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Effects of Dietary Zinc and/or an Herbal Mixture on Intestinal Microbiota and Barrier Integrity in Lambs

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Abstract: The purpose of this experiment was to determine the impact of feed supplementation with organic zinc and/or a medicinal plants mixture on the composition and enzymatic activity of intestinal microflora as well as on the duodenal and jejunal barrier integrity in lambs. A total of 28 lambs were randomly allocated into 4 dietary treatments (n = 7) and were fed an unsupplemented basal diet (BD), or the BD enriched with organic Zn (Zn, 70 mg/kg diet), an herbal mixture (Herbmix, 100 g/day) or a combination of both additives (Zn+Herbmix). The Herbmix contained 33% each of *Fumaria officinalis*, *Malva sylvestris*, *Matricaria chamomilla* and 1% *Artemisia absinthium*. No significant effect on the fecal microbiota composition was observed due to the 35-day or 70-day dietary treatment. The intake of Zn alone resulted in decreased bacterial enzyme activities, such as β-glucuronidase, N-acetyl-glucosaminidase, β-galactosidase and β-glucosidase. The transepithelial electrical resistance of the small intestinal mucosa was not influenced by the dietary treatment, whereas simultaneous feeding of Zn and Herbmix exhibited higher claudin-1 and occludin levels in the jejunal mucosa. These results indicate that dietary intake of organic zinc and/or medicinal plants in the mentioned dosage did not alter the diversity of intestinal bacteria in growing lambs but did significantly influence bacterial enzyme activity. Supplementing the zinc and herbs combination showed the potential to regulate intestinal permeability by increasing the level of tight junction proteins in the jejunal mucosa.

Keywords: zinc; medicinal herbs; gut bacteria; gut integrity; sheep

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1. Introduction

Dietary zinc and medicinal plants belong among the nutraceuticals that provide a wide spectrum of nutritional and health benefits to the animals, including immune system enhancement, antioxidant protection and anti-inflammatory and antimicrobial activity [1,2]. Various dietary factors and sources of zinc in the feed influence the intestinal absorption and bioavailability of zinc [3], which is linked with the growing use of organic mineral sources in the feeding strategy [4]. The study of Reed et al. [5] showed that chronic zinc deficiency conditions are associated with the alteration in the gastrointestinal (GI) microbiota of the host, impaired intestinal permeability, increased intestinal inflammation and decreased GI health. Plant bioactive substances have shown promising perspectives to suppress growth of pathogenic bacteria in the gut, to improve nutrient digestion and absorption and to modulate rumen fermentation parameters in livestock animals [6]. Some evidence suggests that polyphenolic compounds, mainly flavonoids (e.g., naringin and quercetin) and tannins included in the diet of ruminants, can affect the ruminal microbial population towards a lower production of methane emissions [7].

Characterization of the GI microbiome of sheep has revealed the most dominant phyla represented by Firmicutes and Bacteroidetes, which may account from 80 to 90% of the entire gastrointestinal microbiota, followed by the phyla Proteobacteria, Verrucomicrobia and Actinobacteria [8,9]. Nutraceuticals, such as zinc and various herbs, have the potential to regulate gut microbial communities that exhibit a variety of enzymatic reactions involved in the metabolism of dietary and endogenous compounds, either with a positive or negative impact on animal/human health, and this may in turn lead to changes in bacterial metabolic activities [10,11]. For example, some microbial compounds can negatively affect mucus reduction and the release of carcinogens and toxins [12,13]. Therefore, the regulation of some fecal isolates related to *Clostridium* spp. and *Bacteroides* spp. as the highest producers of β-glucuronidase and β-glucosidase activity could contribute to reducing the risk of carcinogenesis. However, bacteria may also have a positive effect on the host, e.g., *Escherichia coli*, which can increase the bioavailability of phosphorus through the enzymatic activity of alkaline phosphatase and phytase [14]. For this reason, the proper modulation of gut microbiota and its related enzymatic activity is a possible way to improve gut wall integrity and thereby gut health [15].

A functional and intact gut barrier allows the absorption of nutrients and fluids but also helps to protect the intestinal mucosa from toxins, allergens or pathogens and is a crucial factor in maintaining homeostasis between the immune system and gut commensal bacteria [16]. Intestinal microbiota can alter tight junction (TJ) protein gene expression and thereby affect gastrointestinal barrier function [17]. Zinc is required for the structure and function of the intestinal mucosal barrier and regeneration of injured gut epithelium [18,19]. Several studies have reported that Zn intake affects intestinal permeability by increasing tight junction-related protein expressions that lead to improvement of intestinal barrier integrity and may also indicate a repair mechanism in monolayer epithelial cells [18–20]. Bioactive phytochemicals have been shown to inhibit the growth of pathogenic bacteria in the intestine and to play a vital role in maintaining epithelial integrity in the gastrointestinal tract [17]. The feeding of various plant bioactive compounds can improve the intestinal microflora by increasing the abundance of beneficial microorganisms; thus, affecting the gastrointestinal barrier functions via regulating the expression of TJ proteins associated with the integrity of gut epithelium in cells as well as in animal experiments [17,21].

The importance of the rumen microbial ecosystem is well established, but modulation of the gut microbiome in ruminants has received far less attention despite its significant influence on overall health, the immune system, carbohydrate metabolism, nutrient absorption and optimal performance [22]. Recently, it has been observed that supplementing sheep diets with organic Zn and an herbal mixture did not influence the ruminal fermentation or the protozoal population in lambs; however, it caused a shift in the relative abundance of cellulolytic and amylolytic bacteria and simultaneously lower total bacteria were observed in animals fed a Zn-enriched diet [23]. Data relating to gut microbial community alterations and bacterial enzymatic activity in ruminants with prolonged feeding (during a 70-day period) of diets supplemented with zinc or herbal nutraceuticals are currently lacking. We hypothesized that the intake of organic zinc and/or a special herbal mixture could affect the gut bacterial population and their enzymatic activity, which may have an impact on the integrity of the small intestine. Therefore, our goal was to explore the effect of dietary zinc from an organic source and an herbal mixture given alone or together for varying durations (35 or 70 days) on the fecal microbiota composition in sheep. Moreover, intestinal barrier integrity was also evaluated by measuring transepithelial electrical resistance (TEER) and TJ protein levels in the small intestine mucosa.

2. Materials and Methods

2.1. Ethical Statement

The experimental design of this study followed the European Community guidelines for the care and use of animals for scientific purposes (EU Directive 2010/63/EU). The experiment was carried out in accordance with the national and institutional standards

(Ethics Committee of the Institute of Animal Physiology of the Slovak Academy of Sciences; State Veterinary and Food Office of the Slovak Republic—resolution no. Ro-4065/18-221/3) for experiments with animals.

