High colonisation by probiotic *Escherichia coli* A0 34/86 strain is associated with a less diverse microbiome related to children's age

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Abstract

Probiotic supplementation in childhood serves as an additional source of bacterial colonisers and represents an opportunity to beneficially manipulate the intestinal microbiome. Differences in the ability of probiotic strains to colonise the gut may be related to the variously diversified gut microbiome. We report the results of the association between composition of the gut microbiome and the colonisation capacity of the probiotic strain *Escherichia coli* A0 34/86 (CNB – Colinfant New Born supplement) in the cases of three healthy children in different development stages (infant, toddler, and pre-school), as a preliminary insight to possible future prospective studies of this subject. Microbiome composition was estimated by 16S rRNA gene sequencing of 55 stool samples collected during approximately 3.5-13 months long periods. Detailed characterisation of the *E. coli* population was performed using colony PCR to detect 33 *E. coli* genetic determinants. In all children, genetic determinants typical for the probiotic *E. coli* A0 34/86 strain were detected immediately after administration of the probiotics. Analysis of the initial sample composition (the last sample taken before the probiotic administration) showed that the gut microbiome of infant and toddler with lower bacterial diversity was more successfully colonised by the probiotic strain. In our case report of three children, we showed for the first that supplementation with CNB probiotics in early infancy and toddlerhood was associated with high *E. coli* A0 34/86 colonisation and a significant change in the composition of the gut microbiome. Our results indicate that administration of CNB for its recommended duration might be efficient only in very early childhood.

1   Introduction

The administration of probiotics has a wide range of beneficial effects and numerous indications for use in the paediatric populations. Probiotics have been suc-cessfully used in preterm and high-risk children to prevent antibiotic-associated diarrhoea, to reduce the risk of necrotising enterocolitis, to lower the incidence of eczema, and in healthy children to prevent a variety of

diarrheal illnesses and allergies (Davis *et al.*, 2020; Irwin *et al.*, 2020; Sansotta *et al.*, 2019; Sjazewska, 2011).

Currently, the most administered probiotic strains belong to *Bifidobacterium, Lactobacillus,* and *Saccharomyces boulardii* (Davis *et al.*, 2020). However, certain *Escherichia coli* probiotics are also available in some European countries (Henker *et al.*, 2008; Wassenaar, 2016). *E. coli* strains form the basis of three commercially available probiotic preparations: Colinfant New Born (CNB), Mutaflor, and Symbioflor (Wassenaar, 2016). CNB supplement containing *E. coli* strain A0 34/86 (O83:K24:H31) is marketed for non-breastfeeding and preterm infants, as well as healthy children for prevention of gastric and intestinal disorders (Lodinová-Zádinová *et al.*, 1991, 2003; Wassenaar, 2016). Although the genus *Escherichia* is considered an opportunistic pathogen due to its pro-inflammatory effects, different epidemiological studies have shown that early-life colonisation with this genus is associated with a lower risk of atopic eczema and asthma and immune modulation (Alsalah *et al.*, 2015; De Muinck *et al.*, 2011; Kuang *et al.*, 2016; Mukhopadhya *et al.*, 2012; Rudi *et al.*, 2012).

However, inconsistent results are observed regarding the influence of probiotics on the gut microbiome composition. The observed differences could be explained by different probiotic dosages, varying duration of administration, or the initial composition of the gut composition (Bazanella *et al.*, 2017; Davis *et al.*, 2020; Vendt *et al.*, 2006; Wu *et al.*, 2016). While the impact of various *Bifidobacterium* and *Lactobacillus* preparations on the composition of the faecal microbiome of children (from newborns to 18 years) has been extensively studied (Davis *et al.*, 2020), information about the effects of administering *E. coli* probiotic strains is limited. Previously, we described the colonisation ability of the *E. coli* CNB probiotic strain and the turnover of *E. coli* strains in stool samples isolated from one infant during the first year of life (Micenková *et al.*, 2020).

