes in protein expression are depicted in volcano plots by fold change ($|\log_2 FC| > 1$, adj. $p < 0.05$), where the biggest difference in protein intensities was observed between MV6 and MV48 (Figures 4e and S6). The 10 most significantly up- and down-regulated proteins are annotated in each combination (Table S5). To sum up, the proteomic characterization of *L. rhamnosus*-derived MVs showed significant differences between MV6 and MV48.

To investigate the cause of the effects we observed *in vitro* (the enhanced pro-inflammatory mediators' and IL-10 production after MV48 treatment), we predicted the immunomodulatory properties of MV protein cargos *in silico*. We analysed PPI between MV proteomes and proteins involved in IL-6, TNF α and IL-10 signalling pathways. The MV48 proteome appears to have stronger immunomodulatory potential than MV6 and MV12, therefore we focused on the interaction of the MV48 up-regulated proteins with the abovementioned pathways. Predicted PPI are summarized in Tables S6–S9. We identified interactions of the following proteins: Aminopeptidase (A0A809MUY0), Aminotransferase class V-fold PLP-dependent enzyme (A0A809MXA9), DUF72 domain-containing protein (A0A809NAH1), Carboxypeptidase (A0A809NCU5), Threonyl-tRNA synthetase (A0A7S7FPR2), Valyl-tRNA synthetase (A0A809N5R9), Non-specific serine/threonine protein kinase (A0A809N7A1), Seryl-tRNA synthetase (A0A7S7FQ03), ABC transporter permease sub-unit (A0A809NDK7), AAA domain-containing proteins (A0A809NOC2, A0A7S7FPQ6), Thioredoxin reductase (A0A7S7FP57), Putative oxidoreductase (A0A809NE16), Glucose-6-phosphate 1-dehydrogenase (A0A7S7FN24) and Malolactic protein (A0A7S7FNR2) (Figures 5 and S7 and Tables S6–S9). The expression of these proteins is at least twice increased in MV48 proteome compared to MV6 and MV12, showing the possible contribution to the functional differences between MV isolated at different times.

3.5 | The LTA participates in the immunomodulatory properties of the *L. rhamnosus* MVs

To expand the search for the possible cause of different immunomodulatory properties of various-time-points MVs beyond the protein composition, we detected the expression of the Gram-positive bacteria marker, LTA, by Western blot and immunodetection. The overall LTA levels in the MVs seemed to increase with the longer periods of maternal bacteria cultivation (Figure 6a). However, only the MV48 exerted a significantly higher expression of LTA compared to the negative control.

3.6 | TLR2 is involved in the recognition of the late-stationary-phase *L. rhamnosus* MVs

Having shown that the MV48 contain the LTA, we went further and attempted to identify the molecular mechanism of the MVs' interaction with the target cells. Generally, bacterial components are recognized by the pattern

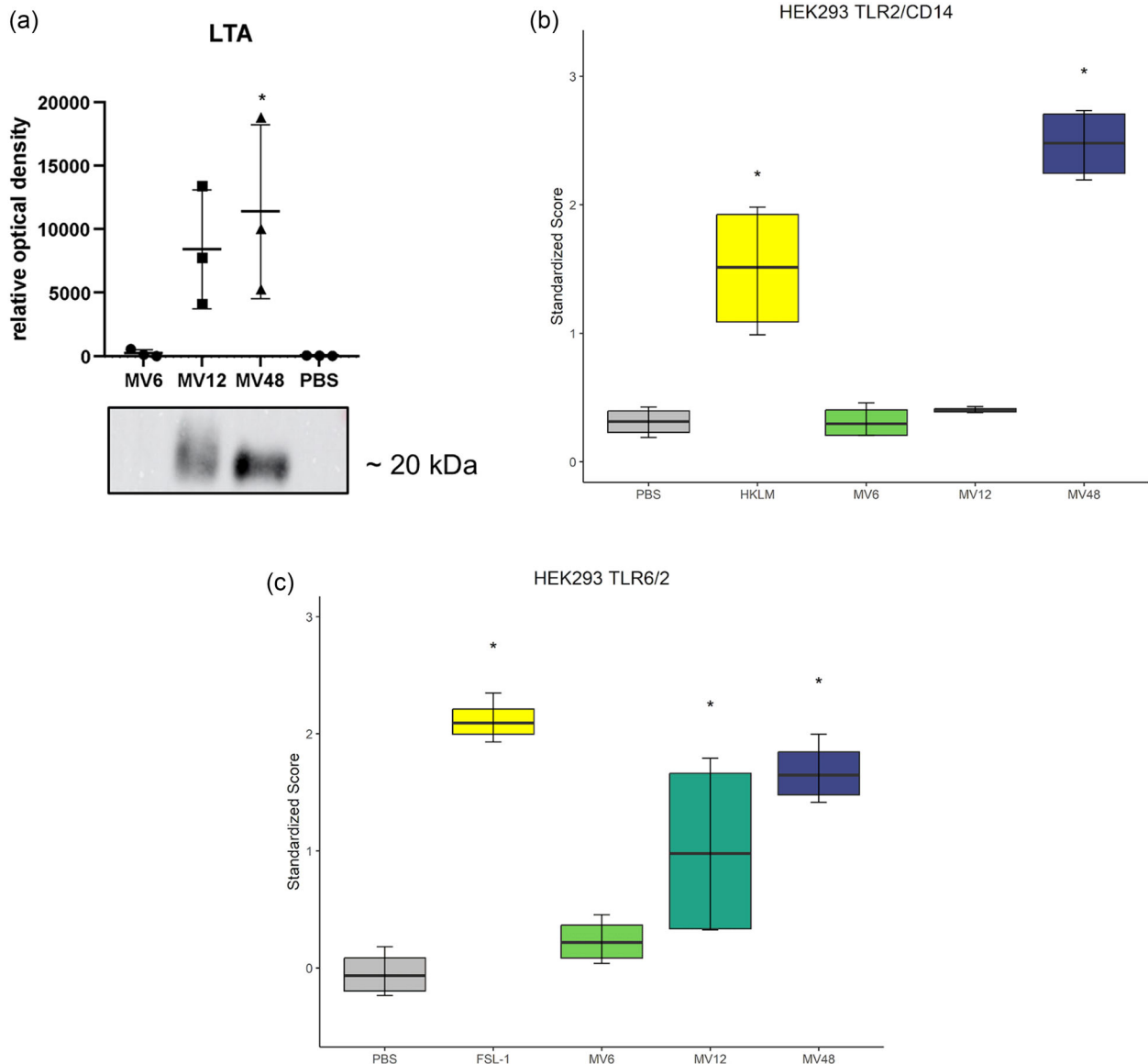


FIGURE 6 (a) Relative optical density and representative Western blot of lipoteichoic acid (LTA) detected in *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs) isolated after 6, 12, 48 h (MV6, MV12, MV48). PBS was used as a negative control. Bars represent means $\pm$ SD; statistical significance was assessed at $*p < 0.05$; ordinary one-way ANOVA was followed by Dunnett's multiple comparison test versus negative control; $n = 3$. (b), (c) Human cell receptors involved in the recognition of *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs). Effects of MVs isolated after 6, 12, 48 h (MV6, MV12, MV48) on activation of TLR2/CD14 (a) and TLR6/2 (b) expressed on human embryonic kidney cells HEK-293 (concentration 10^5 MVs/cell). The recognition abilities are compared to the negative control (PBS), and appropriate positive controls are depicted (HKLM for HEK293 TLR2/CD14, FSL-1 for HEK293 TLR6/2). Results were evaluated based on IL-8 production activity in cell media. The box plots show medians (lines), inter-quartile ranges (boxes) and minimum and maximum values (whiskers); data normalization was performed using Z-score; statistical analysis was performed using the ANOVA and the significance was assessed at $*p < 0.05$; $n = 4$.

recognition receptors (PRRs) of the host cells. To reveal the specific receptors involved in MV recognition in target cells, we analysed the interaction of MV6, MV12 and MV48 with HEK-293 cells over-expressing relevant TLRs and NOD-like receptors.

TLR2 is known to heterodimerize with other TLRs (TLR1/2 and TLR2/6), a property believed to extend the range of PAMPs that TLR2 can recognize, including LTA, peptidoglycan and diacylated mycoplasmal lipopeptide, triacylated lipopeptides and others. Furthermore, pathogen recognition by TLR2 is strongly enhanced by CD14.

Stimulation of HEK-293 cells transfected with TLR2/CD14 and TLR6/2 by MV48 resulted in significant enhancement of IL-8 production compared with vehicle-treated control (Figure 6b,c). None of the MVs isolated from different stages of the *L. rhamnosus* CCM7091 growth curve activated TLR1/2-triggered signalling pathway and scrambled control (293/null cells) (Figure S8a,b).

Additionally, we tested the activation of NOD1 and NOD2 – receptors participating in the recognition of molecular patterns that are derived from peptidoglycan, one of the components of cell walls of Gram-positive bacteria (Kurata et al., 2022). Our results did not confirm the significant stimulation of NOD1 and NOD2 receptors by MV6, MV12 or MV48 (Figure S8c,d).

Further, the Gram-positive bacteria and their vesicles should not contain LPS in their structure. We confirmed this by the absence of TLR4A-MD2-CD14 cell response after treatment with the *L. rhamnosus* CCM7091-derived MVs (Figure S8e). Summarized, TLR2 and TLR2/6 appear to play an exclusive role in the activation of immune response by MVs in the later stages of the *L. rhamnosus* CCM7091 growth curve.

4 | DISCUSSION

The therapeutic potential of bacterial MVs remains to be fully exploited – the most discussed possibilities include postbiotics, drug delivery carriers and vaccine platforms. Compared with OMVs, the MVs produced by *Lactocaseibacillus* spp. and other GRAS Gram-positive bacteria emerge as even more promising, due to the absence of endotoxins (therefore no need to artificially remove them (Kim et al., 2017; Park et al., 2021)) while keeping the general immunoboosting properties. Yet, the studied MVs are being harvested at different stages of bacterial growth (Choi et al., 2022; Han et al., 2023; Keyhani et al., 2022; Kurata et al., 2022) and very little is known about the influence of the bacterial growth phase on the MVs properties. To the best of our knowledge, only three studies on the cultivation time dependence of Gram-positive bacteria-derived MVs have been published to date (da Luz et al., 2022; Lee et al., 2021; Mehanny et al., 2022). The authors compare the effects of two (da Luz et al., 2022; Mehanny et al., 2022) or three (Lee et al., 2021) time points of the growth curve, focusing on either protein content (da Luz et al., 2022), immunomodulatory effects (Mehanny et al., 2022) or antibacterial activity (Lee et al., 2021). However, the effects of the overall growth stages on the composition, uptake by recipient cells, signalling via PRRs and immunomodulatory properties of the MVs, considering the actual growth curve of the studied bacterial species, are lacking.

In our work, we aimed to cover the entire growth curve of *L. rhamnosus* CCM7091: the early exponential, the late exponential and the late stationary phase. We looked at the MVs in a comprehensive manner, describing their biophysical properties, uptake by epithelial cells, proteomic composition, immunomodulatory effects and recognition by PRRs, to evaluate their potential for application as drug carriers.

From a biophysical point of view, MV6 seems to differ the most from the rest of the groups – both the yield and the average diameter were the lowest, whereas the MV48 diameter was significantly bigger compared to the MV6. These findings are in accordance with Mehanny et al. (2022), who showed higher yields and a higher size range of MVs isolated at a later stage of *Streptococcus pneumoniae* growth, although comparing stationary-phase with death-phase MVs (Mehanny et al., 2022). Similarly, Lee et al. (2021) observed an increased particle concentration with increasing cultivation time, although they did not detect any change in the particle diameter of *L. plantarum*-derived MVs (Lee et al., 2021). Their results suggest that the yield of MVs improves with longer cultivation, as the MVs probably cumulate in the medium throughout the longer cultivation. Interestingly, we have shown that the highest increase in MV yield occurs in the late-exponential and not necessarily in the late-stationary-phase MVs. This information may be determinative for the maximization of MV yields for future medical applications. We assume that the decrease in the presence of MVs in the late stationary phase of the growth curve may be explained by their uptake by the maternal bacteria (Kim et al., 2016). Another reason may be their aggregation or fusion, hence the lower numbers and higher average particle diameter. These hypotheses deserve additional experiments to be validated.

To our best knowledge, here we provide for the first time proof that MV uptake efficiency is dependent on the length of the maternal cell cultivation. To reflect the effect of MVs in the context of the intestinal tract, the typical habitat of *L. rhamnosus*, we investigated the route of their uptake by intestinal epithelial cells. We confirmed the previous findings (Champagne-Jorgensen et al., 2021) that the *Lactocaseibacillus* spp. MVs are taken up by intestinal cells likely by clathrin-dependent endocytosis. On top of that, as we employed a wider variety of inhibitors, we showed that the MV uptake routes are not limited to the clathrin-mediated endocytosis, but also the lipid rafts-mediated internalization and micropinocytosis take place. Contrarily to Bajic et al. (2020), in our hands, the predominant uptake route was the caveolae-mediated endocytosis. This may be explained by species specificity (*L. rhamnosus* vs. *L. plantarum*), and target cells diversity (Caco-2 vs. HT29).

It was proven that both Gram-positive and Gram-negative bacteria-derived EVs can modulate the host immune response (Chiba et al., 2010; Kurata et al., 2022; Mehanny et al., 2022); the OMVs containing LPS on the surface may be an even stronger modulator of the innate immune response than the source bacteria (Gilmore et al., 2021). Therefore, we investigated the composition and immunomodulatory effects of the *L. rhamnosus*-derived MVs *In vitro*, as the starting point for future medical tests. We found out that the effects of MVs are dose-dependent, with 10^5 MVs/cell being the concentration revealing the biggest differences between different time points MVs in macrophages. In agreement with Mehanny et al. (2022), at this concentration, we observed a significant increase in the production of TNF α by macrophages when treated with late-stage MVs (Mehanny et al., 2022). Similarly, the early-stages MVs induced TNF α and IL-6 production, in our hands, however, the effect was statistically significant only at the concentration of 10^7 MVs per cell. It is noteworthy that the highest MV concentration resulted in an overall increase in TNF α and RNS in all time-point MVs. This highlights the importance of standardized MV dosing for *in vitro* experiments and

the need for concentration titration. We also measured the release of the immunoregulatory cytokine IL-10, which showed an increase after MV48 treatment, as the cells probably regulate the inflammatory response in a negative feedback loop (Morhardt et al., 2019).

To examine the cause of the different immunomodulatory effects by the specific time-point MVs, we analysed their proteome and the LTA content. The proteomes of *L. rhamnosus*-derived MVs consisted of around 1000 identified proteins, mainly belonging to the cytoplasmic membrane and cytoplasmic proteins. The protein localization is in agreement with (da Luz et al., 2022; Hu et al., 2021; Kim et al., 2016; Tartaglia et al., 2020), whereas the number of identified proteins varies in these studies, probably caused by less sensitive proteomic profiling, more stringent bioinformatic data processing or maternal bacterial strain.

The distribution of MV proteins according to their functional characteristics was similar in all samples with subtle differences. Notably, the MV12 and MV48 proteomes contain a slightly higher percentage of proteins belonging to carbohydrate and amino acid metabolisms compared to the MV6 proteome. This may be attributed to the continued metabolic activity of cells during the transition from exponential to the stationary phase when *L. rhamnosus* cells gain energy from free amino acids or utilize different carbohydrates under environmental stress (De Angelis et al., 2016; Laakso et al., 2011). During the stationary phase of *L. rhamnosus* GG, alternative enzymes related to the tagatose-6-phosphate and Leloir pathways are produced, leading to the hydrolysis of alternative carbohydrates. This is connected with a decrease in glucose concentration at the end of the exponential growth phase (Chubukov & Sauer, 2014). Consistent with this, we observed increased protein intensities in the carbohydrate metabolisms functional group (e.g. tagatose-6-phosphate kinase, tagatose 1,6-diphosphate aldolase, galactose mutarotase, galactokinase) in MV12, reflecting physiological changes of maternal cells at the end of the exponential phase and persisting in MV48. Interestingly, the MV6 proteome contains 43 unique proteins, most of them related to the genetic information processing functional group, in alignment with their reported increased expression during the exponential growth phase and subsequent decrease in the stationary phase (Veselovsky et al., 2022). This confirms that MVs mirror the physiological state of the maternal culture. Besides, the abundance of proteins related to the transfer of various molecules and metabolic processes confirmed the carrier function of the MVs.

In silico PPI analyses revealed potential interactions between certain bacterial proteins and the host proteome. We focused on proteins up-regulated in MV48, as the MV48 elicited the strongest immune reaction. These bacterial proteins include Aminopeptidase (A0A809MUY0) (predicted to interact with IL-6 receptor subunit α and TNF receptor superfamily) and Aminotransferase class V-fold PLP-dependent enzyme (A0A809MXA9) (predicted to interact with a protein associated with endocytosis and vesicle scission (Q05193)). Additionally, we identified DUF72 domain-containing protein (A0A809NAH1) and Carboxypeptidase (A0A809NCU5), which are predicted to interact with NF κ -B subunit, related to multiple cell processes, including inflammation and immune response. Besides, several Aminoacyl-tRNA synthetases (ARSs), such as Threonyl-RS (A0A7S7FPR2), Valyl-RS (A0A809N5R9) and Seryl-RS (A0A7S7FQ03), but also non-specific serine/threonine protein kinase (A0A809N7A1), ABC transporter permease subunit (A0A809NDK7), AAA domain-containing proteins (A0A809N0C2, A0A7S7FPQ6), Thioredoxin reductase (A0A7S7FP57), Putative oxidoreductase (A0A809NE16), Glucose-6-phosphate 1-dehydrogenase (A0A7S7FN24) and Malolactic protein (A0A7S7FNR2) were identified. ARSs are known for their role in protein synthesis, linking amino acids to transfer RNAs. They contribute to bacterial metabolism and virulence (Baddal et al., 2015; Kirubakar et al., 2018; Maloney et al., 2009) and certain bacterial ARSs have been implicated in various immune responses (Nie et al., 2019). *Leishmania donovani* TyrRS could be released from the parasite cytoplasm to the outside of the cell and specifically bind to the host macrophages thereby promoting the secretion of TNF- α and IL-6 (Bhatt et al., 2011). *Brugia malayi* asparaginyl-RS has also been shown to be closely related to the host immune response (Hameed et al., 2019; Ramirez et al., 2006). This corresponds with MV48 effects on murine macrophages we observed *in vitro*, suggesting that ARSs contribute to the MVs' immunomodulatory effects. Briefly, our results and PPI analyses suggest that the changes in the proteomic cargo of the MVs isolated at different growth stages may contribute to their different immunomodulatory capacities by interacting with TNF α , IL-6 and IL-10 signalling pathways.