2.2. Animals and Experimental Design

Twenty-eight castrated male lambs of the Improved Valachian breed (4 months old) were individually kept in stalls for 30 d to adapt to the diet and environment, with free access to drinking water. The lambs were fed with an equal basal diet (BD) composed of 350 g/day of ground barley and 700 g/day of meadow hay. After the adaptation period, all lambs, aged five months and weighing an average of 22.6 ± 2.9 kg, were divided into one of four treatment groups (n = 7) in a completely randomized design. Dietary treatments included an unsupplemented control BD (Control), and the BD was further supplemented either with organic zinc (Zn) or an herbal mixture (Herbmix) or both (Zn+Herbmix). Aliquots of organic zinc Availa-Zn 100 EU (Zinpro Corporation, Eden Prairie, MN, USA) were directly mixed with the ground barley to provide an additional 70 mg Zn/kg into the diet. The herbal mixture consisted of 33% of *Fumaria officinalis* L. (stem), *Malva sylvestris* L. (flower), *Matricaria chamomilla* L. (flower) and 1% *Artemisia absinthium* L. (stem). The individual dry herbs were obtained from commercial sources (AGROKARPATY, Plavnica, Slovak Republic), subsequently mixed daily and offered to the lambs in the amount of 100 g DM/day/animal. Detailed chemical composition of dietary components was published by Petrič et al. [23].

Fecal samples for gut microbiota analysis were collected directly from the rectum on d 35 and d 70 of the experiment in sterile tubes for DNA extraction and stored at −80 °C until processed within 8 weeks of collection. After 70 days of dietary treatment, all animals from each group (n = 7) were humanely slaughtered (abattoir of the Centre of Biosciences of SAS, Institute of Animal Physiology, Košice, Slovakia, No. SK U 06018) according to European Commission rules (Council Regulation 1099/2009) for slaughtering procedures [24]. The fecal samples for bacterial enzyme activity determination were taken from the terminal part of the rectum. The middle sections of the duodenum and jejunum were excised to determine levels of tight junction proteins and approximately 1.5 cm segments of both intestinal parts were immediately separated to measure TEER ex vivo.

2.3. Intestinal Integrity Evaluation

Intestinal integrity was assessed by monitoring the TEER of the duodenal and jejunal tissue in an Ussing chamber system [25,26]. Intestinal sections (0.71 cm^2) were inserted into the chambers with Tyroide's solution and were incubated at 37 °C in 95% O_2 and 5% CO_2. TEER was measured with electrodes using a volt ohmmeter (MXD-5040RS232 Digital Multimeter with True RMS, METEX Instruments, Korea), and its values were monitored at 3 min intervals over a period of 12 min.

The TJ protein level of claudin-1 and -3 (CLDN-1,-3), zonula occludens-1 (ZO-1) and occludin (OCLN) were determined using a commercially available sheep enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource Inc., San Diego, CA, USA). Therefore, the small intestine was cut longitudinally, and the mucosa was carefully scraped using a glass slide on ice and subsequently homogenized in an ice-cold phosphate buffer (pH 7.2) to make a 10% homogenate (w/v). After centrifugation at 1000× g for 20 min at 4 °C, the supernatants were separated, and the samples were processed on a sandwich ELISA plate according to the manufacturer's protocol using an Apollo 11 LB913 ELISA absorbance reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The sensitivity of the ELISA kits was 0.1 ng/mL All samples were analyzed in duplicate, and the values of TJ abundance were expressed as ng per g tissue protein. The mucosal protein level was measured according to the method of Bradford [27].

2.4. Analyses of Bacterial Enzyme Activities

The activity of various bacterial enzymes in feces (collected on d 70 of treatment) was determined using the APIZYM kit (bioMérieux, Marcy-l'Étoile, France) manufacturer's manual. The activities of the 19 enzymes present in the kit were tested. Freshly collected fecal samples (0.1 g) were dissolved in saline (2 mL) and centrifuged for 10 min at 550× *g* to remove debris and were subsequently inoculated (65 µL to each cup) into an APIZYM strip. After 4 h of incubation at 37 °C, Zym A and Zym B reagents were added, and the enzyme activities were read. Color intensity values from 0 to 5 were assigned for each reaction according to the color scale included in this kit.

2.5. Microbiota Composition

The microbial population in feces was determined by sequencing the hypervariable region V4 of the bacterial 16S rRNA gene. Ovine feces ($n = 7$/group) were diluted 5× with molecular-grade water and homogenized. Then, the homogenized feces (260 µL) were used for DNA isolation performed using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

The isolated DNA was used as a template in a polymerase chain reaction (PCR) targeting the hypervariable V4 region (515F–806R) of the bacterial 16S rRNA gene (16S Metagenomic sequencing Library Preparation protocol; Illumina, San Diego, CA, USA). The detailed sequences of primer pairs are shown in Table S1. Sequencing was performed with the MiSeq Reagent Kit v2 using a MiSeq 2000 instrument according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

2.6. Bioinformatics and Data Analysis

Initial processing of paired reads was performed using Python 3's in-house pipeline. This process involved several steps including trimming poor quality 3' end reads, removing read pairs containing unspecified N bases and discarding pairs with very short reads. To minimize potential errors from PCR and sequencing, forward and reverse reads were subjected to denoising using the Divisive Amplicon Denoising Algorithm 2 (DADA2) amplicon denoising R package [28]. After denoising, the forward and reverse reads were merged into a single extended read using the fastq-join read merging tool [29]. In a final step, all chimeric sequences were removed from the merged reads using the remove Bimera function of the DADA2 R package. Subsequent taxonomic assignments were performed using the UCLUST consensus method within the Quantitative Insights Into Microbial Ecology (QIIME) analysis [30] using the Silva v. 132 reference database [31].

In this study, microbial sequencing data were filtered to include only samples with a minimum of 5000 reads for analysis. Then, filtration of taxa was performed, retaining only those with a relative abundance of at least 1% in at least three samples. To test differences in gut microbiota composition among the 4 groups following 35 and 70 days of dietary treatment, PERMANOVA (permutational multivariate analysis of variance) tests were conducted. In addition, differences among the alpha diversity indices were assessed using Kruskal–Wallis tests. All statistical analyses were conducted using the R programming language.