In this follow-up study, we assess the difference in the colonisation capacity of *E. coli* A0 34/86 strain after administration of the same dose of the probiotic CNB to three healthy children of different ages (infant, toddler, and pre-school child). Samples were taken from the infant's stools during the first 13 months of life and from the toddler's and pre-school child's stools during a period of approximately 3.5 months. Stool microbial composition was assessed by 16S rRNA gene sequencing. Colony PCR for the detection of 33 *E. coli* genetic determinants characterised the overall *E. coli* population in detail.

2   Materials and methods

Stool samples

The criteria for inclusion of the children's samples in the project were as follows. The children were selected within the framework of the First Years of Life (FYOL) project, the aim of which was to map the dynamics of oral or gut microbiome composition during the first years of life with respect to selected factors such as antibiotic or probiotic usage, mode of delivery, etc. These variables were recorded in the questionnaires filled in by the mothers at the time of sampling. If the child was receiving the probiotic preparation CNB, he/she was included in this sub-project focusing on the effect of CNB consumption on the composition of the microbiome and the colonisation capacity of the probiotic strain *E. coli* A0 34/86.

Stool samples (n = 56) from three children of different ages (infant, toddler, and pre-school child) were collected at different time points before, during, and after the CNB administration. Twenty-one stool samples were collected from one infant (female) during the first 13 months of life from 31 March 2018 to 14 April 2019 (Brno, Czech Republic) (Supplementary Table S1). Twenty samples were collected from a two-year-old toddler (male; born on the 4th of February 2017) from 3 April to 16 July 2019 (Brno, Czech Republic). Moreover, one sample was collected after more than two years (15th of June 2021 – this sample was used only for the cultivation and PCR experiments). Fourteen stools were collected from a four-year-old pre-school child (male; born on the 3rd of March 2015) from the 3rd of April to the 17th of July 2019 (Brno, Czech Republic). The toddler and the pre-school child are siblings living in the same household. Samples were collected at home by mothers, who were instructed to follow a specific collection protocol. Mothers were given stool sampling kits consisting of FLOQSwabs (Copan, Italy), disposable gloves, and hand and surface disinfectant wipes for more comfortable sampling. The mother of infant was instructed to collect stool samples from the same spot in the diaper, and the mother of toddler and pre-school child was instructed to collect all stool samples from the same spot. Samples were stored immediately at –20 °C. At the same time, we monitored the following variables: child health status and medication, breastfeeding, solid food introduction, probiotic consumption, and mother's antibiotic intake. The study was approved by the ELSPAC Steering Committee (Ref. No: CELSPAC/EK/1/2018), Masaryk University, and all samples were collected after the mother signed informed consent.

Colinfant New Born probiotics dosage

The orally administered probiotic preparation Colinfant New Born (Dyntec, Czech Republic) is designed specially to prevent hospital infections, gastrointestinal diseases of various origins, disorders of the composition of the intestinal microbiota, and others. This probiotic containing the *E. coli* criodesiccata A0 34/86 (O83:K24:H31) (from 0.8×10^8 to 1.6×10^8 bacterial cells per dose) was supplemented to all healthy children in 12 doses for approximately 2 to 4 weeks (Supplementary Table S1). CNB was prescribed by the children's physician and with the mothers' consent for the prevention of various diarrhoeal diseases and allergies. The probiotic dose was recommended by a physician and also discussed by an immunologist. CNB was added to the breastfeeding infant's diet during the 2nd and 3rd months of life. In the infant, the probiotic supplement was administered by syringe without a needle directly to the root of the tongue to prevent the baby from spitting out the probiotics. The infant has been exclusively breastfed for approximately the first five months of life (samples 1-15); starting on the 25th of August 2018 (samples 16-21), the breastfeeding was complemented with solid food. During the CNB studied periods (between, during and after CNB consumption), the infant was exclusively breastfed. All children had a balanced omnivorous diet. The children did not suffer from diarrhoea or other digestive complications that could affect composition of the microbiome during the studied periods. The 4-year-old pre-school child had a pollen allergy (from April to the end of May 2019). Supplementary Table S1 provides detailed information about the monitored external variables (diet, medication, probiotic consumption, etc.).