MVs were isolated from a batch cultivation system, implying that MV48 should encompass information from MV6 and MV12 if present in the medium. Surprisingly, unique proteins found in MV6 and MV12 were not identified in MV48, which raises questions for further investigation. Hypotheses for future research include continuous MV uptake by cells (Kim et al., 2016) serving for a constant exchange of information, potentially affected by nutrient scarcity, leading to decreased MV48 proteomic variety. Alternatively, unique MV surface proteins might undergo increased degradation in the presence of proteolytic enzymes during bacterial growth, especially under stress conditions, possibly resulting in the release of lytic enzymes (Frees et al., 2007) and also because the MVs' biogenesis mechanism of explosive cell lysis (proven in Gram-positive bacteria by Jeong et al. (2022)) may become more eminent when the bacterial culture is under stress conditions in late-stationary phase (Turnbull et al., 2016). This theory aligns with the observed reduced bacterial cell numbers.

To further explore the diverse immunomodulatory effects observed *in vitro*, we were interested in whether the LTA, a Gram-positive bacteria marker, may contribute to the effects of MVs. In *Staphylococcus aureus*, it was confirmed that LTA is more abundant in MVs compared to the secretome (Uppu et al., 2023). The LTA is also known to be built differently in the cell membrane during different growth stages (Shiraishi et al., 2018), however, to the best of our knowledge, no such information was confirmed in the LTA content in MVs isolated at different time points of bacteria cultivation. Here, we have shown for the first time that the LTA amounts in the MVs indeed differ and increase with longer cultivation time. We suppose that the LTA

contributes significantly to the immunoregulatory effects in the murine macrophages, being most abundant and supposedly potent in the *L. rhamnosus*-derived MVs isolated in the late stationary phase of the growth curve.

Based on the LTA contents, we continued the search for the mechanism of action of the MVs by using HEK293 cells over-expressing relevant PRRs. In agreement with Champagne-Jorgensen et al. (2021) and Kurata et al. (2022), we confirmed that not only *L. plantarum*, but also *L. rhamnosus* MVs activate cell response by TLR2. In their work, Kurata et al. (2022) did not detect the LTA in *L. plantarum* and identified different ligands recognized by TLR2, still, it is generally known that the LTA is recognized by TLR2/6 which we confirmed here. The study of Friedrich et al. showed that the LTA isolated from *L. rhamnosus* GG induced dendritic and T cells activation, supposedly by TLR2 signalling pathway (Friedrich et al., 2022). This corresponds with our results that the increased amount of LTA in MV48 correlated with the increased TLR2/CD14 and TLR6/2 signalling, suggesting that the LTA is a key immunomodulatory molecule abundant particularly in the late stages-of-the-growth-curve MVs as we have found out in this research. Contrastingly to Kurata et al. (2022), however, in our hands, none of the MVs induced significant NOD1 nor NOD2 activation. These receptors recognize peptidoglycan-derived molecular patterns, so a possible cause of the discrepancy is that peptidoglycan may be only a co-isolate, which we removed in the MV purification step. To conclude, the late-stationary-phase *L. rhamnosus* MVs have suitable immunoboosting properties for further therapeutical applications.

Overall, here we addressed the pressing need for characterization and standardization of the MVs, to be fully utilized in clinics. We have described the profile of *L. rhamnosus*-derived MVs and how they change throughout bacterial growth. A limitation of our study is related to the incomplete annotation of the proteomic databases in the bacterial proteins we used for proteomic analysis (around 65% of the identified proteins were properly annotated). Therefore, the MV-protein localization and their classification into functional groups may be influenced by the unknown proteins. Also, the nucleic acids and lipids contained in the MVs, which were not at the centre of the interest of our study, may contribute to the effects of MVs on the host cells and we plan to focus on them in our future research. Lastly, in the late stationary phase, a few bacterial cells may start to die, causing a possible small portion of membrane components of the dying cells that may be co-isolated with the MVs (Liu et al., 2022) or bacterial ghosts to be present in the MV48 sample. Bacterial ghosts are empty cell envelopes and have been recently proposed, together with the MVs, as a promising therapeutic delivery system (Alexander & van Pijkeren, 2023; Yu et al., 2022). Incapable of replication, their presence cannot be revealed in a sterility test, so we cannot rule out their presence in the MV samples.

5 | CONCLUSION

In this study, we demonstrated that the growth phase of the maternal bacterial culture impacts the size, composition, biophysical and immunomodulatory properties of *L. rhamnosus*-derived MVs. Therefore, the MV harvest point should be considered and standardized in future research, especially when aiming at reproducibility and therapeutic use of the MVs. Our cultivation and MV isolation procedure is highly reproducible, illustrated by the similitudes of the triplicates, both in MADLS and proteomics results. We further reported that late-stationary-phase *L. rhamnosus* MVs are bigger, taken up predominantly by caveolae-mediated pathway, and stimulate the immune response of murine macrophages. Our proteomic and PPI analyses indicate that the MV protein cargo changes with different isolation time points, probably contributing to their diverse immunomodulatory potentials. Here we showed for the first time that the expression of the LTA in the *L. rhamnosus* MVs increases with a longer cultivation period. The late-stationary MVs further elicit the TLR2-triggered signalling pathway, suggesting the contribution of LTA to the immunomodulatory effects of the MVs. Additionally, the MVs, compared with the maternal bacteria, exhibit much higher safety, and inability to replicate while still being effective immunoboosters. Therefore, for future medical applications of the probiotic bacteria-derived MVs, the late-stationary-phase MVs may be the most suitable for effective immunoboosting while keeping all their safety properties. Further studies are needed to validate our findings on another probiotic Gram-positive bacterial species and to eventually elucidate the contribution of nucleic acids to the MV's immunostimulatory effects.

AUTHOR CONTRIBUTIONS

Miriam Sandanusova: Conceptualization; writing—original draft; writing—review and editing; investigation; data curation; formal analysis. **Kristyna Turkova:** Conceptualization; writing—original draft; writing—review and editing; investigation; project administration. **Eva Pechackova:** Investigation; data curation; methodology. **Jan Kotoucek:** Investigation; data curation; methodology. **Pavel Roudnicky:** Data curation; methodology. **Martin Sindelar:** Methodology; software. **Lukas Kubala:** Supervision; writing—review and editing. **Gabriela Ambrozova:** Conceptualisation; project administration; supervision; methodology; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings in this study are included in the article and are available from the authors upon reasonable request.

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REFERENCES

- Aakko, J., Sanchez, B., Gueimonde, M., & Salminen, S. (2014). Assessment of stress tolerance acquisition in the heat-tolerant derivative strains of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus rhamnosus* GG. *Journal of Applied Microbiology*, 117, 239–248.
- Al-Nedawi, K., Mian, M. F., Hossain, N., Karimi, K., Mao, Y. K., Forsythe, P., Min, K. K., Stanisz, A. M., Kunze, W. A., & Bienenstock, J. (2015). Gut commensal microvesicles reproduce parent bacterial signals to host immune and enteric nervous systems. *FASEB Journal*, 29, 684–695.
- Alexander, L. M., & van Pijkeren, J. P. (2023). Modes of therapeutic delivery in synthetic microbiology. *Trends in Microbiology*, 31, 197–211.
- Ambrozova, G., Pekarova, M., & Lojek, A. (2010). Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages. *European Journal of Nutrition*, 49, 133–139.
- Arias-Negrete, S., Jimenez-Romero, L. A., Solis-Martinez, M. O., Ramirez-Emiliano, J., Avila, E. E., & Cuellar-Mata, P. (2004). Indirect determination of nitric oxide production by reduction of nitrate with a freeze-thawing-resistant nitrate reductase from *Escherichia coli* MC1061. *Analytical Biochemistry*, 328, 14–21.
- Baddal, B., Muzzi, A., Censini, S., Calogero, R. A., Torricelli, G., Guidotti, S., Taddei, A. R., Covacci, A., Pizza, M., Rappuoli, R., Soriani, M., & Pezzicoli, A. (2015). Dual RNA-seq of nontypeable *Haemophilus influenzae* and host cell transcriptomes reveals novel insights into host-pathogen cross talk. *MBio*, 6, e01765–e01715.
- Bajic, S. S., Canas, M. A., Tolinnacki, M., Badia, J., Sanchez, B., Golic, N., Margolles, A., Baldoma, L., & Ruas-Madiedo, P. (2020). Proteomic profile of extracellular vesicles released by *Lactiplantibacillus plantarum* BGAN8 and their internalization by non-polarized HT29 cell line. *Scientific Reports*, 10, 21829.
- Behzadi, E., Mahmoodzadeh Hosseini, H., & Imani Fooladi, A. A. (2017). The inhibitory impacts of *Lactobacillus rhamnosus* GG-derived extracellular vesicles on the growth of hepatic cancer cells. *Microbial Pathogenesis*, 110, 1–6.
- Bhatt, T. K., Khan, S., Dwivedi, V. P., Banday, M. M., Sharma, A., Chandele, A., Camacho, N., Ribas de Pouplana, L., Wu, Y., Craig, A. G., Mikkonen, A. T., Maier, A. G., Yogavel, M., & Sharma, A. (2011). Malaria parasite tyrosyl-tRNA synthetase secretion triggers pro-inflammatory responses. *Nature Communications*, 2, 530.
- Champagne-Jorgensen, K., Mian, M. F., McVey Neufeld, K. A., Stanisz, A. M., & Bienenstock, J. (2021). Membrane vesicles of *Lactocaseibacillus rhamnosus* JB-1 contain immunomodulatory lipoteichoic acid and are endocytosed by intestinal epithelial cells. *Scientific Reports*, 11, 13756.
- Chatterjee, S. N., & Das, J. (1967). Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *Journal of General Microbiology*, 49, 1–11.
- Chen, W., Wu, Y., Deng, J., Yang, Z., Chen, J., Tan, Q., Guo, M., & Jin, Y. (2022). Phospholipid-membrane-based nanovesicles acting as vaccines for tumor immunotherapy: Classification, mechanisms and applications. *Pharmaceutics*, 14, 2446.
- Chiba, Y., Shida, K., Nagata, S., Wada, M., Bian, L., Wang, C., Shimizu, T., Yamashiro, Y., Kiyoshima-Shibata, J., Nanno, M., & Nomoto, K. (2010). Well-controlled proinflammatory cytokine responses of Peyer's patch cells to probiotic *Lactobacillus casei*. *Immunology*, 130, 352–362.
- Choi, J. H., Moon, C. M., Shin, T. S., Kim, E. K., McDowell, A., Jo, M. K., Joo, Y. H., Kim, S. E., Jung, H. K., Shim, K. N., Jung, S. A., & Kim, Y. K. (2020). *Lactobacillus paracasei*-derived extracellular vesicles attenuate the intestinal inflammatory response by augmenting the endoplasmic reticulum stress pathway. *Experimental & Molecular Medicine*, 52, 423–437.
- Choi, J., Kwon, H., Kim, Y. K., & Han, P. L. (2022). Extracellular vesicles from gram-positive and gram-negative probiotics remediate stress-induced depressive behavior in mice. *Molecular Neurobiology*, 59, 2715–2728.
- Chubukov, V., & Sauer, U. (2014). Environmental dependence of stationary-phase metabolism in *Bacillus subtilis* and *Escherichia coli*. *Applied and Environmental Microbiology*, 80, 2901–2909.
- da Luz, B. S. R., de Rezende Rodvalho, V., Nicolas, A., Chabelskaya, S., Jardin, J., Briard-Bion, V., Le Loir, Y., de Carvalho Azevedo, V. A., & Guedon, E. (2022). Impact of environmental conditions on the protein content of *Staphylococcus aureus* and its derived extracellular vesicles. *Microorganisms*, 10, 1808.
- De Angelis, M., Calasso, M., Cavallo, N., Di Cagno, R., & Gobbetti, M. (2016). Functional proteomics within the genus *Lactobacillus*. *Proteomics*, 16, 946–962.
- EV-TRACK Consortium. Van Deun, J., Mestdag, P., Agostinis, P., Akay, O., Anand, S., Anckaert, J., Martinez, Z. A., Baetens, T., Beghein, E., Bertier, L., Berx, G., Boere, J., Boukouris, S., Bremer, M., Buschmann, D., Byrd, J. B., Casert, C., Cheng, L., ... Hendrix, A. (2017). EV-TRACK: Transparent reporting and centralizing knowledge in extracellular vesicle research. *Nature Methods*, 14, 228–232.
- Frees, D., Savijoki, K., Varmanen, P., & Ingmer, H. (2007). Clp ATPases and ClpP proteolytic complexes regulate vital biological processes in low GC, Gram-positive bacteria. *Molecular Microbiology*, 63, 1285–1295.

- Friedrich, A. D., Leoni, J., Paz, M. L., & Gonzalez Maglio, D. H. (2022). Lipoteichoic acid from *Lactocaseibacillus rhamnosus* GG modulates dendritic cells and T cells in the gut. *Nutrients*, *14*, 723.
- Gilmore, W. J., Johnston, E. L., Zavan, L., Bitto, N. J., & Kaparakis-Liaskos, M. (2021). Immunomodulatory roles and novel applications of bacterial membrane vesicles. *Molecular Immunology*, *134*, 72–85.
- Gnopo, Y. M. D., & Putnam, D. (2020). A lipid mixing assay to accurately quantify the fusion of outer membrane vesicles. *Methods (San Diego, California)*, *177*, 74–79.
- Goedhart, J., & Luijsterburg, M. S. (2020). VolcanoR is a web app for creating, exploring, labeling and sharing volcano plots. *Scientific Reports*, *10*, 20560.
- Gonzalez-Lozano, E., Garcia-Garcia, J., Galvez, J., Hidalgo-Garcia, L., Rodriguez-Nogales, A., Rodriguez-Cabezas, M. E., & Sanchez, M. (2022). Novel horizons in postbiotics: Lactobacillaceae extracellular vesicles and their applications in health and disease. *Nutrients*, *14*, 5296.
- Gu, Z., Li, F., Liu, Y., Jiang, M., Zhang, L., He, L., Wilkey, D. W., Merchant, M., Zhang, X., Deng, Z. B., Chen, S. Y., Barve, S., McClain, C. J., & Feng, W. (2021). Exosome-like nanoparticles from *Lactobacillus rhamnosus* GG protect against alcohol-associated liver disease through intestinal aryl hydrocarbon receptor in mice. *Hepatology Communications*, *5*, 846–864.
- Hameed, A., Natarajan, M., Jabbar, S., Dhanasekaran, J. J., Kumar, K., Sivanesan, S., Kron, M., & Dhanasekaran, A. (2019). Immune response to *Brugia malayi* Asparaginyl-tRNA synthetase in Balb/c mice and human clinical samples of lymphatic filariasis. *Lymphatic Research and Biology*, *17*, 447–456.
- Han, F., Wang, K., Shen, K., Wang, J., Han, S., Hu, D., & Wu, G. (2023). Extracellular vesicles from *Lactobacillus druckerii* inhibit hypertrophic scar fibrosis. *Journal of Nanobiotechnology*, *21*, 113.
- He, X., Zeng, Q., Puthiyakunnon, S., Zeng, Z., Yang, W., Qiu, J., Du, L., Boddu, S., Wu, T., Cai, D., Huang, S. H., & Cao, H. (2017). *Lactobacillus rhamnosus* GG supernatant enhance neonatal resistance to systemic *Escherichia coli* K1 infection by accelerating development of intestinal defense. *Scientific Reports*, *7*, 43305.
- Hu, R., Lin, H., Wang, M., Zhao, Y., Liu, H., Min, Y., Yang, X., Gao, Y., & Yang, M. (2021). *Lactobacillus reuteri*-derived extracellular vesicles maintain intestinal immune homeostasis against lipopolysaccharide-induced inflammatory responses in broilers. *Journal of Animal Science and Biotechnology*, *12*, 25.
- Hunt, L., Hacker, D. L., Grosjean, F., De Jesus, M., Uebersax, L., Jordan, M., & Wurm, F. M. (2005). Low-temperature pausing of cultivated mammalian cells. *Biotechnology and Bioengineering*, *89*, 157–163.
- Jeong, D., Kim, M. J., Park, Y., Chung, J., Kweon, H. S., Kang, N. G., Hwang, S. J., Youn, S. H., Hwang, B. K., & Kim, D. (2022). Visualizing extracellular vesicle biogenesis in gram-positive bacteria using super-resolution microscopy. *BMC Biology*, *20*, 270.
- Jones, E. J., Booth, C., Fonseca, S., Parker, A., Cross, K., Miquel-Clopes, A., Hautefort, I., Mayer, U., Wileman, T., Stentz, R., & Carding, S. R. (2020). The uptake, trafficking, and biodistribution of bacteroides thetaiotaomicron generated outer membrane vesicles. *Frontiers in Microbiology*, *11*, 57.
- Kanehisa, M. (2019). Toward understanding the origin and evolution of cellular organisms. *Protein Science*, *28*, 1947–1951.
- Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M., & Ishiguro-Watanabe, M. (2023). KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Research*, *51*, D587–D592.
- Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, *28*, 27–30.
- Kang, J. Y., Nan, X., Jin, M. S., Youn, S. J., Ryu, Y. H., Mah, S., Han, S. H., Lee, H., Paik, S. G., & Lee, J. O. (2009). Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity*, *31*, 873–884.
- Kawai, T., & Akira, S. (2007). Signaling to NF-kappaB by Toll-like receptors. *Trends in Molecular Medicine*, *13*, 460–469.
- Keyhani, G., Mahmoodzadeh Hosseini, H., & Salimi, A. (2022). Effect of extracellular vesicles of *Lactobacillus rhamnosus* GG on the expression of CEA gene and protein released by colorectal cancer cells. *Iranian Journal of Microbiology*, *14*, 90–96.
- Kim, O. Y., Park, H. T., Dinh, N. T. H., Choi, S. J., Lee, J., Kim, J. H., Lee, S. W., & Gho, Y. S. (2017). Bacterial outer membrane vesicles suppress tumor by interferon-gamma-mediated antitumor response. *Nature Communications*, *8*, 626.
- Kim, M. H., Kim, S. Y., Son, J. H., Kim, S. I., Lee, H., Kim, S., Shin, M., & Lee, J. C. (2019). Production of membrane vesicles by *Enterococcus faecium* cultured with or without subinhibitory concentrations of antibiotics and their pathological effects on epithelial cells. *Frontiers in Cellular and Infection Microbiology*, *9*, 295.
- Kim, Y., Edwards, N., & Fenselau, C. (2016). Extracellular vesicle proteomes reflect developmental phases of *Bacillus subtilis*. *Clin Proteomics*, *13*, 6.
- Kirubakar, G., Murugaiyan, J., Schaudinn, C., Dematheis, F., Holland, G., Eravci, M., Weise, C., Roesler, U., & Lewin, A. (2018). Proteome analysis of a *M. avium* mutant exposes a novel role of the bifunctional protein LysX in the regulation of metabolic activity. *Journal of Infectious Diseases*, *218*, 291–299.
- Knapp, S., von Aulock, S., Leendertse, M., Haslinger, I., Draing, C., Golenbock, D. T., & van der Poll, T. (2008). Lipoteichoic acid-induced lung inflammation depends on TLR2 and the concerted action of TLR4 and the platelet-activating factor receptor. *Journal of Immunology*, *180*, 3478–3484.
- Koskenniemi, K., Koponen, J., Kankainen, M., Savijoki, K., Tynkkynen, S., de Vos, W. M., Kalkkinen, N., & Varmanen, P. (2009). Proteome analysis of *Lactobacillus rhamnosus* GG using 2-D DIGE and mass spectrometry shows differential protein production in laboratory and industrial-type growth media. *Journal of Proteome Research*, *8*, 4993–5007.
- Krsek, D., Yara, D. A., Hrbackova, H., Daniel, O., Mancikova, A., Schuller, S., & Bielaszewska, M. (2023). Translocation of outer membrane vesicles from enterohemorrhagic *Escherichia coli* O157 across the intestinal epithelial barrier. *Frontiers in Microbiology*, *14*, 1198945.
- Kurata, A., Kiyohara, S., Imai, T., Yamasaki-Yashiki, S., Zaima, N., Moriyama, T., Kishimoto, N., & Uegaki, K. (2022). Characterization of extracellular vesicles from *Lactiplantibacillus plantarum*. *Scientific Reports*, *12*, 13330.
- Laakso, K., Koskenniemi, K., Koponen, J., Kankainen, M., Surakka, A., Salusjarvi, T., Auvinen, P., Savijoki, K., Nyman, T. A., Kalkkinen, N., Tynkkynen, S., & Varmanen, P. (2011). Growth phase-associated changes in the proteome and transcriptome of *Lactobacillus rhamnosus* GG in industrial-type whey medium. *Microbial Biotechnology*, *4*, 746–766.
- Lee, E. Y., Choi, D. Y., Kim, D. K., Kim, J. W., Park, J. O., Kim, S., Kim, S. H., Desiderio, D. M., Kim, Y. K., Kim, K. P., & Gho, Y. S. (2009). Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. *Proteomics*, *9*, 5425–5436.
- Lee, B. H., Wu, S. C., Shen, T. L., Hsu, Y. Y., Chen, C. H., & Hsu, W. H. (2021). The applications of *Lactobacillus plantarum*-derived extracellular vesicles as a novel natural antibacterial agent for improving quality and safety in tuna fish. *Food Chemistry*, *340*, 128104.
- Liu, H., Zhang, Q., Wang, S. C., Weng, W. Z., Jing, Y. Y., & Su, J. C. (2022). Bacterial extracellular vesicles as bioactive nanocarriers for drug delivery: Advances and perspectives. *Bioactive Materials*, *14*, 169–181.
- Loaiza, C. D., & Kaundal, R. (2021). PredHPI: An integrated web server platform for the detection and visualization of host-pathogen interactions using sequence-based methods. *Bioinformatics*, *37*, 622–624.
- Long, E. M., Millen, B., Kubes, P., & Robbins, S. M. (2009). Lipoteichoic acid induces unique inflammatory responses when compared to other toll-like receptor 2 ligands. *PLoS ONE*, *4*, e5601.
- Ma, L., Shen, Q., Lyu, W., Lv, L., Wang, W., Yu, M., Yang, H., Tao, S., & Xiao, Y. (2022). *Clostridium butyricum* and its derived extracellular vesicles modulate gut homeostasis and ameliorate acute experimental colitis. *Microbiology Spectrum*, *10*, e0136822.