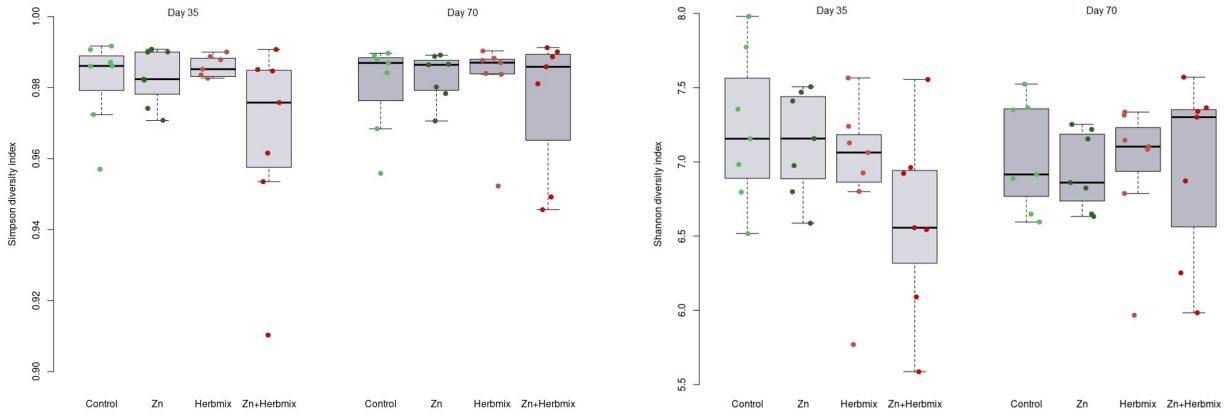
The data for the parameters of intestinal integrity and bacterial enzyme activity were statistically analyzed using the GraphPad Prism 8.4.2 software (GraphPad Software Inc., San Diego, CA, USA). The significant differences among treatments were assessed by one-way ANOVA followed by the Tukey post hoc test. Values were expressed as the means with pooled standard errors of means (SEM). The differences were considered significant at a p-value of <0.05.

3. Results

3.1. Microbiota Composition

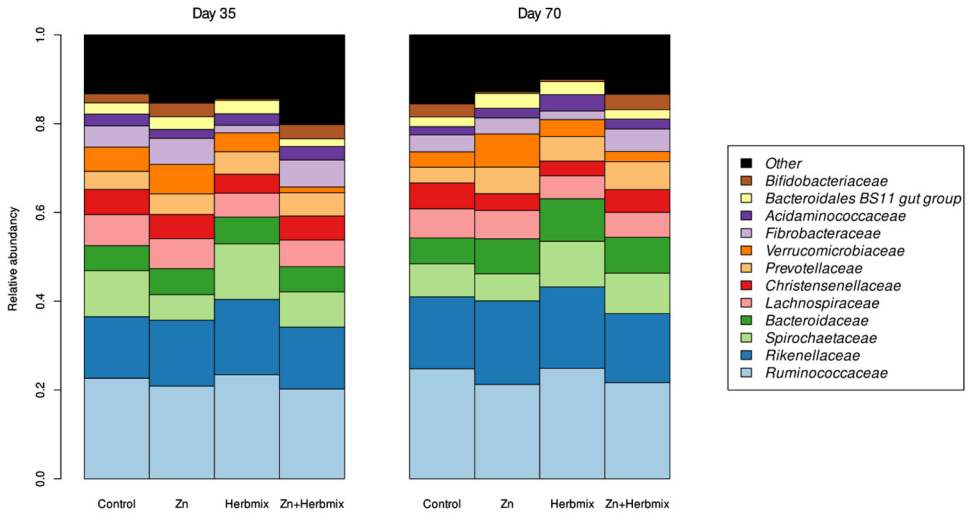
Diversities of the microbial communities were established from the amplicon sequence variants (ASVs) using both the Shannon diversity index and the Simpson diversity index. Neither index is significantly different among the groups, between different times or with

a combination of both factors (Kruskal–Wallis test, all p-values > 0.5). Boxplots of both indices (Figure 1) note higher variability in the Zn+Herbmix group. Barplots of the mean relative abundance are shown in Figure 2a (families) and Figure 2b (genera). Only taxa with a relative abundance of at least 2.5% are shown; the rest are aggregated in "other".



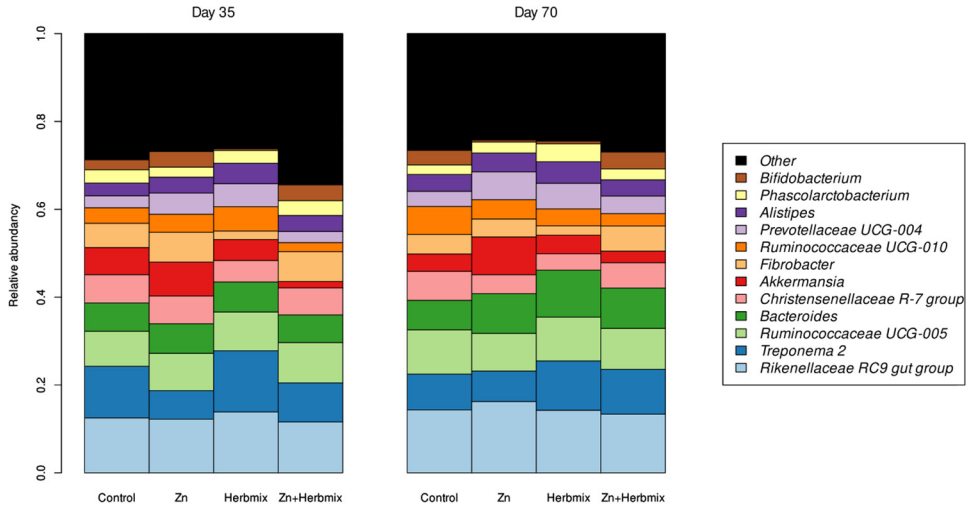
(**a**) (**b**)

Figure 1. Microbial α-diversity in the feces of sheep fed diets supplemented with Zn, Herbmix and Zn+Herbmix on d 35 and d 70 of treatment. (**a**) Simpson index, (**b**) Shannon index. The boxes represent the interquartile range (IQR), with the horizontal line inside representing the median. The whiskers indicate the minimum and maximum values within 1.5 times the IQR. The values for each sample are visualized as colored dots according to experimental groups.



(**a**)

Figure 2. *Cont.*



(**b**)

Figure 2. The microbial community structures (mean relative abundance) in feces of sheep on d 35 and d 70 of treatment, based on family (**a**) and genus (**b**) level.

For further analysis, a simple abundance filter was applied (separately on both genera and family taxonomic levels), as follows: only taxa with at least 0.5% relative abundance in at least 5 samples were kept. This filter kept ~96% of abundance while filtering out ~75% of genera (drop from 203 to 51 genera). On the family level, ~98.5% of the abundance was kept while filtering out ~64% of the families (drop from 81 to 29 families). The most abundant families (over 5%) in all the groups and on both sampling days were Ruminococcaceae (20–22% according to the experimental group at day 35 and 20–24% at day 70), Rikenellaceae (13–16% at day 35 and 15–18% at day 70), followed by Spirochetaceae (6–12% and 6–10%), Bacteroidaceae (5–6% and 5–9%), Lachnospiraceae (5–7% and 5–6%) and Christensellaceae (4–6% and 3–6%).