Characterisation of Escherichia coli isolates

Isolation of *E. coli* from children's stool was performed using selective MacConkey agar. ENTEROtest16 (Erba Lachema, Czech Republic) was used for biochemical identification of enterobacterial species. Approximately one stool sample per month was selected for cultivation (in total, thirteen samples from the infant, eight from the 2-year-old toddler, and six from the 4-year-old pre-school child), covering periods before, during, and after the CNB administration (Supplementary Table S1).

E. coli isolates were confirmed by the detection of chromosomal gene *tonB* (Supplementary Table S2). In addition, the distribution of 32 *E. coli* colonisation/virulence and bacteriocin encoding genes (Supplementary Table S2) was screened to identify individual *E. coli* strains. The most common *E. coli* colicin (types: B, E1,

Ia, Ib, Js, K, L, M, N, and S4) and microcin (types: mB17, mC7, mJ25, mH47, mM, and mV) encoding genes were selected and tested as described previously (Micenková *et al.*, 2014, 2016, 2017, 2020; Šmajs *et al.*, 2010).

Additionally, three cyclomodulin encoding genes (*cnf1*, *cdt*, and *pks*); adhesin-encoding genes (*afaI*, *sfa*, *pap*, and *fimA*); siderophore genes (*aer*, *iucC*, *fyuA*, and *fepC*); α-hemolysin (α-*hly*) gene, and genes associated with diarrheagenic *E. coli* strains (*ial*, *lt*, *st*, and *bfpA*) were tested. Primer pair sequences, PCR product lengths, and PCR protocols used in this study were previously described (Supplementary Table S2) (Gómez-Moreno *et al.*, 2014; Gordon and O'Brien, 2006; Johnson and Stell, 2000; Kuhnert *et al.*, 1997; López-Saucedo *et al.*, 2003; Martínez *et al.*, 1994; Micenková *et al.*, 2014; Okeke *et al.*, 2004; Šmajs and Weinstock, 2001; Yamamoto *et al.*, 1995).

The *E. coli* A0 34/86 (O83:K24:H31) strain, cultivated directly from CNB probiotic preparation, was used as the positive PCR control for the detection of virulence and bacteriocin genes typical for this strain. This strain was positive on virulence genes *fimA*, α-*hly*, *cnf1*, *sfa*, *pap*, *fyuA*, and *fepC* and microcin determinants H47, and M (Micenková *et al.*, 2020). Positive controls for PCR detection of *tonB*, bacteriocin, and virulence encoding genes were taken from our laboratory stock and were previously published (Micenková *et al.*, 2014, 2017; Šmajs *et al.*, 2010).

The previously described triplex PCR method based on the combination of the *chuA* and *yjaA* genes and the TSPE4 DNA fragment was used to assign *E. coli* isolates to one of the four major phylogenetic groups (A, B1, B2, and D) (Clermont *et al.*, 2000).

DNA isolation from stools, PCR amplification, and sequencing of the 16S rRNA gene

DNA isolation of 55 stool samples was performed using a PowerLyzer® PowerSoil® DNA Isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Isolated DNA was used as a template in PCR reactions targeting the hypervariable V4 region (EMP 515-806) of the bacterial 16S rRNA gene according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA, USA) (Supplementary Table S2). Sequencing was performed using MiSeq Reagent Kits v2 on a MiSeq 2000 sequencer according to the manufacturer's instructions (Illumina).

Bioinformatics and statistical analysis

The raw sequence reads were preprocessed by the following pipeline. The first step of the pipeline was

demultiplexing of reads in sequencing pools into individual samples. The next step in the pipeline was trimming of low-quality end of each read.

Both demultiplexing and length filtering were performed by an in-house tool written in Python 3. Forward and reverse reads were denoised using the DADA2 amplicon denoising R package (Callahan *et al.*, 2016). Following denoising, the forward and reverse reads were joined using the fastq-join read joining utility (Aronesty, 2011). Finally, chimeric sequences were removed from the joined reads using the removeBimera function of the DADA2 R package. The taxonomy was determined using the usearch-consensus algorithm from the microbiome analysis toolkit QIIME (v 1.9.1) (Caporaso *et al.*, 2010). For each input sequence, the three closest organisms were found in the Silva v.123 reference database (Quast, 2013). Their taxonomies were combined into the final taxonomic assignment using the least common ancestor (LCA) algorithm. Using the genus-level Silva v. 123 reference database, the taxonomy was assigned down to genus-equivalent level. Clusters that did not contain the sequences of the described genera were named at the lowest taxonomic level that could be assigned as a genus-level group.