- Maloney, E., Stankowska, D., Zhang, J., Fol, M., Cheng, Q. J., Lun, S. C., Bishai, W. R., Rajagopalan, M., Chatterjee, D., & Madiraju, M. V. (2009). The two-domain LysX protein of mycobacterium tuberculosis is required for production of lysinylated phosphatidylglycerol and resistance to cationic antimicrobial peptides. *Plos Pathogens*, 5, e1000534.
- Mata Forsberg, M., Bjorkander, S., Pang, Y., Lundqvist, L., Ndi, M., Ott, M., Escriba, I. B., Jaeger, M. C., Roos, S., & Sverremark-Ekstrom, E. (2019). Extracellular membrane vesicles from lactobacilli dampen IFN-gamma responses in a monocyte-dependent manner. *Scientific Reports*, 9, 17109.
- Mehanny, M., Kroniger, T., Koch, M., Hoppstadter, J., Becher, D., Kiemer, A. K., Lehr, C. M., & Fuhrmann, G. (2022). Yields and immunomodulatory effects of pneumococcal membrane vesicles differ with the bacterial growth phase. *Advanced Healthcare Materials*, 11, e2101151.
- Micoli, F., & MacLennan, C. A. (2020). Outer membrane vesicle vaccines. *Seminars in Immunology*, 50, 101433.
- Milacic, M., Beavers, D., Conley, P., Gong, C., Gillespie, M., Griss, J., Haw, R., Jassal, B., Matthews, L., May, B., Petryszak, R., Ragueneau, E., Rothfels, K., Sevilla, C., Shamovsky, V., Stephan, R., Tiwari, K., Varusai, T., Weiser, J., ... D'Eustachio, P. (2024). The Reactome Pathway Knowledgebase 2024. *Nucleic Acids Research*, 52, D672–D678.
- Morhardt, T. L., Hayashi, A., Ochi, T., Quiros, M., Kitamoto, S., Nagao-Kitamoto, H., Kuffa, P., Atarashi, K., Honda, K., Kao, J. Y., Nusrat, A., & Kamada, N. (2019). IL-10 produced by macrophages regulates epithelial integrity in the small intestine. *Scientific Reports*, 9, 1223.
- Nahui Palomino, R. A., Vanpouille, C., Costantini, P. E., & Margolis, L. (2021). Microbiota-host communications: Bacterial extracellular vesicles as a common language. *Plos Pathogens*, 17, e1009508.
- Nie, A., Sun, B., Fu, Z., & Yu, D. (2019). Roles of aminoacyl-tRNA synthetases in immune regulation and immune diseases. *Cell Death & Disease*, 10, 901.
- Nilsen, N. J., Deininger, S., Nonstad, U., Skjeldal, F., Husebye, H., Rodionov, D., von Aulock, S., Hartung, T., Lien, E., Bakke, O., & Espevik, T. (2008). Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling: Role of CD14 and CD36. *Journal of Leukocyte Biology*, 84, 280–291.
- O'Donoghue, E. J., & Krachler, A. M. (2016). Mechanisms of outer membrane vesicle entry into host cells. *Cellular Microbiology*, 18, 1508–1517.
- Park, K. S., Svennerholm, K., Crescitelli, R., Lasser, C., Griponika, I., & Lotvall, J. (2021). Synthetic bacterial vesicles combined with tumour extracellular vesicles as cancer immunotherapy. *Journal of Extracellular Vesicles*, 10, e12120.
- Perez-Riverol, Y., Bai, J., Bandla, C., Garcia-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., Kundu, D. J., Prakash, A., Frericks-Zipper, A., Eisenacher, M., Walzer, M., Wang, S., Brazma, A., & Vizcaino, J. A. (2022). The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research*, 50, D543–D552.
- Pospichalova, V., Svoboda, J., Dave, Z., Kotrbova, A., Kaiser, K., Klemova, D., Ilkovic, L., Hampl, A., Crha, I., Jandakova, E., Minar, L., Weinberger, V., & Bryja, V. (2015). Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer. *Journal of Extracellular Vesicles*, 4, 25530.
- Ramirez, B. L., Howard, O. M., Dong, H. F., Edamatsu, T., Gao, P., Hartlein, M., & Kron, M. (2006). Brugia malayi asparaginyl-transfer RNA synthetase induces chemotaxis of human leukocytes and activates G-protein-coupled receptors CXCR1 and CXCR2. *Journal of Infectious Diseases*, 193, 1164–1171.
- Rubio, A. P. D., Martinez, J., Palavecino, M., Fuentes, F., Lopez, C. M. S., Marcilla, A., Perez, O. E., & Piuri, M. (2020). Transcytosis of Bacillus subtilis extracellular vesicles through an in vitro intestinal epithelial cell model. *Scientific Reports*, 10, 3120.
- Shiraishi, T., Yokota, S., Sato, Y., Ito, T., Fukiya, S., Yamamoto, S., Sato, T., & Yokota, A. (2018). Lipoteichoic acids are embedded in cell walls during logarithmic phase, but exposed on membrane vesicles in Lactobacillus gasseri JCM 1131(T). *Beneficial Microbes*, 9, 653–662.
- Tartaglia, N. R., Nicolas, A., Rodvalho, V. R., Luz, B., Briard-Bion, V., Krupova, Z., Thierry, A., Coste, F., Burel, A., Martin, P., Jardin, J., Azevedo, V., Le Loir, Y., & Guedon, E. (2020). Extracellular vesicles produced by human and animal Staphylococcus aureus strains share a highly conserved core proteome. *Scientific Reports*, 10, 8467.
- Tashiro, Y., Ichikawa, S., Shimizu, M., Toyofuku, M., Takaya, N., Nakajima-Kambe, T., Uchiyama, H., & Nomura, N. (2010). Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in Pseudomonas aeruginosa. *Applied and Environmental Microbiology*, 76, 3732–3739.
- Tong, L., Zhang, X., Hao, H., Liu, Q., Zhou, Z., Liang, X., Liu, T., Gong, P., Zhang, L., Zhai, Z., Hao, Y., & Yi, H. (2021). Lactobacillus rhamnosus GG derived extracellular vesicles modulate gut microbiota and attenuate inflammatory in DSS-induced colitis mice. *Nutrients*, 13, 3319.
- Turnbull, L., Toyofuku, M., Hynen, A. L., Kurosawa, M., Pessi, G., Petty, N. K., Osvath, S. R., Carcamo-Oyarce, G., Gloag, E. S., Shimoni, R., Omasits, U., Ito, S., Yap, X., Monahan, L. G., Cavaliere, R., Ahrens, C. H., Charles, I. G., Nomura, N., Eberl, L., & Whitchurch, C. B. (2016). Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications*, 7, 11220.
- Uppu, D. S., Wang, X., & Lee, J. C. (2023). Contribution of extracellular membrane vesicles to the secretome of Staphylococcus aureus. *MBio*, 14, e0357122.
- van der Ley, P., & van den Dobbelsteen, G. (2011). Next-generation outer membrane vesicle vaccines against Neisseria meningitidis based on nontoxic LPS mutants. *Human Vaccines*, 7, 886–890.
- Veselovsky, V. A., Dyachkova, M. S., Bespiatykh, D. A., Yunes, R. A., Shitikov, E. A., Polyayeva, P. S., Danilenko, V. N., Olekhovich, E. I., & Klimina, K. M. (2022). The gene expression profile differs in growth phases of the bifidobacterium longum culture. *Microorganisms*, 10, 1683.
- Wagner, T., Joshi, B., Janice, J., Askarian, F., Skalko-Basnet, N., Hagestad, O. C., Mekhlif, A., Wai, S. N., Hegstad, K., & Johannessen, M. (2018). Enterococcus faecium produces membrane vesicles containing virulence factors and antimicrobial resistance related proteins. *Journal of Proteomics*, 187, 28–38.
- Wang, X., Koffi, P. F., English, O. F., & Lee, J. C. (2021). Staphylococcus aureus extracellular vesicles: A story of toxicity and the stress of 2020. *Toxins (Basel)*, 13, 75.
- Wisniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, 6, 359–362.
- Yerneni, S. S., Werner, S., Azambuja, J. H., Ludwig, N., Eutsey, R., Aggarwal, S. D., Lucas, P. C., Bailey, N., Whiteside, T. L., Campbell, P. G., & Hiller, N. L. (2021). Pneumococcal extracellular vesicles modulate host immunity. *MBio*, 12, e0165721.
- Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinalp, S. C., Ester, M., Foster, L. J., & Brinkman, F. S. (2010). PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics*, 26, 1608–1615.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supplementary material

Growth phase matters: Boosting immunity via *Lactocaseibacillus*-derived membrane vesicles and their interactions with TLR2 pathways

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Materials and Methods

Proteomic sample preparation and analysis by liquid chromatography–tandem mass spectrometry

Membrane vesicles of *Lactocaseibacillus rhamnosus* CCM7091 were lysed in SDT buffer (4% SDS, 0.1M DTT, 0.1M Tris/HCl, pH 7.6) in a thermomixer (Eppendorf ThermoMixer® C, 30 min, 95°C, 750 rpm). After that, samples were centrifuged (15 min, 20 000 x g) and the supernatants (ca 50 µg of total protein) used for filter-aided sample preparation (FASP) as described elsewhere [1] using 0.5 µg of trypsin (sequencing grade; Promega). Resulting peptides were analysed by LC-MS/MS.

LC-MS/MS analyses of all peptides were done using UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) connected to timsTOF Pro spectrometer (Bruker). Prior to LC separation, tryptic digests were online concentrated and desalted using trapping column (Acclaim™ PepMap™ 100 C18, dimensions 300 µm ID, 5 mm long, 5 µm particles, Thermo Fisher Scientific). After washing of the trapping column with 0.1% formic acid (FA), the peptides were eluted (flow rate –

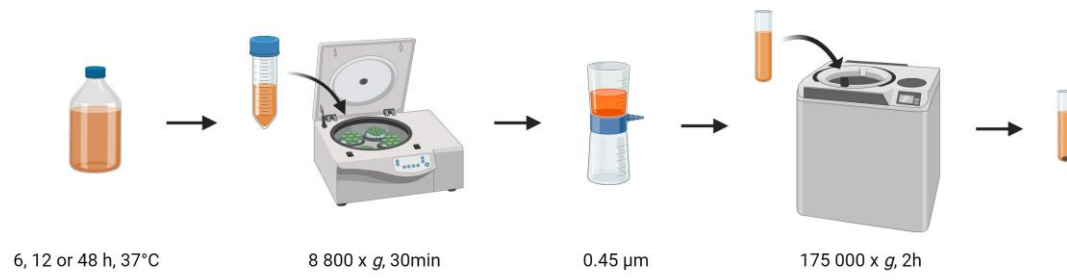
300 nl/min) from the trapping column onto an analytical column (Aurora C18, 75µm ID, 250 mm long, 1.6 µm particles, heated to 50°C, Ion Opticks) by 60 min linear gradient program (3-80% of mobile phase B; mobile phase A: 0.1% FA in water; mobile phase B: 0.1% FA in 80% ACN). Equilibration of the trapping column and the analytical column was done prior to sample injection to sample loop. The analytical column was placed inside the Butterfly Heater (Phoenix S&T) and its emitter side was installed inside the CaptiveSpray ion source (Bruker) according to the manufacturer instructions with the column temperature set to 50 °C.

MSn data were acquired in data independent acquisition (DIA) mode with base method m/z range of 100-1 700 and 1/k0 range of 0.6-1.6 V×s×cm⁻². Precursor range was defined to m/z 400-1 000 with equal windows sizes of 26 Th using two steps each PASEF scan and cycle time of 100ms locked to 100% duty cycle.

DiaPASEF data were processed in DIA-NN (version 1.8)[2] in library free mode against modified cRAP database (based on <http://www.thegpm.org/crap>; 111 sequences in total) and UniProtKB protein database for *Lactobacillus rhamnosus* ATCC 53103 (<https://www.uniprot.org/proteomes/UP000002067>; version 2022/12, number of protein sequences: 2,718). No variable, carbamidomethylation as fixed modification and trypsin/P enzyme with 1 allowed missed cleavages were set during the library preparation. False discovery rate (FDR) control was set to 1% FDR. MS1 and MS2 accuracies as well as scan window parameters were set based on the initial test searches (median value from all samples ascertained parameter values). MBR was switched on.

Protein MaxLFQ intensities reported in the DIA-NN main report file were further processed using the software container environment (<https://github.com/OmicsWorkflows>), version 4.6.3a. Processing workflow is available upon request. Briefly, it covered: a) removal of low-quality precursors and contaminant protein groups, b) protein group intensities log2 transformation and normalization (loessF), c) filtering out of protein groups not quantified in at least 2 replicates of single sample type, d) imputation of missing values using R package imp4p, e) differential expression analysis using LIMMA statistical test.

A



B

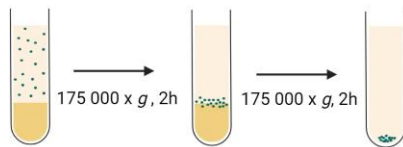


Figure S1: Isolation (A) and purification (B) of *Lacticaseibacillus rhamnosus*-derived membrane vesicles.

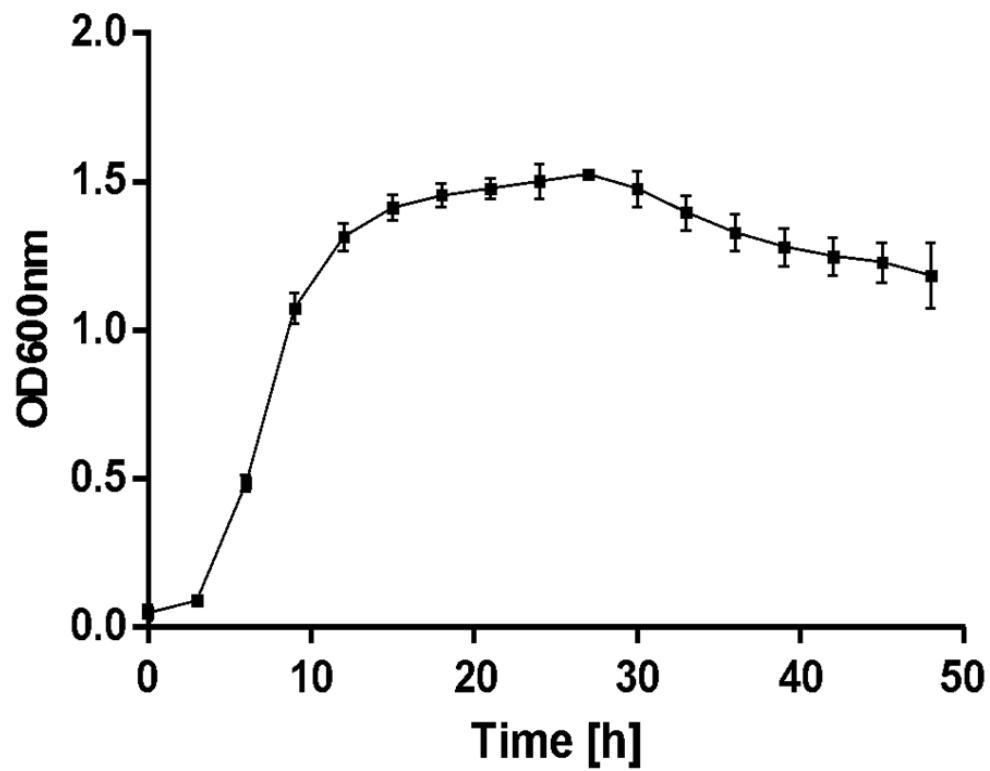


Figure S2: Growth curve of *Lacticaseibacillus rhamnosus* CCM7091 cultivated in MRS broth at 37 °C for 48 h aerobically. Data are shown as mean $\pm$ SD; n=3.