To test the difference in microbiome composition among the 4 groups, a series of PERMANOVA tests (for day 35, day 70, and without time as a factor; based on Bray–Curtis distance) was performed on both the genus and family taxonomic levels. Table 1 presents the *p*-values for the statistical tests conducted. It should be noted that due to multiple hypothesis testing, a more stringent significance level of approximately 0.008 should be considered instead of the conventional threshold of 0.05.

Table 1. The results of PERMANOVA tests conducted using the Bray–Curtis distance metric to compare microbial community composition at the genus and family taxonomic levels are presented. These tests were performed for d 35 and d 70 time points, as well as for an analysis that did not consider time as a factor.

	Day 35	**Day 70**	**Both Times**
Genus	$p = 0.005$	$p = 0.023$	$p < 7 \times 10^{-6}$
Family	$p = 0.113$	$p = 0.017$	$p < 7.1 \times 10^{-5}$

3.2. The Activity of Bacterial Enzymes

Alteration of enzyme activities in the feces of lambs fed dietary Zn and Herbmix alone or in combination are presented in Table 2. The semi-quantitative determination by APIZYM revealed statistically significant differences in 5 out of 19 enzymes (namely: alkaline phosphatase, β-glucuronidase, β-glucosidase, N-acetyl-glucosaminidase and β-galactosidase). Intake of Zn alone influenced some of the monitored enzyme activities; more specifically, the activity of β-galactosidase, β-glucuronidase, β-glucosidase and N-acetyl-glucosaminidase was significantly lower compared to the Control group ($p < 0.05$). The inclusion of the Herbmix into the diet resulted in an elevated activity

of alkaline phosphatase ($p < 0.01$) and β-glucuronidase ($p < 0.05$) compared to the Zn group. The simultaneous treatment with Zn+Herbmix led to significantly lower fecal bacterial β-galactosidase activity compared to the Control group ($p < 0.05$) but higher N-acetyl-glucosaminidase activity compared to the Zn group ($p < 0.05$).

Table 2. Effect of organic zinc and/or herbal mixture on the enzyme activity in the feces of lambs (activity score index 0–5), $n = 7$.

Enzyme	Control	Zn	Herbmix	Zn+Herbmix	SEM	*p*-Value
Alkaline phosphatase	4.29 ^{ab}	3.57 ^{b}	4.86 ^{a}	4.14 ^{ab}	0.149	0.014
β-galactosidase	3.71 ^{a}	2.43 ^{b}	3.14 ^{ab}	2.29 ^{b}	0.181	0.009
β-glucuronidase	4.86 ^{a}	3.86 ^{b}	4.86 ^{a}	4.43 ^{ab}	0.131	0.012
β-glucosidase	4.43 ^{a}	3.00 ^{b}	4.00 ^{ab}	3.43 ^{ab}	0.162	0.004
N-acetyl-glucosaminidase	4.00 ^{a}	2.43 ^{b}	3.43 ^{ab}	3.71 ^{a}	0.188	0.011

^{a,b} Means with different superscript letters in a row are significantly different ($p < 0.05$) and were measured using one-way analysis of variance followed by a Tukey's post hoc test.

3.3. Intestinal Integrity

The TEER values measured over time increased during the first 9 min of intestinal incubation and were stable after this time. However, neither TEER of the duodenal nor the jejunal barrier was influenced by dietary treatment (Figure 3).

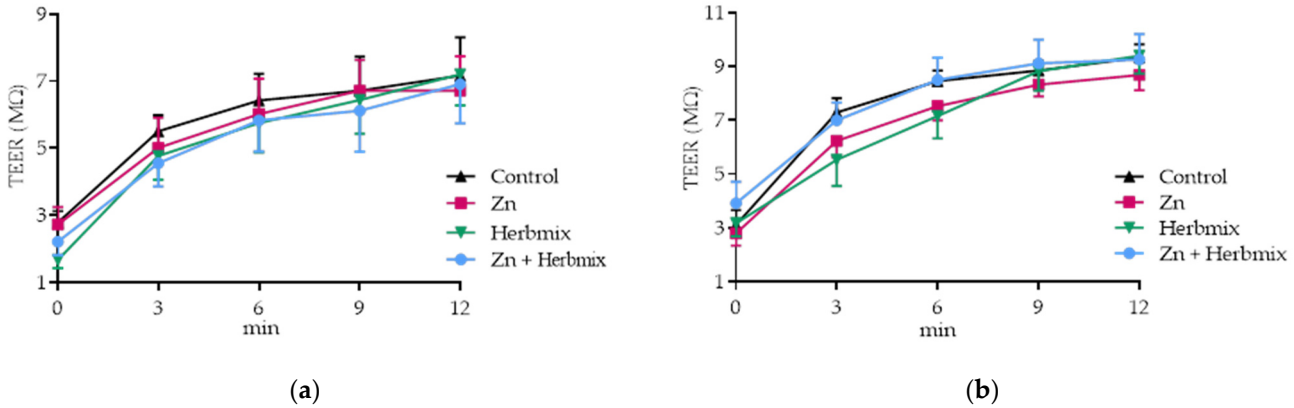


Figure 3. Transepithelial electrical resistance (TEER) values in duodenal (**a**) and jejunal (**b**) tissues of sheep, $n = 7$.

The effects of Zn and/or herbs on intestinal TJ protein levels are shown in Table 3. There was no effect of dietary treatment on duodenal TJ proteins concentration, while the intake of Zn in combination with medicinal herbs mixture significantly increased OCLN and CLDN-1 levels in jejunal mucosa compared to the Control group ($p < 0.05$). No significant effect on jejunal CLDN-3 and ZO-1 levels was recorded due to the treatment.

Table 3. Effect of organic zinc and/or herbal mixture on the concentration of selected tight junction proteins in the intestinal mucosa of lambs, $n = 7$.