After quality filtering and chimeras' removal, the number of reads ranged from 10,841 to 83,542, with a median of 28,833 reads per sample (Supplementary Table S3).

Statistical analysis of bacterial compositions was performed at the genus level. The Shannon diversity index was chosen to estimate the bacterial diversity and uniformity of the microbial community. Data were treated as compositional and before all statistical analyses were transformed using centred log-ratio (CLR) transformation (Aitchison, 1986). All zeroes in original data were replaced using the count zero multiplicative approach (Martin-Fernandez *et al.*, 2015). Only genera with an abundance of minimally 0.3% of the total number of reads of the least sequenced sample, at least in three samples, were included in the transformation process and additional statistical analysis of the microbiome to avoid high sparsity in data.

Non-parametric Kruskal-Wallis test (with Dunns post-hoc; for infant samples) and Mann-Whitney U (for toddler and pre-school child samples) test with Benjamini-Hochberg (BH) adjustment for multiple testing were used to test the differences among periods before, within, and after using CNB probiotics. Results were considered significant at FDR of 5% (q < 0.05).

The samples used for the analysis of microbial differences before, during, and after the administration of the CNB probiotics were taken from timepoints homogenous in terms of monitored external variables across all these periods of interest (Supplementary Table S1). This resulted in a shorter 'after the CNB probiotics administration' period of the infant. We could not collect more than one sample before the CNB administration from the toddler and pre-school child.

All statistical analyses were performed in R, version 4.0.3. (R Core Team 2020) using R packages zCompositions; version 1.3.4 (zero replacement) (Martin-Fernandez *et al.*, 2015), compositions; version 2.0-1 (CLR transformation) (Van den Boogaart *et al.*, 2019), ggplot2; version 3.3.2 (Wickham, 2016), UpSetR; version 1.4.0 (2019). UpSetR: A More Scalable Alternative to Venn and Euler Diagrams for Visualizing Intersecting Sets. R package) and ggpubr; version 0.4.0 (2020). ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. (https://CRAN.R-project.org/package=ggpubr) (version 1.4.0. https://CRAN.R-project.org/package=UpSetR).

3　　　　Results

The probiotic supplement CNB containing *E. coli* A0 34/86 strain (O83:K24:H31) was administrated in twelve doses over a period of approximately 2-4 weeks to three healthy children of different ages (infant, toddler, and pre-school child) to prevent various diarrheal illnesses and allergies. Stool samples were collected during the first 13 months of the infant's life and from the toddler's and pre-school child's stools during a period of approximately 3.5 months. In addition, one toddler's stool sample was collected more than two years after CNB administration and used only for the cultivation experiments.

Influence of the initial gut microbial composition and diversity on the colonisation capacity of the CNB probiotic E. coli A0 34/86 strain

Our objective was to assess the differences in the initial microbial composition among children and identify the children who were successfully colonised by probiotic strains. To this end, we focused on the microbial composition, diversity, and abundance of genus *Escherichia* in the initial samples (the last samples collected before the CNB administration) estimated by 16S rRNA gene sequencing with the *E. coli* population composition characterised by the cultivation experiments. *E. coli* isolates were cultivated from children's stool samples approximately once a month (Supplementary Table S1). A set of 33 *E. coli* genetic determinants was

TABLE 1 Bacterial composition of the initial stool sample – last sample before the Colinfant New Born (CNB) administration