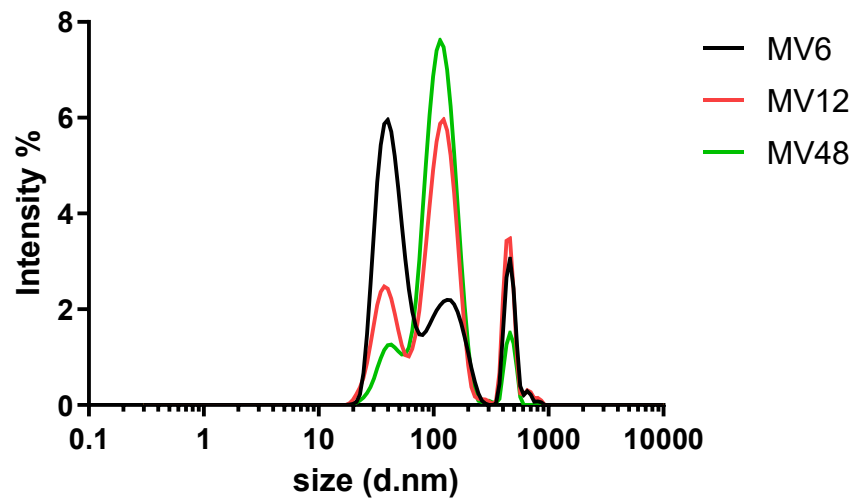


Figure S3: Size distribution by Intensity of *Lacticaseibacillus rhamnosus* CCM7091 membrane vesicles (MVs) isolated throughout bacterial growth (MV6, MV12, MV48).

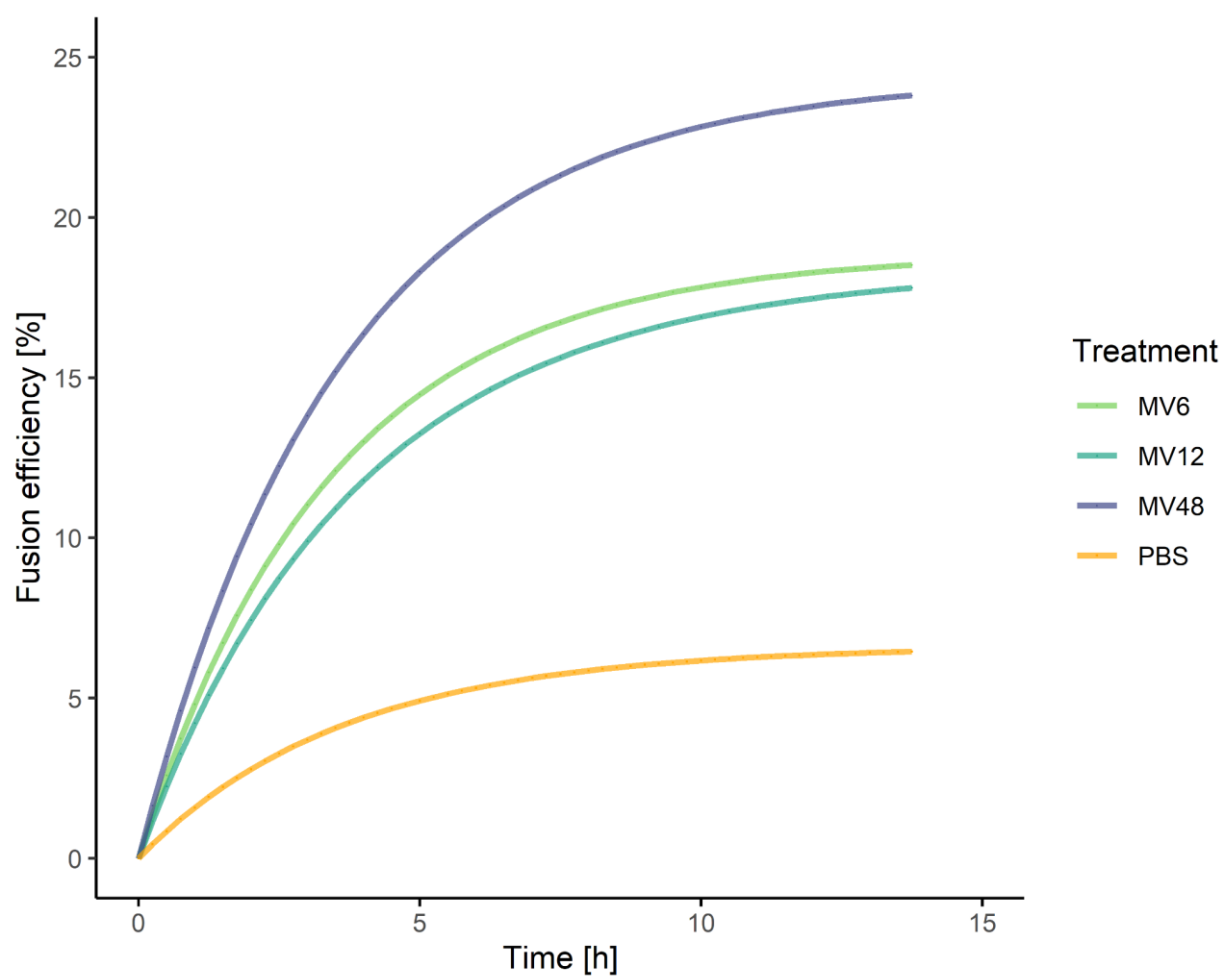


Figure S4: Time-course dynamics of internalization of MVs into Caco-2 cells. The efficiency of fusion events within Caco-2 cells (calculated according to Equation 1). The increase of the fluorescence signal was observed after addition of R-18 labeled MVs from different times of isolation (MV6, MV12, MV48) to Caco-2 cells in time. The vehicle alone, subjected to the same labeling protocol, was used as negative control (PBS).

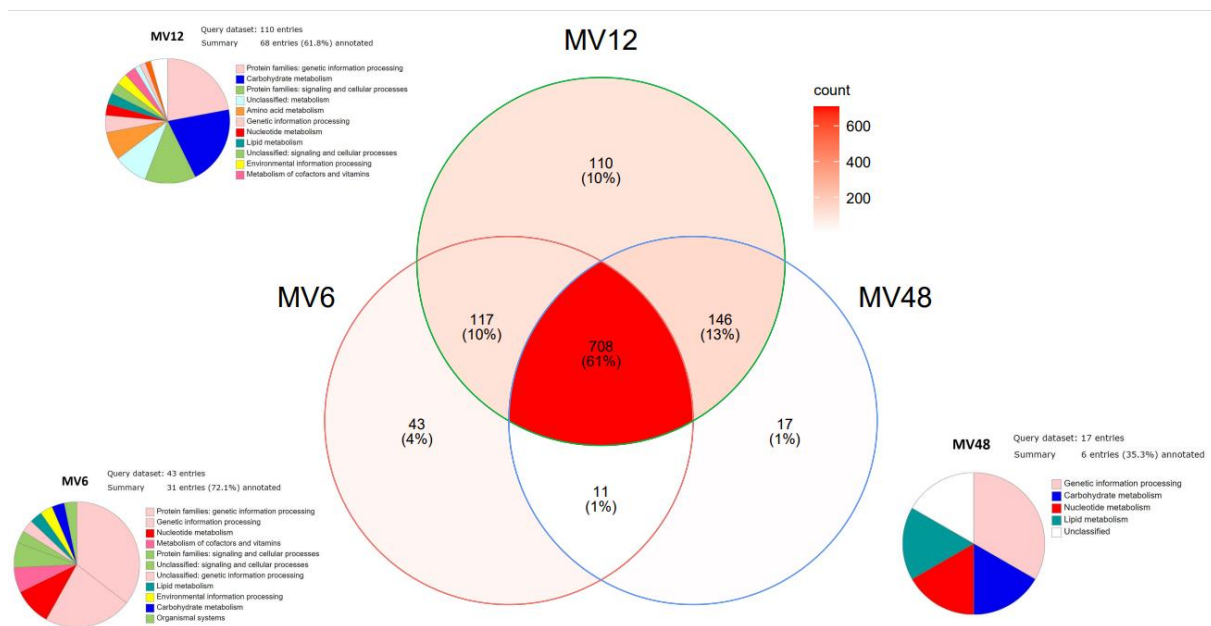
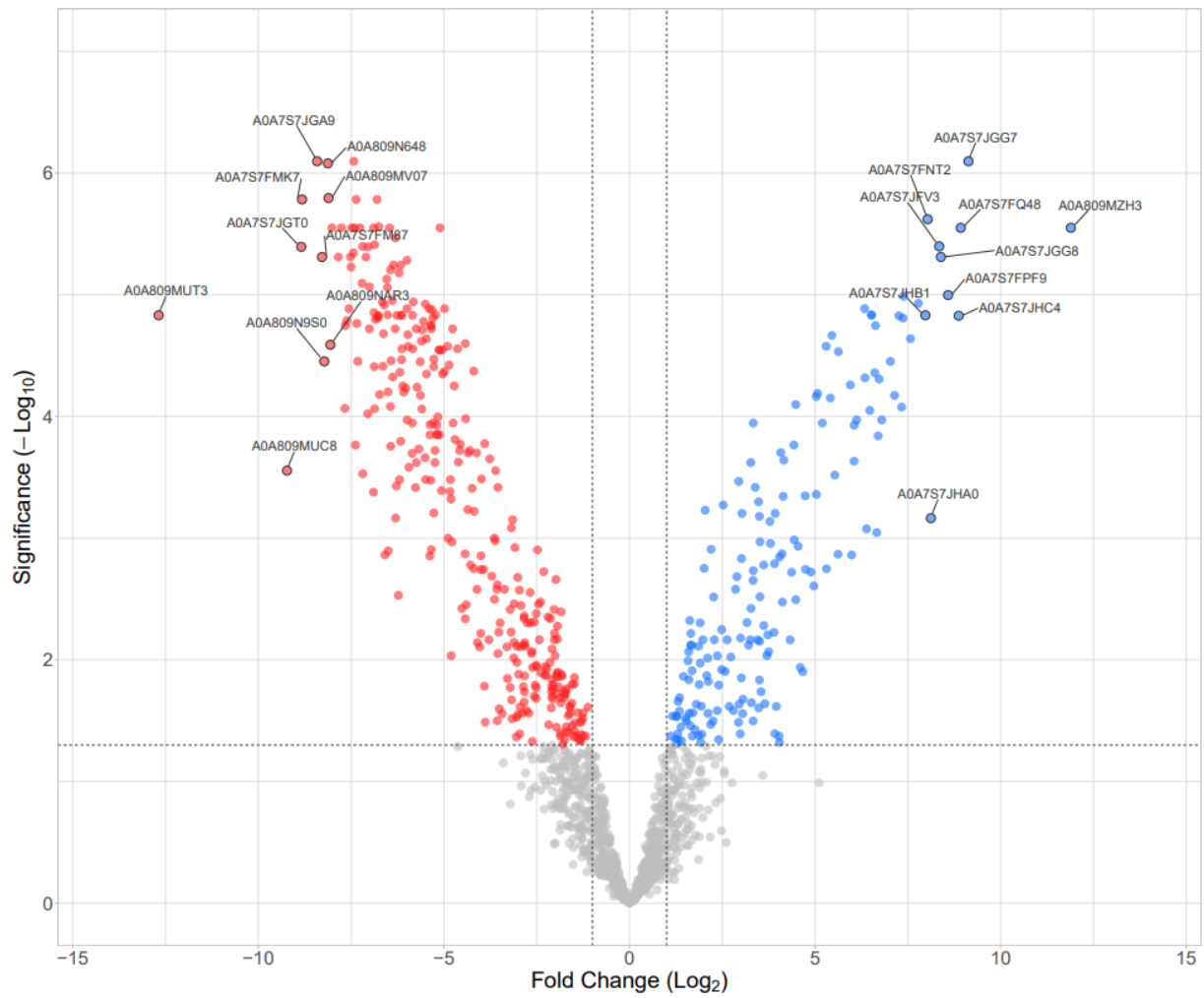


Figure S5: Venn diagram depicting protein overlap of the membrane vesicles (MVs) samples. The diagram was generated using the ggVennDiagram package in R. Functional protein categories are visualized using pie charts generated by KEGG Mapper. Number of entries reflects the information available in databases.

A

MV6 vs MV12, $|\log FC| > 1$, $\text{adj.}P < 0.05$



B

MV12 vs MV48, $|\log FC| > 1$, adj.P < 0.05

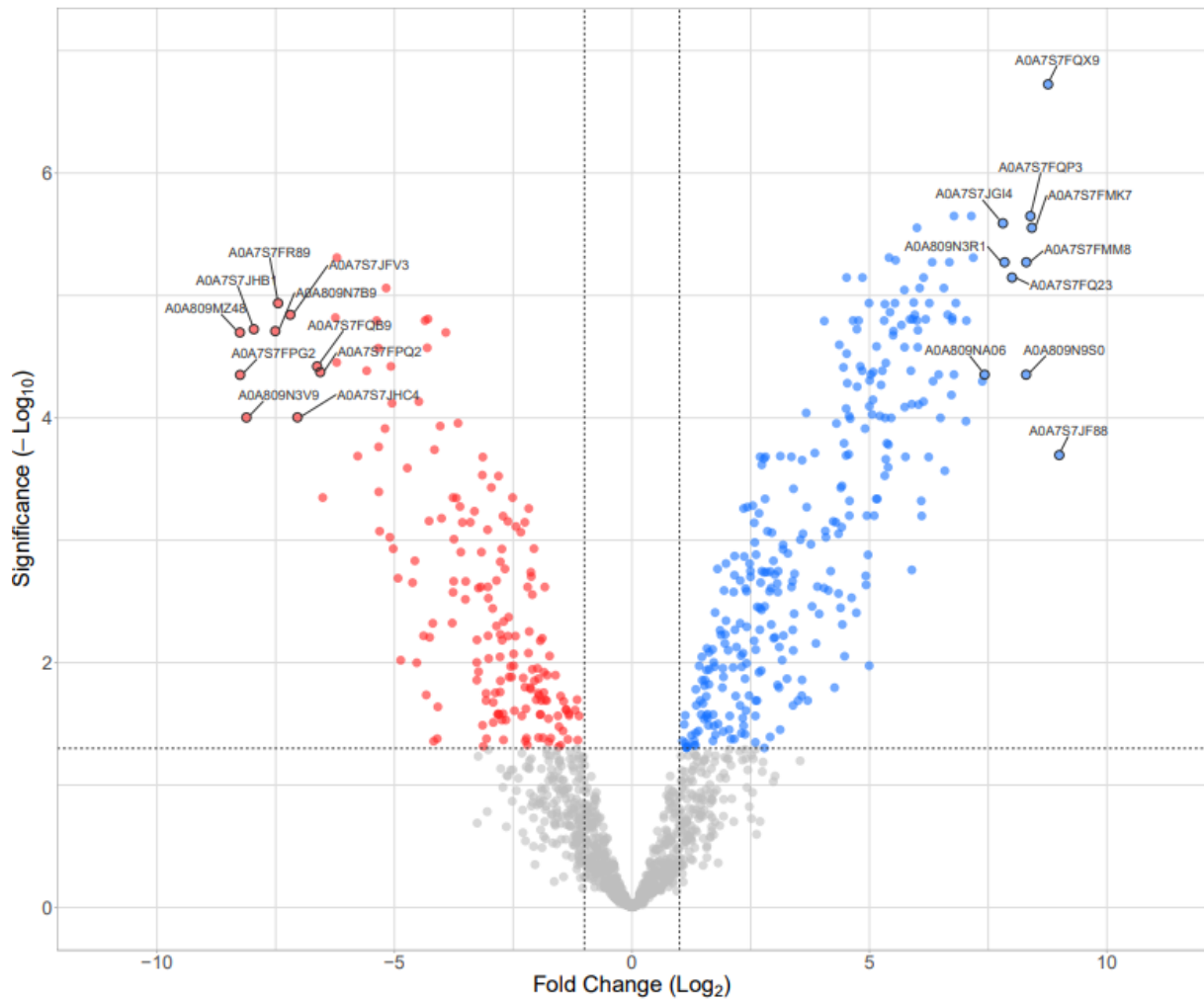


Figure S6: Volcano plots showing proteomics data from membrane vesicles (MVs): MV6 vs MV12 (A) and MV12 vs MV48 (B). The dots indicate different proteins that display both large magnitude fold changes (x axis) and high statistical significance ($-\log_{10}$ of adjusted p values, y axis). The LIMMA package in R was utilized to calculate statistical significance. A log fold change greater than 1 and an adjusted P value less than 0.05 were chosen as the thresholds to determine differential expression (grey – unchanged, red – decreased, blue – increased).