Parameter	Control	Zn	Herbmix	Zn+Herbmix	SEM	*p*-Value
Duodenal mucosa						
OCLN (ng/g protein)	294.8	266.9	331.4	292.4	12.258	0.315
CLDN-1 (ng/g protein)	230.1	181.7	235.9	227.5	9.312	0.142
CLDN-3 (ng/g protein)	658.0	522.4	648.2	670.7	23.348	0.072
ZO-1 (ng/g protein)	254.4	182.7	199.0	260.0	12.554	0.055
Jejunal mucosa						
OCLN (ng/g protein)	280.8 ^{b}	297.3 ^{ab}	337.8 ^{ab}	452.9 ^{a}	23.161	0.034
CLDN-1 (ng/g protein)	221.0 ^{b}	239.2 ^{ab}	230.3 ^{ab}	315.1 ^{a}	12.548	0.021
CLDN-3 (ng/g protein)	581.8	606.7	546.3	631.8	27.576	0.748
ZO-1 (ng/g protein)	180.6	186.9	223.1	184.5	10.146	0.470

OCLN: occludin, CLDN-1: claudin-1, CLDN-3: claudin-3, ZO-1: zonula occludens-1. ^{a,b} Means with different superscript letters in a row are significantly different ($p < 0.05$) and were measured using one-way analysis of variance followed by a Tukey's post hoc test.

4. Discussion

The gut bacterial composition can be modified by various dietary components or feed supplementation [19,32]. One of them is dietary Zn, an important factor in the modulation of the microbiota community [10,33], related to competition for Zn among bacteria [34]. Analysis of microbial community diversity using Shannon and Simpson diversity indices did not reveal any significant differences among the groups, either between different time points or in the combined effect of both factors (Kruskal–Wallis test, all *p*-values > 0.5). Most data on the effect of dietary zinc on the intestinal microflora are obtained from studies of weaned piglets. The influence of dietary zinc on the gut microbiome of other animals is less established. Regarding ruminants, only a few experiments were performed, more specifically, in neonatal calves and lactating dairy cattle with a minimal effect on the diversity or richness of the gut microbiota, and in growing beef cattle with some changes in the relative abundance of certain phyla such as *Firmicutes*, *Actinobacteria* and *Tenericutes* [35]. Regarding the studies in non-ruminant animals, for example, Xia et al. [36] used zinc oxide nanoparticles in weaned piglets, and their results showed increased vs. decreased bacterial richness and diversity in the ileum vs. the cecum and colon. In contrast to these results, Barszcz et al. [14] demonstrated no effect of dietary zinc supplementation on bacterial population analyses in the large intestine of pigs. Some other existing information illustrates a close relationship between Zn metabolism and gut microbiota [19,36,37]. The frequent high heterogeneity of these findings may be related to the different biological impact of various chemical sources of zinc and, in addition, to the dose- and the host-species-specific response of the intestinal bacteria to this microelement [10]; further, the methodology used for bacterial quantification cannot be ignored.

Members of the gut microbiota exhibit a variety of metabolic reactions of structurally diverse endogenous/exogenous compounds with a potential impact on animal/human health. Thus, variation in the microbial population could also be responsible for alterations in enzyme activities; however, it appears that decreased activity of bacterial enzymes connected with colon pathogenesis (namely N-acetyl-glucosaminidase, β-glucuronidase and β-glucosidase) in this experiment, after the Zn diet was administered in the lambs, does not correlate with the results of the microbial population profiling. Analysis of the microbiome composition differences among the 4 groups on day 70, when enzymatic activity was monitored, showed *p*-values of 0.023 and 0.017 at the genus and family taxonomic levels, respectively (Table 1). However, it should be noted that multiple hypothesis testing was performed; therefore, a more stringent significance level of ~0.008 (rather than 0.05) should be considered. Nevertheless, we cannot conclude that statistically significant differences exist in the gut microbiome composition among the groups. However, there are studies describing the impact of metal ions (including Zn^{2+}) on bacterial lignocellulolytic enzyme

activities (including β-glucosidase) [38] and N-acetyl-glucosaminidase activity [39], either in terms of their activation or inhibition. In contrast to our results, Barszcz et al. [14] demonstrated no effect of Zn supplements ($ZnSO_4$ or Zn glycine chelate) on β-glucuronidase activity in the large intestine of pigs together with no effect on the relative abundance of bacteria that produce this enzyme, i.e., Bacteroides and *E. coli.*

Although no differences were observed in fecal bacterial richness or α-diversity estimated by the Shannon and Simpson index, supplementation of the diet with Zn could result in changes in the relative abundance of several genera. As Flores et al. [40] evaluated in their work, the activity of β-glucuronidase and β-glucosidase was directly connected with a higher abundance of particular genera, specifically with five *Clostridia* genera in the phylum Firmicutes, mainly with *Ruminococcaceae*, *Subdoligranulum* and non-*Clostridiales*. Their findings indicate that these considerable roles are predominantly carried out by specialist organisms rather than by the community as a whole. Another important cellulose and other carbohydrate degraders contributing to β-glucosidase and/or β-glucuronidase activity are the *Bifidobacterium* spp. population and Bacteroides [41,42]. Taking a look at the relative abundance of these populations, we can see changes in the proportion of these bacteria, which could contribute to the reduction in the aforementioned enzymatic activity. Another possible discrepancy in the explanation of the differences in microbiome composition and changes in enzymatic activities is the inability of DNA-based 16S rRNA gene sequencing for microbial community analyses to distinguish live (metabolically active) microorganisms and dead (metabolically inactive) bacteria [43].

Intestinal alkaline phosphatase (Zn-containing metalloenzyme) attenuates inflammation by modifying the gut microflora and dephosphorylating nucleotides and endotoxin of bacteria as lipopolysaccharides [44]. Therefore, any change in alkaline phosphatase levels and activity leads to an increase in inflammation susceptibility and the risk of colon cancer. When Zn^{2+} occupies all three metal-binding sites, alkaline phosphatase activity reaches its maximum. For this reason, Zn^{2+} concentrations in the normal range allow for the physiological function of alkaline phosphatase [45]. A previously published study provides evidence that a prolonged zinc-deficient diet or dietary supplementation of various zinc sources alters the activity of alkaline phosphatase [46]. In this experiment, no significant difference in fecal alkaline phosphatase activity was demonstrated in the Zn-fed lambs in comparison with the Control group. The *Bacteroides/Prevotella*, *Bifidobacterium* and *Eubacterium rectale/Clostridium coccoides* group, the *Atopobium* group and *Streptococcus/Lactococcus* and *Lactobacillus/Enterococcus* are considered to be the major producers of β-galactosidase [47], the enzyme that catalyzes the first step of lactose fermentation in the colon: the hydrolysis of lactose into glucose and galactose. Our results showed that fecal β-galactosidase activity was significantly reduced in both groups (Zn and Zn+Herbmix) supplemented with Zn compared to the Control group.