Initial stool sample (last sample before the CNB administration)	Infant	2-year-old toddler	4-year-old pre-school child
Number of genera	12	55	65
Diversity and uniformity index (Shannon)	0.5	4.7	5.7
Escherichia genus abundance	1.04%	0.00026%	0.004%
Initial *E. coli* population genetic content	phyloB2, *fimA*, colicin Ia	not detected	phyloB2, *fimA*
Resident colonisation of the probiotic CNB strain after supplementation?	Yes	Yes	Yes
Three most dominant bacterial genera detected in the initial stool sample[1]	*Bifidobacterium* (85.4%) *Streptococcus* (11.2%) *Lactobacillus* (1.2%)	*Bacteroides* (33.9%) *Bifidobacterium* (7.1%) *Streptococcus* (6.8%)	*Faecalibacterium* (17.4%) *Bacteroides* (16.8%) *Lachnospiraceae* un. (7.9%)

[1] For all sequencing results, see Supplementary Table S4.

used to detect probiotic *E. coli* A0 34/86 and other *E. coli* isolates with different genetic content.

The microbial diversity and uniformity of the initial samples (as estimated by the Shannon index) and the number of identified genera increased from infant to pre-school child (Table 1).

Only 12 genera were detected in the infant's initial sample, with *Bifidobacterium* predominating (85.4%), followed by *Streptococcus* (11.2%) and *Lactobacillus* (1.2%) (Table 1). The observed abundance of the genus *Escherichia* was 1.04%. An *E. coli* isolate containing genetic determinants (phyloB2; *fimA*, colicin Ia) was detected in the initial sample and in all stool samples (used for cultivation) from birth to the beginning of the CNB administration period (Figure 1A). During the administration of the CNB probiotics, this original *E. coli* isolate was replaced by the probiotic strain. The CNB strain containing genetic determinants (phyloB2; *fimA*, α-*hly*, *cnf1*, *sfa*, *pap*, *fyuA*, *fepC*, mH47, mM, and mV) colonised the infant's intestinal tract from the start of CNB supplementation (2nd month of life) until the end of the study (13th month of life).

In the initial toddler sample, 55 genera were detected with the dominance of *Bacteroides* (33.9%), *Bifidobacterium* (7.1%), and *Streptococcus* (6.8%) (Table 1). The proportion of *Escherichia* genus was lower compared to the infant (0.00026%), and no *E. coli* were successfully cultivated. During the CNB administration period, the probiotic strain colonised the intestinal tract and was detected in all subsequent samples, including the sample collected more than two years later (Figure 1B).

In the initial samples of the pre-school child, 65 genera were detected, with three dominant genera *Faecalibacterium* (17.4%), *Bacteroides* (16.8%), and *Lachnospiraceae* – unassigned (7.9%) (Table 1). The genus *Escherichia* accounted for 0.004% of the sample (Table 1), and an *E. coli* isolate belonging to phylogroup B2 containing gene *fimA* was identified (Figure 1C). Both *E. coli* (phylogroup B2, *fimA*) and CNB *E. coli* were identified during the consumption of the probiotics, but after this period no *E. coli* isolates were successfully cultivated until the end of the study. In this case, the probiotic strain was not capable of resident/longitudinal colonisation.

The most striking differences between the children's initial microbiome composition were in the higher occurrence of different genera (*Eubacterium*, *Ruminiclostridium*, *Ruminococcaceae*, and *Lachnospiraceae* groups) from *Clostridia* class, which were enriched in the pre-school child's initial stool sample (Figure 2A). The increase of the microbial diversity, as represented by increased uniformity and the number of new unique genera in the initial sample, was linked to children age. More unique bacterial genera were identified in pre-school child compared to infant and toddler (Figure 2B).

Effect of Colinfant New Born administration on the microbial composition and diversity