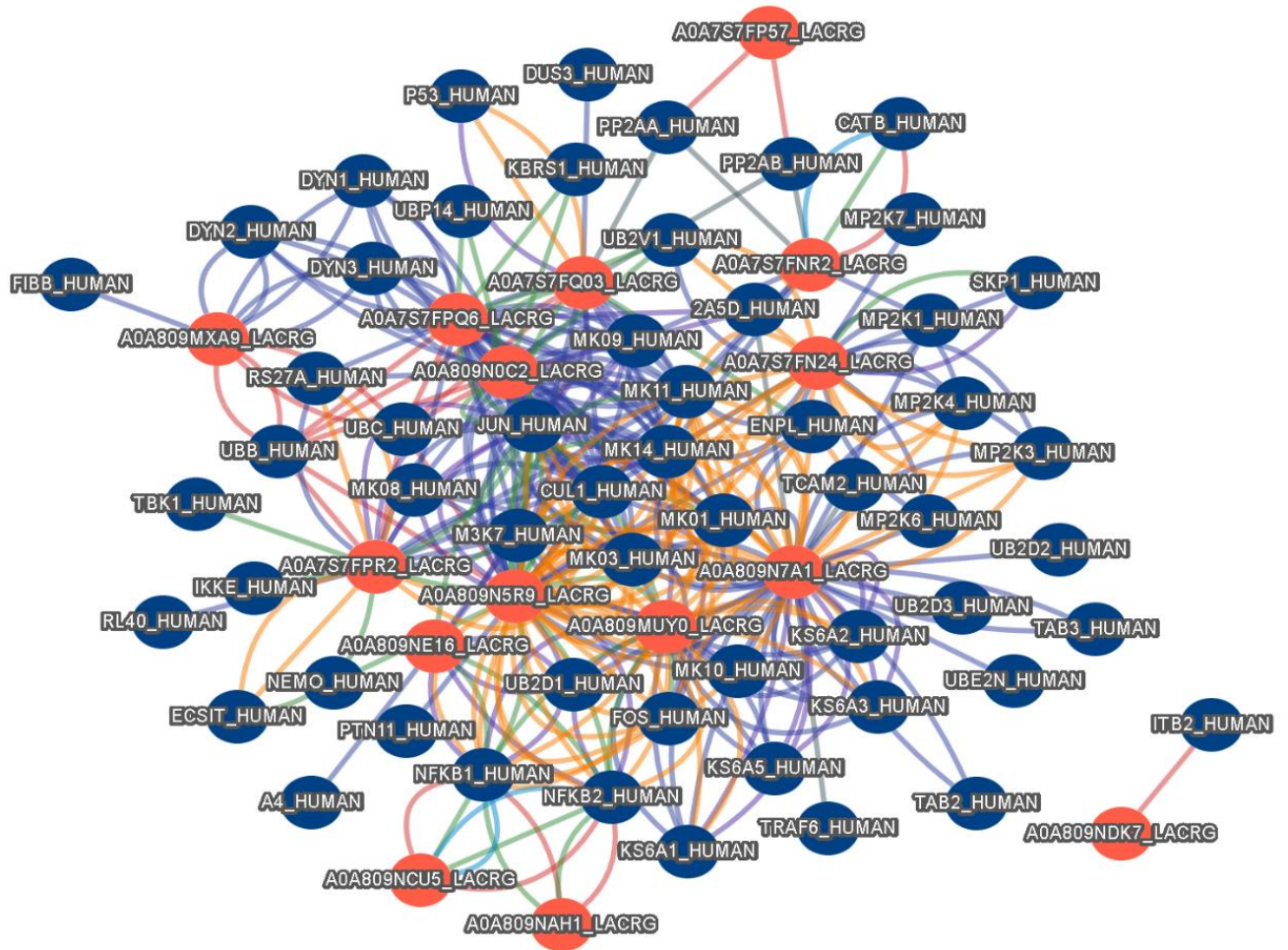
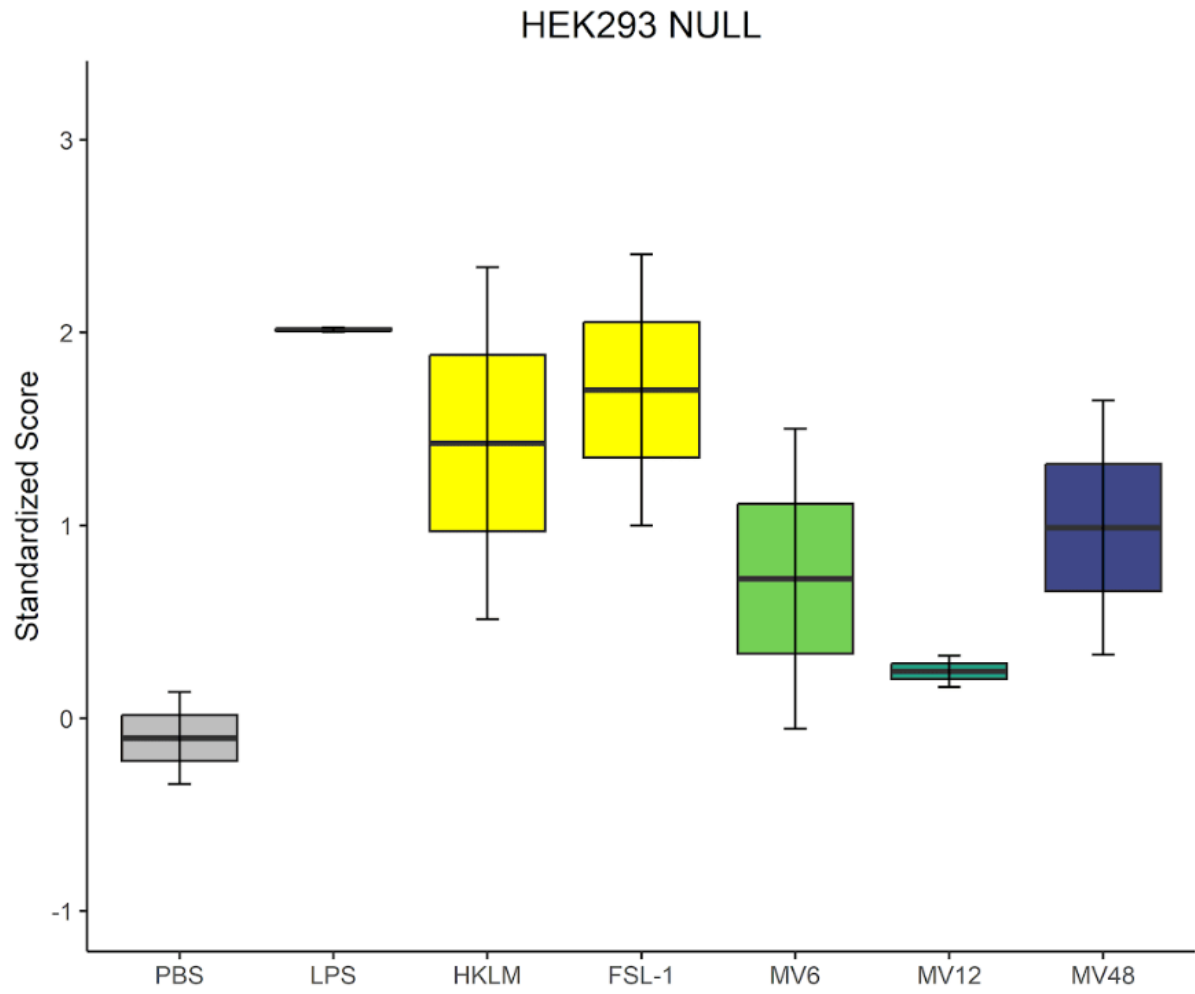
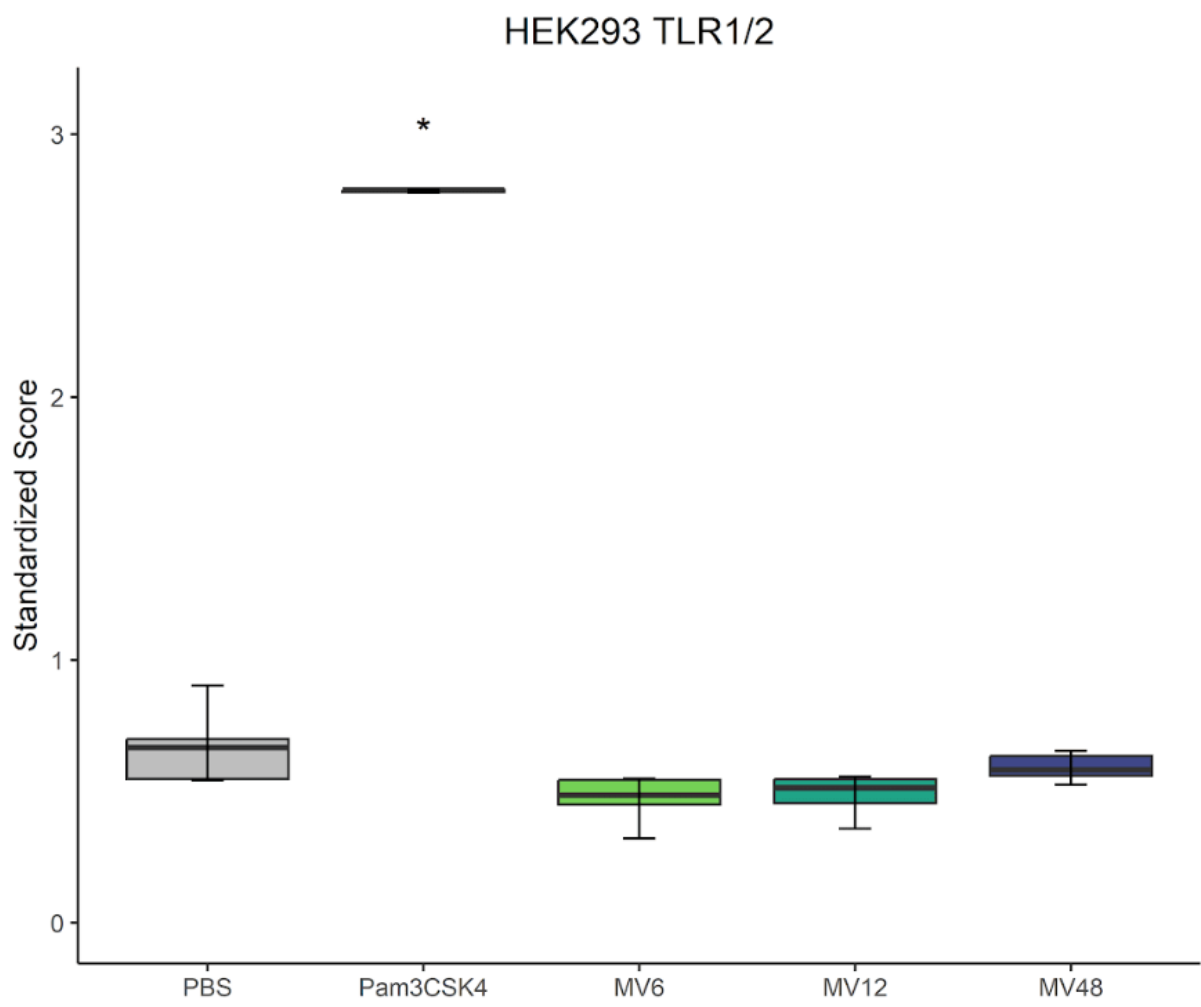


Figure S7: Predicted interaction network of upregulated proteins from MV48 with human TLR cascade. Red dots represent *L.rhamnosus* CCM7091 proteins; Uniprot ID is used as protein identifier. Blue dots represent host proteins; Uniprot name is used as a protein identifier. The interactions were predicted with proteins from the human TLR cascade, which were retrieved from the Reactome database [3]. The interolog prediction on the PredHPI Webserver [4] was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