Other important nutraceuticals are secondary metabolites of plants with a key role in a wide range of biological and pharmacological properties, including antimicrobial activity [48]. The medicinal herbs used in our trial had high concentrations of flavonoids, especially quercetin, together with phenolic acids and alkaloids, as was previously described by Petrič et al. [23]. The application of some flavonoids with antimicrobial properties can influence the intestinal microbial population. For example, Lin et al. [49] found that dietary quercetin increased gut microbial diversity; more specifically, it enhanced the populations of *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Clostridia* and significantly reduced those of *Enterococcus* and *Fusobacterium*. Under our experimental condition, no significant differences in microbial diversity were observed after the application of Herbmix or Zn+Herbmix. Moreover, some of the flavonoids, including quercetin, silymarin and kaempferol, displayed strong to moderate in vitro and in vivo inhibitory effects against bacterial β-glucuronidase activity [50] as an important object to mitigate the toxicity or intestinal disturbance caused by the hydrolysis of β-D-glucuronides. Some of the flavonoids—luteolin, for example—have been reported as a chemopreventive and anticarcinogenic agent, plausibly by inhibiting bacterial β-glucuronidases-mediated enterohepatic

circulation of colonic carcinogens. However, no changes in this monitored fecal enzyme activity were revealed after Herbmix treatment in comparison to the Control group.

Fecal alkaline phosphatase excretion was determined as an indicator for intestinal damage in rats and recognized as a potent regulator of inflammation and gut microbiota homeostasis [51]. Kumar et al. [52] reviewed the efficiency of flavonoids in acetic acid-induced colitis models and observed that application of some flavonoids significantly attenuated inflammatory responses and demonstrated that, among others, alkaline phosphatase concentration was significantly decreased after treatment with respect to the control group. Fecal alkaline phosphatase under our experimental condition was non-significantly increased in lambs fed with herbs. Our results fill the missing gap of research, since, to the best of our knowledge, there is a paucity of information in data concerning microbial alteration and bacterial enzymatic activity in ruminants with prolonged feeding of diets supplemented with zinc and medicinal herbs.

Intestinal bacteria and their metabolites play a considerable role in the maintenance of epithelial barrier integrity and homeostasis. Microbiota composition may directly influence intestinal permeability by affecting TJ protein properties or indirectly by inflammatory modulation, leading to intestinal permeability impairment [53]. In general, the measurement of TEER is considered to be an important parameter for assessing intestinal tight junction integrity [54]. The results of this experiment showed no differences in the TEER values between the treatments, even though a significantly increased level of OCLN and CLDN-1 in the jejunal mucosa was recorded in sheep fed zinc and the herbal mixture. Claudin proteins are essential for the pore pathway to allow the transport of small molecules and ions, while OCLN and ZO-1 are important in the leaky pathway mediating the movement of large uncharged macromolecules [55,56]. More animal studies have indicated the importance of claudins in the integrity of TJs, and an enhanced level of occludin is important in damage-preventing and -improving tight junction barrier functions [16,56]. In terms of the intestinal barrier function, our findings suggest that simultaneous zinc and herbal feed supplementation may offer greater potential for the improvement of intestinal barrier integrity through enhancing TJ protein expression in the jejunum and for adjustment of intestinal permeability. Moreover, our results did not show dietary treatment-associated microbial dysbiosis that could cause impairment of intestinal permeability. Most of the polyphenols tested in vitro and in vivo, including quercetin, catechin, kaempferol, etc., have shown the ability to increase the expression of numerous TJ proteins and TEER across a cellular monolayer [57]. Our findings are partially consistent with previous studies in piglets and poultry that zinc dietary supplementation is important in the regulation of intestinal permeability and maintaining epithelial cell integrity [19,20,58]; however, no information is available for ruminants. Therefore, further research should be conducted to elucidate the synergistic action of dietary zinc and plant bioactive compounds resulting in the improvement of intestinal integrity in growing ruminants.

5. Conclusions

Regardless of the unrecorded changes in microbial community diversities, our research pointed out the potential of organic Zn intake for the reduction in the activity of bacterial enzymes connected with colon pathogenesis, i.e., β-glucuronidase, β-glucosidase and N-acetyl-glucosaminidase. Moreover, feed supplementation with Zn in combination with a medicinal herbs mixture may improve intestinal barrier function by increasing the expression of TJ proteins, especially OCLN and CLDN-1 in the jejunal mucosa of growing lambs. Both of these factors can contribute significantly to the amelioration of gut health through the improvement of intestinal barrier integrity and adjustment of intestinal permeability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13091819/s1. Table S1: The sequences of the primers used in this study.

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Data Availability Statement: The data presented in this study are available from the corresponding author upon reasonable request.

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References

1. Christaki, E.; Bonos, E.; Giannenas, I.; Florou-Paneri, P. Aromatic plants as a source of bioactive compounds. *Agriculture* **2012**, *2*, 228–243. [CrossRef]
2. Gammoh, N.Z.; Rink, L. Zinc in infection and inflammation. *Nutrients* **2017**, *9*, 624. [CrossRef]
3. Maares, M.; Haase, H. A guide to human zinc absorption: General overview and recent advances of in vitro intestinal models. *Nutrients* **2020**, *12*, 762. [CrossRef] [PubMed]
4. Grešáková, Ľ.; Tokarčíková, K.; Čobanová, K. Bioavailability of Dietary Zinc Sources and Their Effect on Mineral and Antioxidant Status in Lambs. *Agriculture* **2021**, *11*, 1093. [CrossRef]
5. Reed, S.; Neuman, H.; Moscovich, S.; Glahn, R.P.; Koren, O.; Tako, E. Chronic zinc deficiency alters chick gut microbiota composition and function. *Nutrients* **2015**, *7*, 9768–9784. [CrossRef]
6. Valenzuela-Grijalva, N.V.; Pinelli-Saavedra, A.; Muhlia-Almazan, A.; Domíniguez-Díaz, D.; González-Ríos, H. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J. Anim. Sci. Technol.* **2017**, *59*, 8. [CrossRef]
7. Króliczewska, B.; Pecka-Kiełb, E.; Bujok, J. Strategies Used to Reduce Methane Emissions from Ruminants: Controversies and Issues. *Agriculture* **2023**, *13*, 602. [CrossRef]
8. Forcina, G.; Pérez-Pardal, L.; Carvalheira, J.; Beja-Pereira, A. Gut Microbiome Studies in Livestock: Achievements, Challenges, and Perpectives. *Animals* **2022**, *12*, 3375. [CrossRef]
9. Wang, J.; Fan, H.; Han, Y.; Zhao, J.; Zhou, Z. Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis. *Asian-Australas. J. Anim. Sci.* **2017**, *30*, 100–110. [CrossRef]
10. Skalny, A.V.; Aschner, M.; Lei, X.G.; Gritsenko, V.A.; Santamaria, A.; Alekseenko, S.I.; Prakash, N.T.; Chang, J.-S.; Sizova, E.A.; Chao, J.C.J.; et al. Gut microbiota as a mediator of essential and toxic effects of zinc in the intestines and other tissues. *Int. J. Mol. Sci.* **2021**, *22*, 13074. [CrossRef]
11. An, X.; Bao, Q.; Di, S.; Zhao, Y.; Zhao, S.; Zhang, H.; Lian, F.; Tong, X. The interaction between the gut microbiota and herbal medicines. *Biomed. Pharmacother.* **2019**, *118*, 109252. [CrossRef]
12. Desrouilleres, K.; Millette, M.; Vu, K.D.; Touja, R.; Lacroix, M. Cancer preventive effects of a specific probiotic fermented milk containing *Lactobacillus acidophilus* CL1285, *L. casei* LBC80R and L. rhamnosus CLR2 on male F344 rats treated with 1,2-dimethylhydrazine. *J. Funct. Foods* **2015**, *17*, 816–827. [CrossRef]
13. Valeri, M.; Rossi Paccani, S.; Kasendra, M.; Nesta, B.; Serino, L.; Pizza, M.; Soriani, M. Pathogenic *E. coli* exploits SsIE mucinase activity to translocate through the mucosal barrier and get access to host cells. *PLoS ONE* **2015**, *10*, e0117486. [CrossRef] [PubMed]
14. Barszcz, M.; Taciak, M.; Tuśnio, A.; Święch, E.; Skomiał, J.; Čobanová, K.; Grešáková, Ľ. The effect of organic and inorganic zinc source, used with lignocellulose or potato fiber, on microbiota composition, fermentation, and activity of enzymes involved in dietary fiber breakdown in the large intestine of pigs. *Livest. Sci.* **2021**, *245*, 104429. [CrossRef]