We were also interested in whether CNB administration affected overall microbial composition and diversity, as measured by 16S rRNA gene sequencing. A limitation of this analysis was that we have only one sample in the
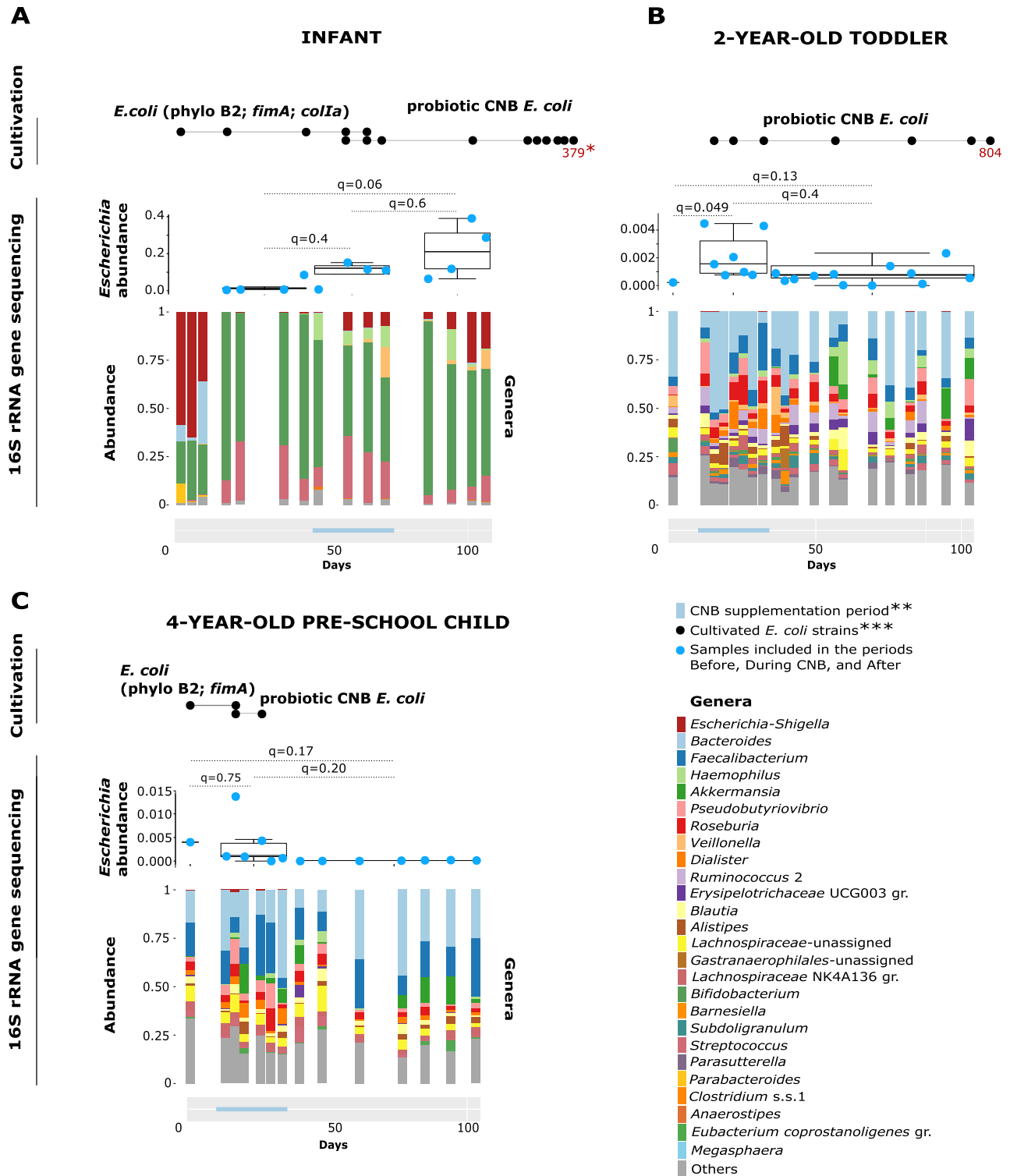
FIGURE 1   The effect of Colinfant New Born (CNB) administration on the microbiome composition, diversity, *Escherichia* abundance, and colonisation capacity of the probiotic strain *Escherichia coli* A0 34/86. (A) Infant, (B) Toddler, (C) Pre-school child. Cultivation – analysis of *E. coli* isolates turnover. Sequencing – the abundance of genus *Escherichia* – in the periods 'before, during, and after' the CNB administration, and dynamics of the relative bacterial abundance at the genus level. Genus *Escherichia* plus genera with relative abundance higher than 5% are visualised (taxa lower than 5% are displayed as 'Others'; all sequencing results are listed in Supplementary Table S4). * Stool samples used only for the cultivation experiments. ** Period of CNB administration – CNB was supplemented in 12 doses to all children. *** *E. coli* isolates were cultivated from stool samples collected in approximately month periods and analysed by colony PCR.