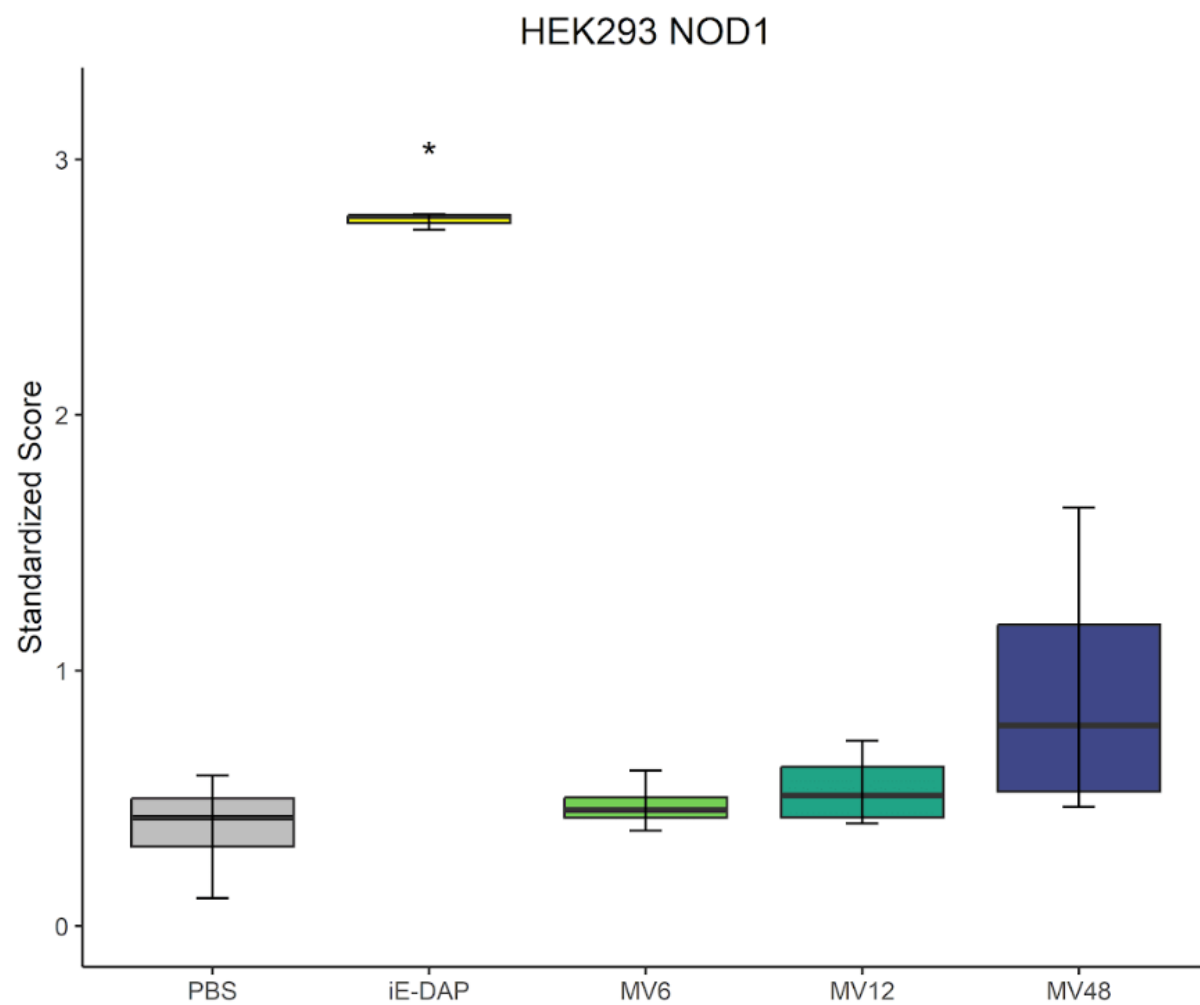
A



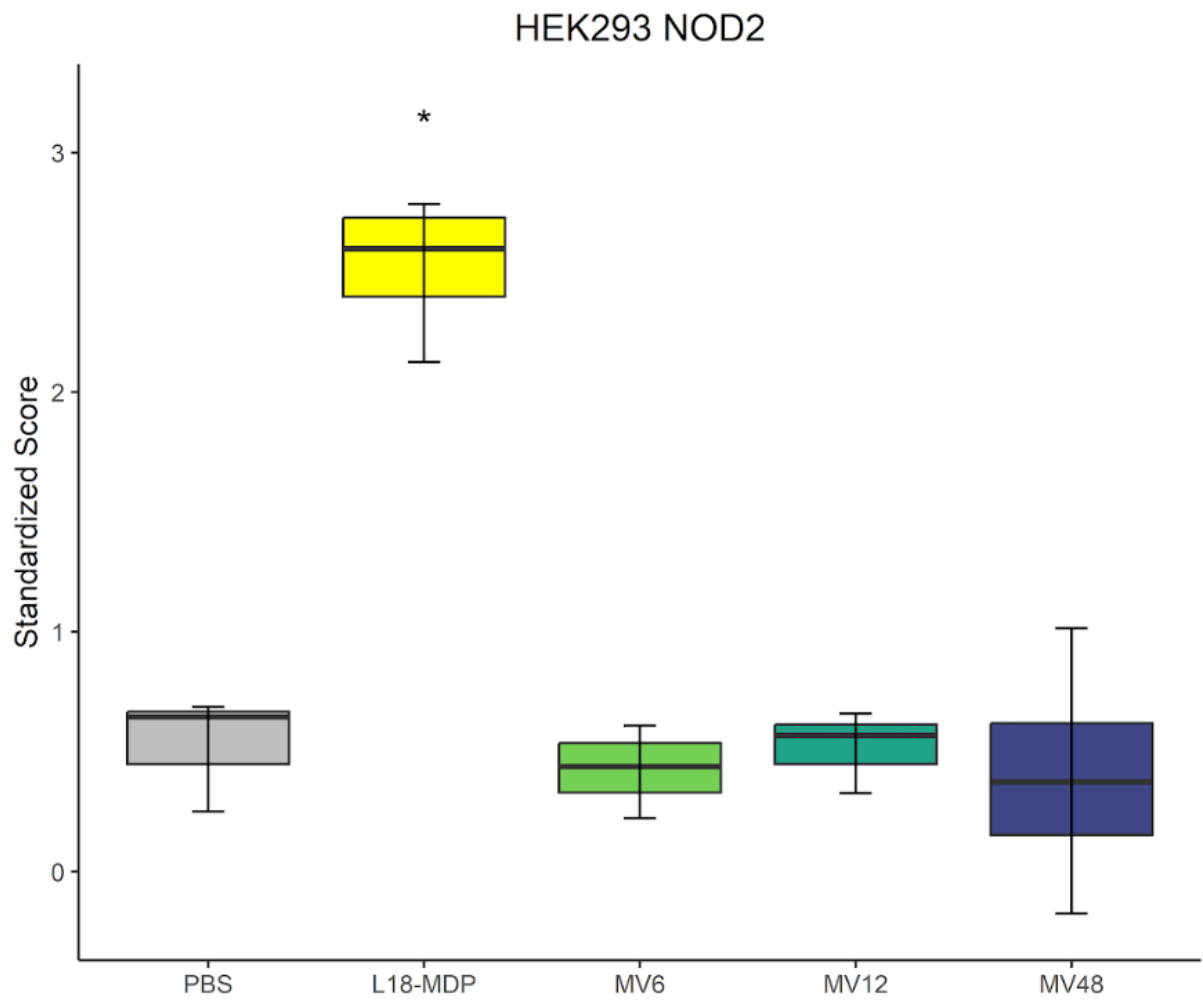
B



C



D



E

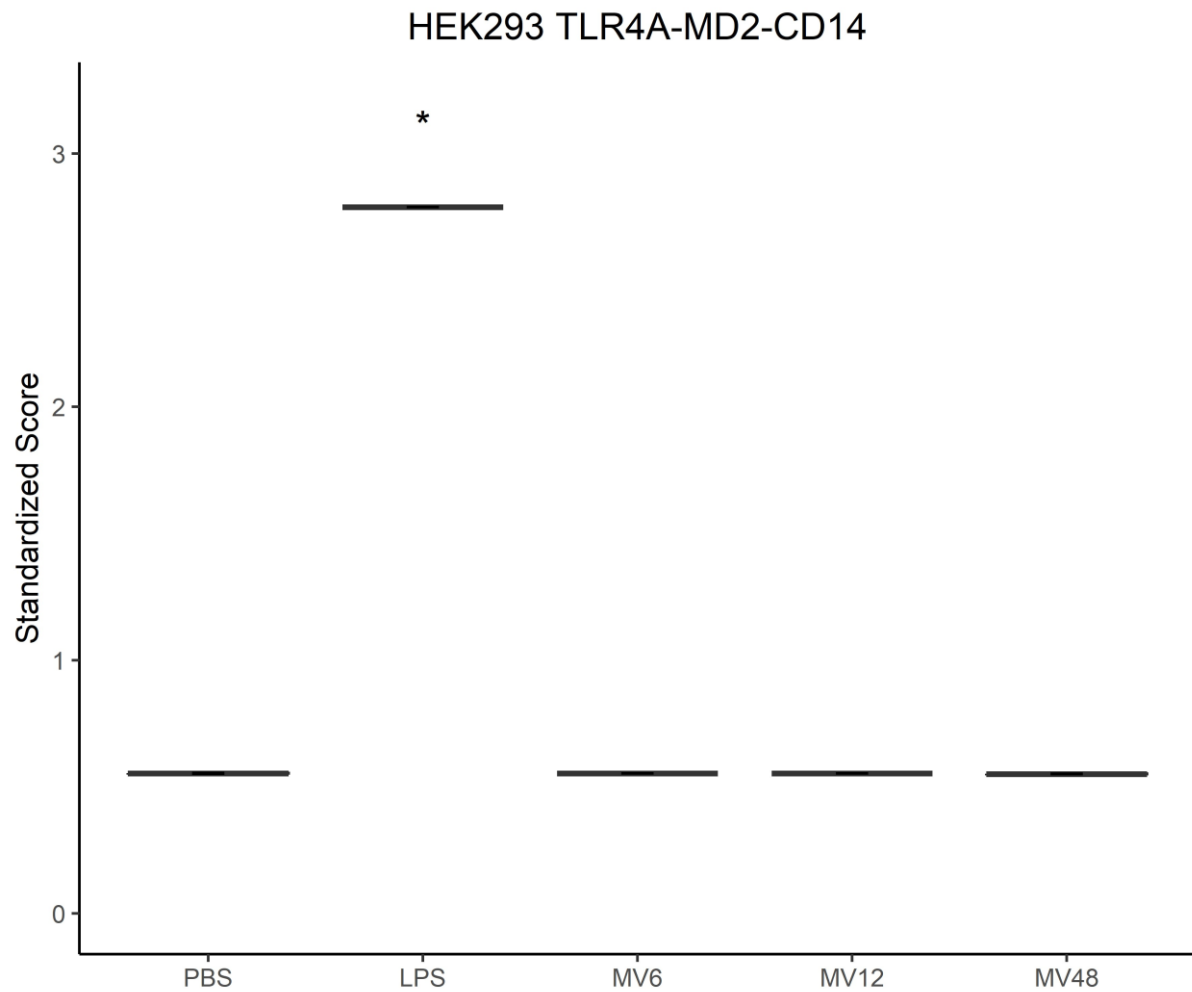


Figure S8: Human cell receptors not involved in the recognition of membrane vesicles (MVs). MVs isolated after 6, 12, and 48 h (MV6, MV12 and MV48) did not affect scrambled control (A) and activation of TLR1/2 (B), NOD1 (C), NOD2 (D), and TLR4A-MD2-CD14 (E), expressed on human embryonic kidney cells HEK-293 (concentration 10^5 MVs per cell). The recognition abilities were compared to negative control (PBS), appropriate positive control is depicted (Pam3CSK4 for HEK293 TLR1/2, iE-DAP for HEK293 NOD1, L18-LDP for HEK293 NOD2, LPS for HEK293 TLR4A-MD2-CD14). Results were evaluated based on IL-8 production activity in cell culture media. The box plots show medians (lines), interquartile ranges (boxes), and minimum and maximum values (whiskers); data normalization was performed using Z-score; statistical analysis was performed using the ANOVA and the significance was assessed at * $p < 0.05$; $n=4$.

Table S1: Colony forming units per milliliter (CFUml⁻¹). Data are shown as mean $\pm$ SD; n=3.

Collection time (h)	CFU/ml
0	$1.64 \cdot 10^7 \pm 4.33 \cdot 10^6$
6	$5.16 \cdot 10^8 \pm 2.88 \cdot 10^8$
12	$5.07 \cdot 10^9 \pm 2.52 \cdot 10^8$
48	$3.31 \cdot 10^8 \pm 8.82 \cdot 10^7$

Table S2: Kinetic parameters of *Lacticaseibacillus rhamnosus*-derived membrane vesicles (MVs) uptake by Caco-2 cells at 4 °C. The parameters encompass the saturation plateau, kinetic rate constant (K), half-life (defined as the time required to attain half of the saturation level), and tau (signifying the time constant of saturation).

	MV6	MV12	MV48
Plateau [%]	0.744	0.359	0.702
K [h⁻¹]	1.727	1.866	1.688
Half-life [h]	0.401	0.371	0.411
Tau [h]	0.579	0.536	0.592

Table S3: Protein localization prediction using PSORTb software.

Localization				Standardized values [%]		
	MV6	MV12	MV48	MV6	MV12	MV48
Cytoplasmic membrane	429	491	393	48.8	45.4	44.6
Cytoplasmic	285	364	302	32.4	33.7	34.2
Cell wall	17	20	20	1.9	1.9	2.3
Extracellular	8	10	9	0.9	0.9	1
Unknown	140	196	158	15.9	18.1	17.9

Table S4: The number of proteins in each functional group annotated using KEGG Mapper. The color scale indicates the ratio of protein representation in the individual groups (green – the largest, red – the smallest). The percentage values presented are relative to the total number of proteins.

	MV6	MV12	MV48	Standardized values [%]		
				MV6	MV12	MV48
Number of proteins in total	879	1081	882	100	100	100
Number of classified proteins	579	707	582	65.9	65.4	66
Environmental Information Processing	93	97	83	10.6	9	9.4
Protein families: signaling and cellular processes	73	85	63	8.3	7.9	7.1
Genetic Information Processing	98	98	92	11.1	9.1	10.4
Carbohydrate metabolism	61	91	73	6.9	8.4	8.3
Protein families: metabolism	20	32	31	2.3	3	3.5
Protein families: genetic information processing	55	65	43	6.3	6	4.9
Glycan biosynthesis and metabolism	19	20	18	2.2	1.9	2
Unclassified: metabolism	28	41	30	3.2	3.8	3.4
Unclassified	23	33	30	2.6	3.1	3.4
Energy metabolism	13	16	15	1.5	1.5	1.7
Cellular Processes	15	15	14	1.7	1.4	1.6
Lipid metabolism	21	22	18	2.4	2	2
Unclassified: signaling and cellular processes	17	20	16	1.9	1.9	1.8
Nucleotide metabolism	20	23	21	2.3	2.1	2.4
Metabolism of other amino acids	4	10	9	0.5	0.9	1
Organismal systems	4	3	3	0.5	0.3	0.3
Metabolism of cofactors and vitamins	7	13	9	0.8	1.2	1
Human diseases	1	0	1	0.1	0	0.1
Unclassified: genetic information processing	7	7	5	0.8	0.6	0.6
Amino acid metabolism	6	20	14	0.7	1.9	1.6
Metabolism of terpenoids and polyketides	1	2	1	0.1	0.2	0.1
Xenobiotics biodegradation and metabolism	0	0	0	0	0	0



Table S5: Ten most significantly up- and down-regulated proteins annotated in each combination of membrane vesicles (MVs) (MV6-MV12, MV6-MV48, MV12-MV48).

MV6-MV12

Uniprot ID	Description
UP	
A0A7S7JGG7	DUF2325 domain-containing protein
A0A809MZH3	Recombination factor protein RarA
A0A7S7FQ48	DUF2087 domain-containing protein (Transcriptional regulator)
A0A7S7FNT2	tRNA-specific 2-thiouridylase MnmA (EC 2.8.1.13)
A0A7S7JHB1	GTP diphosphokinase (EC 2.7.6.5)
A0A7S7JFV3	UvrABC system protein B (Protein UvrB) (Excinuclease ABC subunit B)
A0A7S7JGG8	IS5-like element ISLrh2 family transposase (Putative transposase)
A0A7S7JHC4	DNA polymerase III PolC-type (PolIII) (EC 2.7.7.7)
A0A7S7FPF9	Phage protein
A0A7S7JHA0	Chaperone protein ClpB
DOWN	
A0A809N648	Cytosolic protein
A0A809MV07	Putative cell surface protein (WxL domain-containing protein)
A0A7S7JGA9	Ribonucleoside-diphosphate reductase subunit beta (EC 1.17.4.1)
A0A809NAR3	DUF1819 family protein
A0A809MUC8	ABC transporter ATP-binding protein
A0A809N9S0	DUF1129 domain-containing protein
A0A7S7FMK7	Secreted protein
A0A7S7FM87	SDR family NAD(P)-dependent oxidoreductase (Sorbitol-6-phosphate 2-dehydrogenase)
A0A809MUT3	S-adenosylmethionine:tRNA ribosyltransferase-isomerase (EC 2.4.99.17) (Queuosine biosynthesis protein QueA)
A0A7S7JGT0	Leucine--tRNA ligase (EC 6.1.1.4) (Leucyl-tRNA synthetase) (LeuRS)

MV12-MV48

Uniprot ID	Description
UP	
A0A7S7JGI4	ATP-binding cassette domain-containing protein (Multidrug ABC transporter ATP-binding component)
A0A7S7FQX9	Cobalt ABC transporter ATP-binding component (Energy-coupling factor ABC transporter ATP-binding protein)
A0A7S7FMK7	Secreted protein
A0A7S7FQP3	Amino acid permease (Amino acid transporter protein)
A0A809N3R1	Alpha-L-fucosidase
A0A7S7FQ23	Phage protein (Phage tail protein)
A0A7S7FMM8	Fructuronate reductase (Mannitol dehydrogenase family protein)
A0A809N9S0	DUF1129 domain-containing protein
A0A809NA06	PadR family transcriptional regulator (Transcriptional regulator)
A0A7S7JF88	Anaerobic ribonucleoside-triphosphate reductase (EC 1.17.4.2)
DOWN	
A0A7S7JHB1	GTP diphosphokinase (EC 2.7.6.5)
A0A7S7FR89	Adenosine deaminase (EC 3.5.4.4) (Adenosine aminohydrolase)
A0A809MZ48	Phage protein (Recombinase RecT)
A0A7S7JFV3	UvrABC system protein B (Protein UvrB) (Excinuclease ABC subunit B)
A0A809N3V9	Putative pyrophosphatase
A0A7S7FPG2	Coenzyme A biosynthesis bifunctional protein CoaBC (DNA/pantothenate metabolism flavoprotein) (Phosphopantothenoylcysteine synthetase/decarboxylase) (PPCS-PPCDC)
A0A809N7B9	ATP synthase subunit a (ATP synthase F0 sector subunit a) (F-ATPase subunit 6)
A0A7S7FQB9	UPF0246 protein GM657_10425
A0A7S7FPQ2	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.-)
A0A7S7JHC4	DNA polymerase III PolC-type (PolIII) (EC 2.7.7.7)

MV6-MV48

Uniprot ID	Description
UP	
A0A7S7FQX9	Cobalt ABC transporter ATP-binding component (Energy-coupling factor ABC transporter ATP-binding protein)
A0A809N3R1	Alpha-L-fucosidase
A0A7S7JG14	ATP-binding cassette domain-containing protein (Multidrug ABC transporter ATP-binding component)
A0A7S7FQ48	DUF2087 domain-containing protein (Transcriptional regulator)
A0A809NBT7	Peptide chain release factor 3 (RF-3)
A0A7S7FP39	UvrABC system protein A (UvrA protein) (Excinuclease ABC subunit A)
A0A7S7FRH4	Alpha/beta fold hydrolase (Putative cell surface hydrolase)
A0A809MY00	DEAD-box ATP-dependent RNA helicase CshB (EC 3.6.4.13)
A0A7S7JF88	Anaerobic ribonucleoside-triphosphate reductase (EC 1.17.4.2)
A0A7S7FPQ0	Ribonuclease J (RNase J) (EC 3.1.-.-)
DOWN	
A0A809MYT0	Putative cell surface protein
A0A809NE16	Putative oxidoreductase
A0A809MV07	Putative cell surface protein (WxL domain-containing protein)
A0A7S7FPL2	Methionyl-tRNA formyltransferase (EC 2.1.2.9)
A0A830Q8M2	Transporter
A0A830TYX9	Helix-turn-helix domain-containing protein
A0A809MW20	UPF0237 protein GM657_04305
A0A7S7JGB4	Sulfonate ABC transporter substrate-binding protein (Taurine ABC transporter substrate binding component)
A0A809MUC8	ABC transporter ATP-binding protein
A0A809N3V9	Putative pyrophosphatase

Table S6: Predicted interaction network of upregulated proteins from *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles isolated throughout bacterial growth after 48 h (MV48) with murine TNF α signaling cascade. Uniprot ID is used as a protein identifier for *L.rhamnosus* CCM7091 proteins (bacterial proteins). Uniprot name is used as a protein identifier for host protein. The interactions were predicted with host proteins, which were retrieved from the Reactome database [3]. The interolog prediction on the PredHPI Webserver [4] was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

Host Protein	Bacterial Protein	Homologous Proteins Interacting	Protein-Protein Database
CHIP_MOUSE	A0A7S7FN24_LACRG	10273 with 2539	biogriddb
CYLD_MOUSE	A0A809N5R9_LACRG	1540 with 7407	biogriddb
IKKE_MOUSE	A0A7S7FPR2_LACRG	P26639 with Q14164	intactdb
M3K7_MOUSE	A0A809NE16_LACRG	54101 with 25979	biogriddb
M3K7_MOUSE	A0A7S7FQ03_LACRG	54101 with 6301	biogriddb
M3K7_MOUSE	A0A7S7FPR2_LACRG	54101 with 6897	biogriddb
NEMO_MOUSE	A0A809N5R9_LACRG	P26640 with Q9Y6K9	intactdb
RACK1_MOUSE	A0A7S7FPR2_LACRG	854732 with 855143	biogriddb
RACK1_MOUSE	A0A7S7FQ03_LACRG	177859 with 177895	biogriddb
RACK1_MOUSE	A0A7S7FNR2_LACRG	P36013 with P38011	intactdb
RL40_MOUSE	A0A7S7FPR2_LACRG	854732 with 853969	biogriddb
RS27A_MOUSE	A0A7S7FPR2_LACRG	6233 with 6897	biogriddb
RS27A_MOUSE	A0A7S7FPQ6_LACRG	850633 with 850864	biogriddb
RS27A_MOUSE	A0A809N0C2_LACRG	850633 with 850864	biogriddb
RS27A_MOUSE	A0A7S7FPR2_LACRG	9606.ENSPO0000387710 with 9606.ENSPO0000272317	stringdb
RS27A_MOUSE	A0A7S7FPR2_LACRG	9606.ENSPO0000272317 with 9606.ENSPO0000387710	stringdb
TBK1_MOUSE	A0A7S7FPR2_LACRG	P26639 with Q14164	intactdb
TNFA_MOUSE	A0A7S7FN24_LACRG	7124 with 2539	biogriddb
TNR1A_MOUSE	A0A809MUY0_LACRG	P19438 with Q9NZ08	intactdb
TNR1A_MOUSE	A0A809MUY0_LACRG	9606.ENSPO0000296754 with 9606.ENSPO0000162749	stringdb
TNR1A_MOUSE	A0A809MUY0_LACRG	9606.ENSPO0000162749 with 9606.ENSPO0000296754	stringdb
TRAF2_MOUSE	A0A7S7FN24_LACRG	9606.ENSPO0000377192 with 9606.ENSPO0000262395	stringdb
TRAF2_MOUSE	A0A7S7FN24_LACRG	9606.ENSPO0000262395 with 9606.ENSPO0000377192	stringdb
UB2D1_MOUSE	A0A809NE16_LACRG	Q02207 with P14682	mintdb
UB2D1_MOUSE	A0A809NE16_LACRG	P14682 with Q02207	mintdb
UBB_MOUSE	A0A7S7FQ03_LACRG	7316 with 851587	hpiddb
UBB_MOUSE	A0A809MXA9_LACRG	7316 with 851010	hpiddb
UBB_MOUSE	A0A809N5R9_LACRG	7316 with 852986	hpiddb
UBB_MOUSE	A0A7S7FPR2_LACRG	7316 with 854732	hpiddb
UBB_MOUSE	A0A7S7FPQ6_LACRG	7316 with 850633	hpiddb
UBB_MOUSE	A0A809N0C2_LACRG	7316 with 850633	hpiddb
UBB_MOUSE	A0A809MXA9_LACRG	7316 with 851221	hpiddb
UBC_MOUSE	A0A7S7FQ03_LACRG	7316 with 851587	hpiddb
UBC_MOUSE	A0A809MXA9_LACRG	7316 with 851010	hpiddb
UBC_MOUSE	A0A809N5R9_LACRG	7316 with 852986	hpiddb
UBC_MOUSE	A0A7S7FPR2_LACRG	7316 with 854732	hpiddb
UBC_MOUSE	A0A7S7FPQ6_LACRG	7316 with 850633	hpiddb
UBC_MOUSE	A0A809N0C2_LACRG	7316 with 850633	hpiddb
UBC_MOUSE	A0A809MXA9_LACRG	7316 with 851221	hpiddb
UBC_MOUSE	A0A7S7FNR2_LACRG	50522 with 24552	biogriddb
UBP4_MOUSE	A0A7S7FQ03_LACRG	9958 with 54938	biogriddb
UBP4_MOUSE	A0A7S7FNR2_LACRG	Q9Y4E8 with P23368	intactdb

Table S7: Predicted interaction network of upregulated proteins from *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles isolated throughout bacterial growth after 48 h (MV48) with murine IL-6 signaling cascade. Uniprot ID is used as a protein identifier for *L.rhamnosus* CCM7091 proteins (bacterial proteins). Uniprot name is used as a protein identifier for host protein. The interactions were predicted with host proteins, which were retrieved from the Reactome database [3]. The interolog prediction on the PredHPI Webserver [4] was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

Host Protein	Bacterial Protein	Homologous Proteins Interacting	Protein-Protein Database
IL6RA_MOUSE	A0A809MUY0_LACRG	Q9NZ08 with P08887	intactdb
IL6RA_MOUSE	A0A809MUY0_LACRG	9606.ENSPO0000357470 with 9606.ENSPO0000296754	stringdb
IL6RA_MOUSE	A0A809MUY0_LACRG	9606.ENSPO0000296754 with 9606.ENSPO0000357470	stringdb
PTN11_MOUSE	A0A809N5R9_LACRG	5777 with 7407	biogriddb
STAT1_MOUSE	A0A7S7FQ03_LACRG	6772 with 6301	biogriddb
STAT3_MOUSE	A0A7S7FQ03_LACRG	6772 with 6301	biogriddb

Table S8: Predicted interaction network of upregulated proteins from *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles isolated throughout bacterial growth after 48 h (MV48) with murine IL-10 signaling cascade. Uniprot ID is used as a protein identifier for *L.rhamnosus* CCM7091 proteins (bacterial proteins). Uniprot name is used as a protein identifier for host protein. The interactions were predicted with host proteins, which were retrieved from the Reactome database [3]. The interolog prediction on the PredHPI Webserver [4] was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
STAT3 MOUSE	A0A7S7FQ03 LACRG	6772 with 6301	biogriddb

Table S9: Predicted interaction network of upregulated proteins from *Lactocaseibacillus rhamnosus* CCM7091 membrane vesicles isolated throughout bacterial growth after 48 h (MV48) with human TLR cascade. Uniprot ID is used as a protein identifier for *L.rhamnosus* CCM7091 proteins (bacterial proteins). Uniprot name is used as a protein identifier for host protein. The interactions were predicted with host proteins, which were retrieved from the Reactome database [3]. The interolog prediction on the PredHPI Webserver [4] was employed to predict interactions, with the criteria set to a minimum coverage of 60% and a sequence identity threshold of 30% for each interacting partner.