15. Ferraris, C.; Elli, M.; Tagliabue, A. Gut microbiota for health: How can diet maintain a healthy gut microbiota? *Nutrients* **2020**, *12*, 3596. [CrossRef]
16. Suzuki, T. Regulation of intestinal epithelial permeability by tight junctions. *Cell. Mol. Life Sci.* **2013**, *70*, 631–659. [CrossRef]
17. Patra, A.K.; Amasheh, S.; Aschenbach, J.R. Modulation of gastrointestinal barrier and nutrient transport function in farm animals by natural plant bioactive compounds—A comprehensive review. *Crit. Rev. Food Sci.* **2019**, *59*, 3237–3266. [CrossRef] [PubMed]
18. Wan, Y.; Zhang, B. The impact of zinc and zinc homeostasis on the intestinal mucosal barrier and instestinal diseases. *Biomolecules* **2022**, *12*, 900. [CrossRef]
19. Wang, W.; Van Noten, N.; Degroote, J.; Romeo, A.; Vermeir, P.; Michiels, J. Effect of zinc oxide sources and dosages on gut microbiota and integrity of weaned piglets. *J. Anim. Physiol. Anim. Nutr.* **2019**, *103*, 231–241. [CrossRef]
20. Barzegar, M.; Zaghari, M.; Zhandi, M.; Sadeghi, M. Effects of zinc dosage and particle size on gut morphology, tight junctions and TNF-α expression in broiler breeder hens. *J. Anim. Physiol. Anim. Nutr.* **2022**, *106*, 772–782. [CrossRef]
21. Jin, L.; Deng, L.; Bartlett, M.; Ren, Y.; Lu, J.; Chen, Q.; Pan, Y.; Wang, H.; Guo, X.; Liu, C. A novel herbal extract blend product prevents particulate matters-induced inflammation by improving gut microbiota and maintaining the integrity of the intestinal barrier. *Nutrients* **2022**, *14*, 2010. [CrossRef]
22. Wang, X.; Hu, L.; Liu, H.; Xu, T.; Zhao, N.; Zhang, X.; Geng, Y.; Kang, S.; Xu, S. Characterization of the bacterial microbiota across the different intestinal segments of the Qinghai semi-fine wool sheep on the Qinghai-Tibetian Plateau. *Anim. Biosci.* **2021**, *34*, 1921–1929. [CrossRef]
23. Petrič, V.; Mravčáková, D.; Kucková, K.; Kišidayová, S.; Cieslak, A.; Szumacher-Strabel, M.; Huang, H.; Kolodziejski, P.; Lukomska, A.; Slusarczyk, S.; et al. Impact of zinc and/or herbal mixture on ruminal fermentation, microbiota, and histopathology in lambs. *Front. Vet. Sci.* **2021**, *8*, 630971. [CrossRef] [PubMed]
24. European Commission (EC). Council Regulation (EC) 1099/2009 of 24 September 2009 on the Protection of Animals at the Time of Killing. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32009R1099 (accessed on 18 November 2009).
25. Ussing, H.H.; Zerahn, K. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **1951**, *23*, 110–127. [CrossRef] [PubMed]
26. Placha, I.; Ryzner, M.; Cobanova, K.; Faixova, Z.; Faix, S. Effects of dietary supplementation with sage (*Salvia officinalis* L.) essential oil on antioxidant status and duodenal wall integrity of laying strain growers. *Pol. J. Vet. Sci.* **2015**, *18*, 741–749. [CrossRef] [PubMed]
27. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef] [PubMed]
28. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
29. Aronesty, E. Ea-Utils: Command-Line Tools for Processing Biological Sequencing Data. Available online: https://github.com/ExpressionAnalysis/ea-utils (accessed on 1 January 2011).
30. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Peña, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef]
31. Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F.O. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Res.* **2013**, *41*, D590–D596. [CrossRef]
32. Beam, A.; Clinger, E.; Hao, L. Effect of diet and dietary components on the composition of the gut microbiota. *Nutrients* **2021**, *13*, 2795. [CrossRef]
33. Zackular, J.P.; Moore, J.L.; Jordan, A.T.; Juttukonda, L.J.; Noto, M.J.; Nicholson, M.R.; Crews, J.D.; Semler, M.W.; Zhang, Y.; Ware, L.B.; et al. Dietary zinc alters the microbiota and decreases resistance to *Clostridium difficile* infection. *Nat. Med.* **2016**, *22*, 1330–1334. [CrossRef] [PubMed]
34. Gielda, L.M.; DiRita, V.J. Zinc competition among the intestinal microbiota. *mBio* **2012**, *3*, e00171-12. [CrossRef]
35. Duffy, R.; Yin, M.; Redding, L.E. A review of the impact of dietary zinc on livestock health. *J. Trace Elem. Med. Biol.* **2023**, *5*, 100085. [CrossRef]
36. Xia, T.; Lai, W.; Han, M.; Han, M.; Ma, X.; Zhang, L. Dietary ZnO nanoparticles alters intestinal microbiota and inflammation response in weaned piglets. *Oncotarget* **2017**, *8*, 64878–64891. [CrossRef] [PubMed]
37. Oh, H.J.; Kim, M.H.; Yun, W.; Lee, J.H.; An, J.S.; Kim, Y.J.; Kim, M.J.; Kim, H.B.; Cho, J.H. Effect of nano zinc oxide or chelated zinc as alternatives to medical zinc oxide on growth performance, faecal scores, nutrient digestibility, blood profiles and faecal *Escherichia coli* and *Lactobacillus* concentrations in weaned piglets. *Ital. J. Anim. Sci.* **2022**, *21*, 708–716. [CrossRef]
38. Pereira, J.C.; Giese, E.C.; Moretti, M.M.S.; Gomes, A.C.S.; Perrone, O.M.; Boscolo, M.; Da Silva, R.; Gomes, E.; Martins, D.A.B. Effect of Metal Ions, Chemical Agents and Organic Compounds on Lignocellulolytic Enzymes Activities. In *Enzyme Inhibitors and Activators*; Senturk, M., Ed.; InTech: London, UK, 2017. [CrossRef]
39. Schomburg, D.; Salzmann, M. N-Acetyl-beta-glucosaminidase. In *Enzyme Handbook 4*; Schomburg, D., Salzmann, M., Eds.; Springer: Berlin/Heidelberg, Germany, 1991; pp. 195–201. [CrossRef]
40. Flores, R.; Shi, J.; Gail, M.H.; Gajer, P.; Ravel, J.; Goedert, J.J. Association of fecal microbial diversity and taxonomy with selected enzymatic functions. *PLoS ONE* **2012**, *7*, e39745. [CrossRef] [PubMed]