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
2A5D_HUMAN	A0A809N7A1_LACRG	P00546 with P38903	dipdb
2A5D_HUMAN	A0A7S7FPQ6_LACRG	P38903 with P31539	mintdb
2A5D_HUMAN	A0A809N0C2_LACRG	P38903 with P31539	mintdb
A4_HUMAN	A0A809NE16_LACRG	25979 with 351	biogriddb
CATB_HUMAN	A0A7S7FNR2_LACRG	P07858 with Q8ZG09	hpdb
CATB_HUMAN	A0A7S7FNR2_LACRG	P07858 with Q8ZG09	intactdb
CATB_HUMAN	A0A7S7FNR2_LACRG	P07858 with Q8ZG09	phisto
CUL1_HUMAN	A0A7S7FPQ6_LACRG	8452 with 81570	biogriddb
CUL1_HUMAN	A0A809N0C2_LACRG	8452 with 81570	biogriddb
CUL1_HUMAN	A0A7S7FQ03_LACRG	8452 with 54938	biogriddb
CUL1_HUMAN	A0A809MUY0_LACRG	8452 with 9520	biogriddb
CUL1_HUMAN	A0A7S7FPR2_LACRG	8452 with 80222	biogriddb
CUL1_HUMAN	A0A809N5R9_LACRG	8452 with 7407	biogriddb
CUL1_HUMAN	A0A7S7FPR2_LACRG	8452 with 6897	biogriddb
CUL1_HUMAN	A0A7S7FPR2_LACRG	8454 with 6897	biogriddb
CUL1_HUMAN	A0A7S7FNR2_LACRG	8452 with 4199	biogriddb
CUL1_HUMAN	A0A7S7FPQ6_LACRG	850633 with 851424	biogriddb
CUL1_HUMAN	A0A809N0C2_LACRG	850633 with 851424	biogriddb
CUL1_HUMAN	A0A7S7FPR2_LACRG	854732 with 851424	biogriddb
CUL1_HUMAN	A0A7S7FN24_LACRG	855480 with 851424	biogriddb
CUL1_HUMAN	A0A7S7FPR2_LACRG	9606.ENSP00000387710 with 9606.ENSP00000326804	stringdb
CUL1_HUMAN	A0A7S7FPR2_LACRG	9606.ENSP00000326804 with 9606.ENSP00000387710	stringdb
DUS3_HUMAN	A0A7S7FQ03_LACRG	63904 with 6301	biogriddb
DYN1_HUMAN	A0A809MXA9_LACRG	853870 with 851221	biogriddb
DYN1_HUMAN	A0A809MXA9_LACRG	851221 with 853870	biogriddb
DYN1_HUMAN	A0A7S7FPQ6_LACRG	850633 with 850686	biogriddb
DYN1_HUMAN	A0A809N0C2_LACRG	850633 with 850686	biogriddb
DYN1_HUMAN	A0A7S7FPQ6_LACRG	853870 with 850633	biogriddb
DYN1_HUMAN	A0A809N0C2_LACRG	853870 with 850633	biogriddb
DYN2_HUMAN	A0A809MXA9_LACRG	853870 with 851221	biogriddb
DYN2_HUMAN	A0A809MXA9_LACRG	851221 with 853870	biogriddb
DYN2_HUMAN	A0A7S7FPQ6_LACRG	850633 with 850686	biogriddb
DYN2_HUMAN	A0A809N0C2_LACRG	850633 with 850686	biogriddb
DYN2_HUMAN	A0A7S7FPQ6_LACRG	853870 with 850633	biogriddb
DYN2_HUMAN	A0A809N0C2_LACRG	853870 with 850633	biogriddb
DYN3_HUMAN	A0A809MXA9_LACRG	853870 with 851221	biogriddb
DYN3_HUMAN	A0A809MXA9_LACRG	851221 with 853870	biogriddb
DYN3_HUMAN	A0A7S7FPQ6_LACRG	850633 with 850686	biogriddb
DYN3_HUMAN	A0A809N0C2_LACRG	850633 with 850686	biogriddb
DYN3_HUMAN	A0A7S7FPQ6_LACRG	853870 with 850633	biogriddb
DYN3_HUMAN	A0A809N0C2_LACRG	853870 with 850633	biogriddb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
ECSIT_HUMAN	A0A7S7FPR2_LACRG	A2RTX5 with Q9BQ95	intactdb
ECSIT_HUMAN	A0A7S7FPR2_LACRG	9606.ENSP00000338093 with 9606.ENSP00000270517	stringdb
ECSIT_HUMAN	A0A7S7FPR2_LACRG	9606.ENSP00000270517 with 9606.ENSP00000338093	stringdb
ENPL_HUMAN	A0A809N7A1_LACRG	3320 with 6196	biogriddb
ENPL_HUMAN	A0A809N5R9_LACRG	3320 with 7407	biogriddb
ENPL_HUMAN	A0A7S7FQ03_LACRG	P08238 with Q9NP81	intactdb
ENPL_HUMAN	A0A809N7A1_LACRG	9606.ENSP00000360609 with 9606.ENSP00000360290	stringdb
ENPL_HUMAN	A0A809N7A1_LACRG	9606.ENSP00000360290 with 9606.ENSP00000360609	stringdb
FIBB_HUMAN	A0A809MXA9_LACRG	2244 with 1491	biogriddb
FOS_HUMAN	A0A809N5R9_LACRG	P01100 with P26640	mintdb
FOS_HUMAN	A0A809N5R9_LACRG	2353 with 7407	biogriddb
FOS_HUMAN	A0A809N5R9_LACRG	P01100 with P26640	intactdb
FOS_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000364815 with 9606.ENSP00000264716	stringdb
FOS_HUMAN	A0A809N7A1_LACRG	9606.ENSP00000360290 with 9606.ENSP0000036245	stringdb
FOS_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000364815 with 9606.ENSP0000036245	stringdb
FOS_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000441000 with 9606.ENSP0000036245	stringdb
FOS_HUMAN	A0A809N7A1_LACRG	9606.ENSP00000360290 with 9606.ENSP00000264716	stringdb
FOS_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000364815 with 9606.ENSP0000036245	stringdb
FOS_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000364815 with 9606.ENSP00000441000	stringdb
IKKE_HUMAN	A0A7S7FPR2_LACRG	P26639 with Q14164	intactdb
ITB2_HUMAN	A0A809NDK7_LACRG	P16144 with A0A0H2W8G4	hpdb
JUN_HUMAN	A0A7S7FPQ6_LACRG	P05412 with A0A0J1HLL8	hpdb
JUN_HUMAN	A0A809N0C2_LACRG	P05412 with A0A0J1HLL8	hpdb
JUN_HUMAN	A0A7S7FPR2_LACRG	P05412 with A2RTX5	mintdb
JUN_HUMAN	A0A7S7FPR2_LACRG	P05412 with P26639	mintdb
JUN_HUMAN	A0A809N5R9_LACRG	P05412 with P26640	mintdb
JUN_HUMAN	A0A809MUY0_LACRG	P05412 with P55786	mintdb
JUN_HUMAN	A0A809NE16_LACRG	P05412 with Q6IAN0	mintdb
JUN_HUMAN	A0A7S7FQ03_LACRG	P05412 with P49591	mintdb
JUN_HUMAN	A0A7S7FQ03_LACRG	P05412 with Q9NP81	mintdb
JUN_HUMAN	A0A7S7FPR2_LACRG	P05412 with Q9BW92	mintdb
JUN_HUMAN	A0A809N5R9_LACRG	3725 with 7407	biogriddb
JUN_HUMAN	A0A7S7FPR2_LACRG	P05412 with A2RTX5	intactdb
JUN_HUMAN	A0A7S7FPR2_LACRG	P05412 with P26639	intactdb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
JUN_HUMAN	A0A809N5R9_LACRG	P05412 with P26640	intactdb
JUN_HUMAN	A0A7S7FQ03_LACRG	P05412 with P49591	intactdb
JUN_HUMAN	A0A809MUY0_LACRG	P05412 with P55786	intactdb
JUN_HUMAN	A0A809NE16_LACRG	P05412 with Q6IAN0	intactdb
JUN_HUMAN	A0A7S7FPQ6_LACRG	P05412 with Q81VV9	intactdb
JUN_HUMAN	A0A809N0C2_LACRG	P05412 with Q81VV9	intactdb
JUN_HUMAN	A0A7S7FPR2_LACRG	P05412 with Q9BW92	intactdb
JUN_HUMAN	A0A7S7FQ03_LACRG	P05412 with Q9NP81	intactdb
JUN_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000360266 with 9606.ENSP00000320324	stringdb
JUN_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000320324 with 9606.ENSP00000360266	stringdb
JUN_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000364815 with 9606.ENSP00000360266	stringdb
JUN_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000360266 with 9606.ENSP00000364815	stringdb
KBR1_HUMAN	A0A7S7FPQ6_LACRG	P33416 with P01119	intactdb
KBR1_HUMAN	A0A809N0C2_LACRG	P33416 with P01119	intactdb
KS6A1_HUMAN	A0A809N7A1_LACRG	Q02156 with Q15349	mintdb
KS6A1_HUMAN	A0A809N7A1_LACRG	Q15349 with Q02156	mintdb
KS6A1_HUMAN	A0A809N7A1_LACRG	6196 with 6196	biogridb
KS6A1_HUMAN	A0A809MUY0_LACRG	9520 with 6195	biogridb
KS6A1_HUMAN	A0A809MUY0_LACRG	9520 with 6197	biogridb
KS6A1_HUMAN	A0A809N7A1_LACRG	6195 with 6196	biogridb
KS6A1_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000368884 with 9606.ENSP00000320324	stringdb
KS6A1_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000320324 with 9606.ENSP00000368884	stringdb
KS6A2_HUMAN	A0A809N7A1_LACRG	Q02156 with Q15349	mintdb
KS6A2_HUMAN	A0A809N7A1_LACRG	Q15349 with Q02156	mintdb
KS6A2_HUMAN	A0A809N7A1_LACRG	6196 with 6196	biogridb
KS6A2_HUMAN	A0A809MUY0_LACRG	9520 with 6195	biogridb
KS6A2_HUMAN	A0A809MUY0_LACRG	9520 with 6197	biogridb
KS6A2_HUMAN	A0A809N7A1_LACRG	6195 with 6196	biogridb
KS6A2_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000368884 with 9606.ENSP00000320324	stringdb
KS6A2_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000320324 with 9606.ENSP00000368884	stringdb
KS6A3_HUMAN	A0A809N7A1_LACRG	Q02156 with Q15349	mintdb
KS6A3_HUMAN	A0A809N7A1_LACRG	Q15349 with Q02156	mintdb
KS6A3_HUMAN	A0A809N7A1_LACRG	6196 with 6196	biogridb
KS6A3_HUMAN	A0A809MUY0_LACRG	9520 with 6195	biogridb
KS6A3_HUMAN	A0A809MUY0_LACRG	9520 with 6197	biogridb
KS6A3_HUMAN	A0A809N7A1_LACRG	6195 with 6196	biogridb
KS6A3_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000368884 with 9606.ENSP00000320324	stringdb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
KS6A3_HUMAN	A0A809MUY0_LACRG	9606.ENSP00000320324 with 9606.ENSP00000368884	stringdb
KS6A5_HUMAN	A0A809N7A1_LACRG	Q02156 with Q15349	mintdb
KS6A5_HUMAN	A0A809N7A1_LACRG	Q15349 with Q02156	mintdb
KS6A5_HUMAN	A0A809N7A1_LACRG	6196 with 6196	biogriddb
KS6A5_HUMAN	A0A809MUY0_LACRG	9520 with 6195	biogriddb
KS6A5_HUMAN	A0A809MUY0_LACRG	9520 with 6197	biogriddb
KS6A5_HUMAN	A0A809N7A1_LACRG	6195 with 6196	biogriddb
M3K7_HUMAN	A0A809N7A1_LACRG	150094 with 6885	biogriddb
M3K7_HUMAN	A0A809N7A1_LACRG	6885 with 150094	biogriddb
M3K7_HUMAN	A0A7S7FPQ6_LACRG	54101 with 81570	biogriddb
M3K7_HUMAN	A0A809N0C2_LACRG	54101 with 81570	biogriddb
M3K7_HUMAN	A0A809NE16_LACRG	54101 with 25979	biogriddb
M3K7_HUMAN	A0A7S7FQ03_LACRG	54101 with 6301	biogriddb
M3K7_HUMAN	A0A7S7FPR2_LACRG	54101 with 6897	biogriddb
M3K7_HUMAN	A0A809N7A1_LACRG	10090.ENSMUSP00000040307 with 10090.ENSMUSP00000030243	stringdb
M3K7_HUMAN	A0A809N7A1_LACRG	10090.ENSMUSP00000030243 with 10090.ENSMUSP00000040307	stringdb
M3K7_HUMAN	A0A809N7A1_LACRG	10090.ENSMUSP00000063166 with 10090.ENSMUSP00000040307	stringdb
M3K7_HUMAN	A0A809N7A1_LACRG	10090.ENSMUSP00000040307 with 10090.ENSMUSP00000063166	stringdb
MK01_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	mintdb
MK01_HUMAN	A0A809N7A1_LACRG	5595 with 6196	biogriddb
MK01_HUMAN	A0A809N7A1_LACRG	5594 with 6196	biogriddb
MK01_HUMAN	A0A7S7FPQ6_LACRG	817052 with 823706	biogriddb
MK01_HUMAN	A0A809N0C2_LACRG	817052 with 823706	biogriddb
MK01_HUMAN	A0A7S7FPQ6_LACRG	817052 with 828151	biogriddb
MK01_HUMAN	A0A809N0C2_LACRG	817052 with 828151	biogriddb
MK01_HUMAN	A0A7S7FPQ6_LACRG	817052 with 826735	biogriddb
MK01_HUMAN	A0A809N0C2_LACRG	817052 with 826735	biogriddb
MK01_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	intactdb
MK01_HUMAN	A0A809N5R9_LACRG	10116.ENSRNOP00000026627 with 10116.ENSRNOP00000001160	stringdb
MK01_HUMAN	A0A809N5R9_LACRG	10116.ENSRNOP00000001160 with 10116.ENSRNOP00000026627	stringdb
MK01_HUMAN	A0A809N5R9_LACRG	10116.ENSRNOP00000046565 with 10116.ENSRNOP00000026627	stringdb
MK01_HUMAN	A0A809N5R9_LACRG	10116.ENSRNOP00000026627 with 10116.ENSRNOP00000046565	stringdb
MK01_HUMAN	A0A7S7FN24_LACRG	9606.ENSP00000377192 with 9606.ENSP00000263025	stringdb
MK01_HUMAN	A0A7S7FN24_LACRG	9606.ENSP00000263025 with 9606.ENSP00000377192	stringdb
MK03_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	mintdb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
MK03_HUMAN	A0A809N7A1_LACRG	5595 with 6196	biogriddb
MK03_HUMAN	A0A809N7A1_LACRG	5594 with 6196	biogriddb
MK03_HUMAN	A0A7S7FPQ6_LACRG	817052 with 823706	biogriddb
MK03_HUMAN	A0A809N0C2_LACRG	817052 with 823706	biogriddb
MK03_HUMAN	A0A7S7FPQ6_LACRG	817052 with 828151	biogriddb
MK03_HUMAN	A0A809N0C2_LACRG	817052 with 828151	biogriddb
MK03_HUMAN	A0A7S7FPQ6_LACRG	817052 with 826735	biogriddb
MK03_HUMAN	A0A809N0C2_LACRG	817052 with 826735	biogriddb
MK03_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	intactdb
MK03_HUMAN	A0A809N5R9_LACRG	10116.ENSARNOP00000026627 with 10116.ENSARNOP00000001160	stringdb
MK03_HUMAN	A0A809N5R9_LACRG	10116.ENSARNOP00000001160 with 10116.ENSARNOP00000026627	stringdb
MK03_HUMAN	A0A809N5R9_LACRG	10116.ENSARNOP00000046565 with 10116.ENSARNOP00000026627	stringdb
MK03_HUMAN	A0A809N5R9_LACRG	10116.ENSARNOP00000026627 with 10116.ENSARNOP00000046565	stringdb
MK03_HUMAN	A0A7S7FN24_LACRG	9606.ENSPO00000377192 with 9606.ENSPO00000263025	stringdb
MK03_HUMAN	A0A7S7FN24_LACRG	9606.ENSPO00000263025 with 9606.ENSPO00000377192	stringdb
MK08_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	mintdb
MK08_HUMAN	A0A809N7A1_LACRG	5594 with 6196	biogriddb
MK08_HUMAN	A0A809N5R9_LACRG	5600 with 7407	biogriddb
MK08_HUMAN	A0A7S7FPQ6_LACRG	817052 with 826735	biogriddb
MK08_HUMAN	A0A809N0C2_LACRG	817052 with 826735	biogriddb
MK09_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	mintdb
MK09_HUMAN	A0A7S7FNR2_LACRG	2541652 with 2542710	biogriddb
MK09_HUMAN	A0A809N5R9_LACRG	5600 with 7407	biogriddb
MK09_HUMAN	A0A7S7FPQ6_LACRG	817052 with 828151	biogriddb
MK09_HUMAN	A0A809N0C2_LACRG	817052 with 828151	biogriddb
MK09_HUMAN	A0A7S7FPQ6_LACRG	817052 with 826735	biogriddb
MK09_HUMAN	A0A809N0C2_LACRG	817052 with 826735	biogriddb
MK10_HUMAN	A0A809N5R9_LACRG	P21708 with Q04462	mintdb
MK10_HUMAN	A0A809N7A1_LACRG	5594 with 6196	biogriddb
MK10_HUMAN	A0A809N5R9_LACRG	5600 with 7407	biogriddb
MK11_HUMAN	A0A7S7FPR2_LACRG	850803 with 853640	biogriddb
MK11_HUMAN	A0A7S7FNR2_LACRG	2541652 with 2542710	biogriddb
MK11_HUMAN	A0A809N5R9_LACRG	5600 with 7407	biogriddb
MK11_HUMAN	A0A7S7FPQ6_LACRG	850633 with 850803	biogriddb
MK11_HUMAN	A0A809N0C2_LACRG	850633 with 850803	biogriddb
MK11_HUMAN	A0A7S7FN24_LACRG	855480 with 850803	biogriddb
MK11_HUMAN	A0A809N5R9_LACRG	850803 with 852986	biogriddb
MK11_HUMAN	A0A7S7FQ03_LACRG	850803 with 851587	biogriddb
MK11_HUMAN	A0A7S7FPQ6_LACRG	817052 with 823706	biogriddb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
MK11_HUMAN	A0A809N0C2_LACRG	817052 with 823706	biogriddb
MK11_HUMAN	A0A7S7FPQ6_LACRG	817052 with 828151	biogriddb
MK11_HUMAN	A0A809N0C2_LACRG	817052 with 828151	biogriddb
MK11_HUMAN	A0A7S7FPQ6_LACRG	817052 with 826735	biogriddb
MK11_HUMAN	A0A809N0C2_LACRG	817052 with 826735	biogriddb
MK11_HUMAN	A0A809N5R9_LACRG	Q15759 with P26640	intactdb
MK11_HUMAN	A0A809N7A1_LACRG	4932.YLR113W with 4932.YDR477W	stringdb
MK11_HUMAN	A0A809N5R9_LACRG	4932.YLR113W with 4932.YGR094W	stringdb
MK11_HUMAN	A0A809N7A1_LACRG	4932.YDR477W with 4932.YLR113W	stringdb
MK11_HUMAN	A0A809N5R9_LACRG	4932.YGR094W with 4932.YLR113W	stringdb
MK11_HUMAN	A0A7S7FN24_LACRG	4932.YNL241C with 4932.YLR113W	stringdb
MK11_HUMAN	A0A7S7FN24_LACRG	4932.YLR113W with 4932.YNL241C	stringdb
MK11_HUMAN	A0A809N7A1_LACRG	9606.ENSP00000262891 with 9606.ENSP00000229795	stringdb
MK11_HUMAN	A0A809N7A1_LACRG	9606.ENSP00000229795 with 9606.ENSP00000262891	stringdb
MK11_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000364815 with 9606.ENSP00000333685	stringdb
MK11_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000441000 with 9606.ENSP00000333685	stringdb
MK11_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000333685 with 9606.ENSP00000364815	stringdb
MK11_HUMAN	A0A809N5R9_LACRG	9606.ENSP00000333685 with 9606.ENSP00000441000	stringdb
MK14_HUMAN	A0A7S7FPR2_LACRG	850803 with 853640	biogriddb
MK14_HUMAN	A0A7S7FNR2_LACRG	2541652 with 2542710	biogriddb
MK14_HUMAN	A0A809N7A1_LACRG	5594 with 6196	biogriddb
MK14_HUMAN	A0A809N5R9_LACRG	5600 with 7407	biogriddb
MK14_HUMAN	A0A7S7FPQ6_LACRG	850633 with 850803	biogriddb
MK14_HUMAN	A0A809N0C2_LACRG	850633 with 850803	biogriddb
MK14_HUMAN	A0A7S7FN24_LACRG	855480 with 850803	biogriddb
MK14_HUMAN	A0A809N5R9_LACRG	850803 with 852986	biogriddb
MK14_HUMAN	A0A7S7FQ03_LACRG	850803 with 851587	biogriddb
MK14_HUMAN	A0A7S7FPQ6_LACRG	817052 with 823706	biogriddb
MK14_HUMAN	A0A809N0C2_LACRG	817052 with 823706	biogriddb
MK14_HUMAN	A0A7S7FPQ6_LACRG	817052 with 828151	biogriddb
MK14_HUMAN	A0A809N0C2_LACRG	817052 with 828151	biogriddb
MK14_HUMAN	A0A7S7FPQ6_LACRG	817052 with 826735	biogriddb
MK14_HUMAN	A0A809N0C2_LACRG	817052 with 826735	biogriddb
MK14_HUMAN	A0A809N5R9_LACRG	Q15759 with P26640	intactdb
MK14_HUMAN	A0A809N7A1_LACRG	4932.YLR113W with 4932.YDR477W	stringdb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
MK14_HUMAN	A0A809N5R9_LACRG	4932.YLR113W with 4932.YGR094W	stringdb
MK14_HUMAN	A0A809N7A1_LACRG	4932.YDR477W with 4932.YLR113W	stringdb
MK14_HUMAN	A0A809N5R9_LACRG	4932.YGR094W with 4932.YLR113W	stringdb
MK14_HUMAN	A0A7S7FN24_LACRG	4932.YNL241C with 4932.YLR113W	stringdb
MK14_HUMAN	A0A7S7FN24_LACRG	4932.YLR113W with 4932.YNL241C	stringdb
MK14_HUMAN	A0A809N7A1_LACRG	9606.ENSPO00000262891 with 9606.ENSPO00000229795	stringdb
MK14_HUMAN	A0A809N7A1_LACRG	9606.ENSPO00000229795 with 9606.ENSPO00000262891	stringdb
MK14_HUMAN	A0A809N5R9_LACRG	9606.ENSPO00000364815 with 9606.ENSPO00000333685	stringdb
MK14_HUMAN	A0A809N5R9_LACRG	9606.ENSPO00000441000 with 9606.ENSPO00000333685	stringdb
MK14_HUMAN	A0A809N5R9_LACRG	9606.ENSPO00000333685 with 9606.ENSPO00000364815	stringdb
MK14_HUMAN	A0A809N5R9_LACRG	9606.ENSPO00000333685 with 9606.ENSPO00000441000	stringdb
MP2K1_HUMAN	A0A7S7FNR2_LACRG	2540919 with 2542710	biogriddb
MP2K1_HUMAN	A0A809N7A1_LACRG	853313 with 855953	biogriddb
MP2K1_HUMAN	A0A7S7FN24_LACRG	855480 with 853313	biogriddb
MP2K1_HUMAN	A0A7S7FN24_LACRG	5604 with 2539	biogriddb
MP2K3_HUMAN	A0A809N7A1_LACRG	853313 with 855953	biogriddb
MP2K3_HUMAN	A0A7S7FN24_LACRG	855480 with 853313	biogriddb
MP2K3_HUMAN	A0A7S7FN24_LACRG	5604 with 2539	biogriddb
MP2K3_HUMAN	A0A809N7A1_LACRG	4932.YJL128C with 4932.YDR477W	stringdb
MP2K3_HUMAN	A0A809N7A1_LACRG	4932.YDR477W with 4932.YJL128C	stringdb
MP2K3_HUMAN	A0A7S7FN24_LACRG	4932.YNL241C with 4932.YJL128C	stringdb
MP2K3_HUMAN	A0A7S7FN24_LACRG	4932.YJL128C with 4932.YNL241C	stringdb
MP2K4_HUMAN	A0A809N7A1_LACRG	853313 with 855953	biogriddb
MP2K4_HUMAN	A0A7S7FN24_LACRG	855480 with 853313	biogriddb
MP2K4_HUMAN	A0A7S7FN24_LACRG	5604 with 2539	biogriddb
MP2K4_HUMAN	A0A809N7A1_LACRG	4932.YJL128C with 4932.YDR477W	stringdb
MP2K4_HUMAN	A0A809N7A1_LACRG	4932.YDR477W with 4932.YJL128C	stringdb
MP2K4_HUMAN	A0A7S7FN24_LACRG	4932.YNL241C with 4932.YJL128C	stringdb
MP2K4_HUMAN	A0A7S7FN24_LACRG	4932.YJL128C with 4932.YNL241C	stringdb
MP2K6_HUMAN	A0A809N7A1_LACRG	853313 with 855953	biogriddb
MP2K6_HUMAN	A0A7S7FN24_LACRG	855480 with 853313	biogriddb
MP2K6_HUMAN	A0A7S7FN24_LACRG	5604 with 2539	biogriddb
MP2K7_HUMAN	A0A7S7FN24_LACRG	5604 with 2539	biogriddb
NEMO_HUMAN	A0A809N5R9_LACRG	P26640 with Q9Y6K9	intactdb
NFKB1_HUMAN	A0A809NCU5_LACRG	P19838 with Q8CZJ8	hpiddb
NFKB1_HUMAN	A0A809NAH1_LACRG	P19838 with A0A1Q4M3L5	hpiddb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
NFKB1_HUMAN	A0A809MUY0_LACRG	P19838 with P55786	mintdb
NFKB1_HUMAN	A0A809N5R9_LACRG	P19838 with P26640	mintdb
NFKB1_HUMAN	A0A809NE16_LACRG	P19838 with Q6IAN0	mintdb
NFKB1_HUMAN	A0A809N5R9_LACRG	P19838 with P26640	intactdb
NFKB1_HUMAN	A0A809MUY0_LACRG	P19838 with P55786	intactdb
NFKB1_HUMAN	A0A809NE16_LACRG	P19838 with Q6IAN0	intactdb
NFKB1_HUMAN	A0A809NAH1_LACRG	P19838 with Q81T13	intactdb
NFKB1_HUMAN	A0A809NCU5_LACRG	P19838 with Q8CZJ8	intactdb
NFKB1_HUMAN	A0A809MUY0_LACRG	9606.ENSPO0000320324 with 9606.ENSPO0000226574	stringdb
NFKB1_HUMAN	A0A809N5R9_LACRG	9606.ENSPO0000364815 with 9606.ENSPO0000226574	stringdb
NFKB1_HUMAN	A0A809MUY0_LACRG	9606.ENSPO0000226574 with 9606.ENSPO0000320324	stringdb
NFKB1_HUMAN	A0A809N5R9_LACRG	9606.ENSPO0000226574 with 9606.ENSPO0000364815	stringdb
NFKB1_HUMAN	A0A809NCU5_LACRG	P19838 with Q8CZJ8	phisto
NFKB2_HUMAN	A0A809NCU5_LACRG	P19838 with Q8CZJ8	hpidb
NFKB2_HUMAN	A0A809NAH1_LACRG	P19838 with A0A1Q4M3L5	hpidb
NFKB2_HUMAN	A0A809MUY0_LACRG	P19838 with P55786	mintdb
NFKB2_HUMAN	A0A809N5R9_LACRG	P19838 with P26640	mintdb
NFKB2_HUMAN	A0A809NE16_LACRG	P19838 with Q6IAN0	mintdb
NFKB2_HUMAN	A0A809N5R9_LACRG	P19838 with P26640	intactdb
NFKB2_HUMAN	A0A809MUY0_LACRG	P19838 with P55786	intactdb
NFKB2_HUMAN	A0A809NE16_LACRG	P19838 with Q6IAN0	intactdb
NFKB2_HUMAN	A0A809NAH1_LACRG	P19838 with Q81T13	intactdb
NFKB2_HUMAN	A0A809NCU5_LACRG	P19838 with Q8CZJ8	intactdb
NFKB2_HUMAN	A0A809MUY0_LACRG	9606.ENSPO0000320324 with 9606.ENSPO0000226574	stringdb
NFKB2_HUMAN	A0A809N5R9_LACRG	9606.ENSPO0000364815 with 9606.ENSPO0000226574	stringdb
NFKB2_HUMAN	A0A809MUY0_LACRG	9606.ENSPO0000226574 with 9606.ENSPO0000320324	stringdb
NFKB2_HUMAN	A0A809N5R9_LACRG	9606.ENSPO0000226574 with 9606.ENSPO0000364815	stringdb
NFKB2_HUMAN	A0A809NCU5_LACRG	P19838 with Q8CZJ8	phisto
P53_HUMAN	A0A7S7FQ03_LACRG	P04637 with Q9NP81	mintdb
P53_HUMAN	A0A7S7FQ03_LACRG	9606.ENSPO0000472847 with 9606.ENSPO0000269305	stringdb
P53_HUMAN	A0A7S7FQ03_LACRG	9606.ENSPO0000269305 with 9606.ENSPO0000472847	stringdb
PP2AA_HUMAN	A0A7S7FP57_LACRG	P62140 with A0A384L3E0	hpidb
PP2AA_HUMAN	A0A7S7FQ03_LACRG	P38705 with P32345	dipdb
PP2AA_HUMAN	A0A7S7FNR2_LACRG	P20604 with P36013	dipdb
PP2AB_HUMAN	A0A7S7FP57_LACRG	P62140 with A0A384L3E0	hpidb
PP2AB_HUMAN	A0A7S7FQ03_LACRG	P38705 with P32345	dipdb
PP2AB_HUMAN	A0A7S7FNR2_LACRG	P20604 with P36013	dipdb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
PTN11_HUMAN	A0A809N5R9_LACRG	5777 with 7407	biogriddb
RL40_HUMAN	A0A7S7FPR2_LACRG	854732 with 853969	biogriddb
RS27A_HUMAN	A0A7S7FPR2_LACRG	6233 with 6897	biogriddb
RS27A_HUMAN	A0A7S7FPQ6_LACRG	850633 with 850864	biogriddb
RS27A_HUMAN	A0A809N0C2_LACRG	850633 with 850864	biogriddb
RS27A_HUMAN	A0A7S7FPR2_LACRG	9606.ENSP00000387710 with 9606.ENSP00000272317	stringdb
RS27A_HUMAN	A0A7S7FPR2_LACRG	9606.ENSP00000272317 with 9606.ENSP00000387710	stringdb
SKP1_HUMAN	A0A7S7FN24_LACRG	O00091 with Q9Y709	mintdb
SKP1_HUMAN	A0A7S7FN24_LACRG	2540917 with 2543200	biogriddb
SKP1_HUMAN	A0A7S7FN24_LACRG	O00091 with Q9Y709	intactdb
TAB2_HUMAN	A0A809N7A1_LACRG	150094 with 23118	biogriddb
TAB2_HUMAN	A0A809N7A1_LACRG	23118 with 150094	biogriddb
TAB3_HUMAN	A0A809N7A1_LACRG	150094 with 23118	biogriddb
TAB3_HUMAN	A0A809N7A1_LACRG	23118 with 150094	biogriddb
TBK1_HUMAN	A0A7S7FPR2_LACRG	P26639 with Q14164	intactdb
TCAM2_HUMAN	A0A809N7A1_LACRG	Q02156 with Q86XR7	dipdb
TRAF6_HUMAN	A0A809N7A1_LACRG	Q9Y4K3 with O75385	dipdb
UB2D1_HUMAN	A0A809NE16_LACRG	Q02207 with P14682	mintdb
UB2D1_HUMAN	A0A809NE16_LACRG	P14682 with Q02207	mintdb
UB2D1_HUMAN	A0A809N7A1_LACRG	855953 with 852376	biogriddb
UB2D2_HUMAN	A0A809N7A1_LACRG	855953 with 852376	biogriddb
UB2D3_HUMAN	A0A809N7A1_LACRG	855953 with 852376	biogriddb
UB2V1_HUMAN	A0A7S7FN24_LACRG	7335 with 2539	biogriddb
UB2V1_HUMAN	A0A7S7FPQ6_LACRG	P33416 with P53152	intactdb
UB2V1_HUMAN	A0A809N0C2_LACRG	P33416 with P53152	intactdb
UB2V1_HUMAN	A0A7S7FN24_LACRG	9606.ENSP00000377192 with 9606.ENSP00000340305	stringdb
UB2V1_HUMAN	A0A7S7FN24_LACRG	9606.ENSP00000340305 with 9606.ENSP00000377192	stringdb
UBB_HUMAN	A0A7S7FQ03_LACRG	7316 with 851587	hpdb
UBB_HUMAN	A0A809MXA9_LACRG	7316 with 851010	hpdb
UBB_HUMAN	A0A809N5R9_LACRG	7316 with 852986	hpdb
UBB_HUMAN	A0A7S7FPR2_LACRG	7316 with 854732	hpdb
UBB_HUMAN	A0A7S7FPQ6_LACRG	7316 with 850633	hpdb
UBB_HUMAN	A0A809N0C2_LACRG	7316 with 850633	hpdb
UBB_HUMAN	A0A809MXA9_LACRG	7316 with 851221	hpdb
UBC_HUMAN	A0A7S7FQ03_LACRG	7316 with 851587	hpdb
UBC_HUMAN	A0A809MXA9_LACRG	7316 with 851010	hpdb
UBC_HUMAN	A0A809N5R9_LACRG	7316 with 852986	hpdb
UBC_HUMAN	A0A7S7FPR2_LACRG	7316 with 854732	hpdb
UBC_HUMAN	A0A7S7FPQ6_LACRG	7316 with 850633	hpdb
UBC_HUMAN	A0A809N0C2_LACRG	7316 with 850633	hpdb

Host Protein	Pathogen Protein	Homologous Proteins Interacting	Protein-Protein Database
UBC_HUMAN	A0A809MXA9_LACRG	7316 with 851221	hpiddb
UBE2N_HUMAN	A0A809N7A1_LACRG	855953 with 852376	biogriddb
UBP14_HUMAN	A0A7S7FPQ6_LACRG	P33416 with P43593	intactdb
UBP14_HUMAN	A0A809N0C2_LACRG	P33416 with P43593	intactdb

References

1. Wisniewski, J. R., Zougman, A., Nagaraj, N. & Mann, M. (2009) Universal sample preparation method for proteome analysis, *Nat Methods*. **6**, 359-62.
2. Demichev, V., Messner, C. B., Vernardis, S. I., Lilley, K. S. & Ralser, M. (2020) DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput, *Nat Methods*. **17**, 41-44.
3. Milacic, M., Beavers, D., Conley, P., Gong, C., Gillespie, M., Griss, J., Haw, R., Jassal, B., Matthews, L., May, B., Petryszak, R., Ragueneau, E., Rothfels, K., Sevilla, C., Shamovsky, V., Stephan, R., Tiwari, K., Varusai, T., Weiser, J., Wright, A., Wu, G., Stein, L., Hermjakob, H. & D'Eustachio, P. (2024) The Reactome Pathway Knowledgebase 2024, *Nucleic Acids Res*. **52**, D672-D678.
4. Loaiza, C. D. & Kaundal, R. (2021) PredHPI: an integrated web server platform for the detection and visualization of host-pathogen interactions using sequence-based methods, *Bioinformatics*. **37**, 622-624.