41. Finlayson-Trick, E.C.L.; Getz, L.J.; Slaine, P.D.; Thornbury, M.; Lamoureux, E.; Cook, J.; Langille, M.G.I.; Murray, L.E.; McCormick, C.; Rohde, J.R.; et al. Taxonomic differences of gut microbiomes drive cellulolytic enzymatic potential within hind-gut fermenting mammals. *PLoS ONE* **2017**, *12*, e0189404. [CrossRef]
42. Modrackova, N.; Vlkova, E.; Tejnecky, V.; Schwab, C.; Neuzil-Bunesova, V. *Bifidobacterium* β-Glucosidase Activity and Fermentation of Dietary Plant Glucosides Is Species and Strain Specific. *Microorganisms* **2020**, *8*, 839. [CrossRef]
43. Li, R.; Tun, H.M.; Jahan, M.; Zhang, Z.; Kumar, A.; Dilantha Fernando, W.G.; Farenhorst, A.; Khafipour, E. Comparison of DNA-, PMA-, and RNA-based 16S rRNA Illumina sequencing for detection of live bacteria in water. *Sci. Rep.* **2018**, *8*, 17427. [CrossRef]
44. Singh, S.B.; Lin, H.C. Role of intestinal alkaline phosphatase in innate immunity. *Biomolecules* **2021**, *11*, 1784. [CrossRef]
45. Cheng, Y.; Chen, H. Aberrance of zinc metalloenzymes-induced human diseases and its potential mechanisms. *Nutrients* **2021**, *13*, 4456. [CrossRef]
46. Cho, J.H.; Upadhaya, S.D.; Kim, I.H. Effects of dietary supplementation of modified zinc oxide on growth performance, nutrient digestibility, blood profiles, fecal microbial shedding and fecal score in weanling pigs. *Anim. Sci. J.* **2015**, *86*, 617–623. [CrossRef] [PubMed]
47. He, T.; Priebe, M.G.; Vonk, R.J.; Welling, G.W. Identification of bacteria with β-galactosidase activity in faeces from lactase non-persistent subjects. *FEMS Microbiol. Ecol.* **2005**, *54*, 463–469. [CrossRef] [PubMed]
48. Alibi, S.; Crespo, D.; Navas, J. Plant-Derivatives Small Molecules with Antibacterial Activity. *Antibiotics* **2021**, *10*, 231. [CrossRef] [PubMed]
49. Lin, R.; Piao, M.; Song, Y. Dietary Quercetin Increases Colonic Microbial Diversity and Attenuates Colitis Severity in *Citrobacter rodentium*—Infected Mice. *Front. Microbiol.* **2019**, *10*, 1092. [CrossRef]
50. Awolade, P.; Cele, N.; Kerru, N.; Gummidi, L.; Oluwakemi, E.; Singh, P. Therapeutic significance of β-glucuronidase activity and its inhibitors: A review. *Eur. J. Med. Chem.* **2020**, *187*, 111921. [CrossRef]
51. Lallès, J.-P. Intestinal alkaline phosphatase: Novel functions and protective effects. *Nutr. Rev.* **2014**, *72*, 82–94. [CrossRef]
52. Kumar, V.S.; Rajmane, A.R.; Adil, M.; Kandhare, A.D.; Ghosh, P.; Bodhankar, S.L. Naringin ameliorates acetic acid induced colitis through modulation of endogenous oxido-nitrosative balance and DNA damage in rats. *J. Biomed. Res.* **2014**, *28*, 132–145. [CrossRef]
53. Gieryńska, M.; Szulc-Dąbrowska, L.; Struzik, J.; Mielcarska, M.B.; Gregorczyk-Zboroch, K.P. Integrity of the Intestinal Barrier: The Involvement of Epithelial cells and Microbiota—A Mutual Relationship. *Animals* **2022**, *12*, 145. [CrossRef]
54. Srinivasan, B.; Kolli, A.R.; Esch, M.B.; Abaci, H.E.; Shuler, M.L.; Hickman, J.J. TEER measurement techniques for *in vitro* barrier model system. *J. Lab. Autom.* **2015**, *20*, 107–126. [CrossRef]
55. Monaco, A.; Ovryn, B.; Axis, J.; Amsler, K. The epithelial cell leak pathway. *Int. J. Mol. Sci.* **2021**, *22*, 7677. [CrossRef]
56. Lee, B.; Moon, K.M.; Kim, C.Y. Tight Junction in the Intestinal Epithelium: Its Association with Diseases and Regulation by Phytochemicals. *J. Immunol. Res.* **2018**, *2018*, 2645465. [CrossRef] [PubMed]
57. Bernardi, S.; Del Bo, C.; Marino, M.; Gargari, G.; Cherubini, A.; Andrés-Lacueva, C.; Hidalgo-Liberona, N.; Peron, G.; González-Dominguez, R.; Kroon, P.; et al. Polyphenols and Intestinal Permeability: Rationale and Future Perspectives. *J. Agric. Food Chem.* **2020**, *68*, 1816–1829. [CrossRef] [PubMed]
58. Zhang, B.; Guo, Y. Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *Br. J. Nutr.* **2009**, *102*, 687–693. [CrossRef] [PubMed]