A

Unique bacterial genera identified in the initial stool sample

Infant (I)

Rothia (0.34%)
Gemella (0.001%)
Staphylococcus (0.09%)
Enterococcus (0.21%)
Enterobacter (0.07%)

Toddler (T)

Eggerthella (0.25%)
Erysipelotrichaceae i.s. (0.03%)
Anaeroglobus (0.09%)
Varibaculum (0.02%)
Corynebacterium (0.005%)
Hungatella (0.05%)*
Tyzzerella (0.02%)
Tyzzerella 4 (0.04%)
Ruminococcaceae UCG014 gr. (0.01%)
Ruminococcus 2 (3.0%)

Pre-school child (P)

Coprobacter (0.08%)
Eubacterium brachy gr. (0.02%)
Clostridiales AD3011 gr. (0.01%)
Eubacterium hallii gr. (2.34%)
Coprococcus 1 (0.02%)
Lachnospiraceae FCS020 gr. (0.41%)
Lachnospiraceae ND3007 gr. (1.80%)
Lachnospiraceae UCG005 gr. (0.15%)
Lachnospiraceae UCG010 gr. (0.03%)
Lachnospiraceae uncultured (1.85%)
Eubacterium coprostanoligenes gr. (0.41%)
Oscillospira (0.03%)
Ruminiclostridium 9 (0.22%)
Ruminococcaceae NK4A214 gr. (0.01%)
Ruminococcaceae UCG003 gr. (0.18%)
Leptotrichia (0.01%)
Thalassospira (0.12%)

Unique bacteria abudance

SUMMARY
Shannon index
No. of unique genera
Children age

B

An Upset plot showing shared and unique bacterial genera
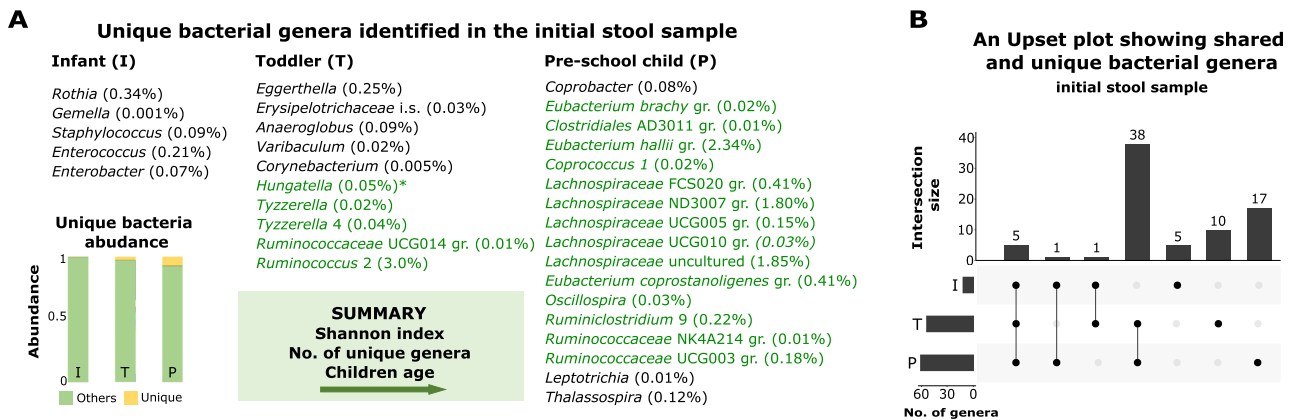**initial stool sample**

FIGURE 2　Influence of the initial gut microbiome composition and diversity on the colonisation capacity of the Colinfant New Born (CNB) probiotic *Escherichia coli* A0 34/86 strain. (A) Detailed characterisation of the initial stool sample (last sample before the CNB administration) in three children. (B) An Upset plot showing shared and unique bacterial genera between the three children and individual unique bacterial genera identified in the initial sample. * Different genera from the *Clostridia* class are highlighted in green.

Comparison (p-values)

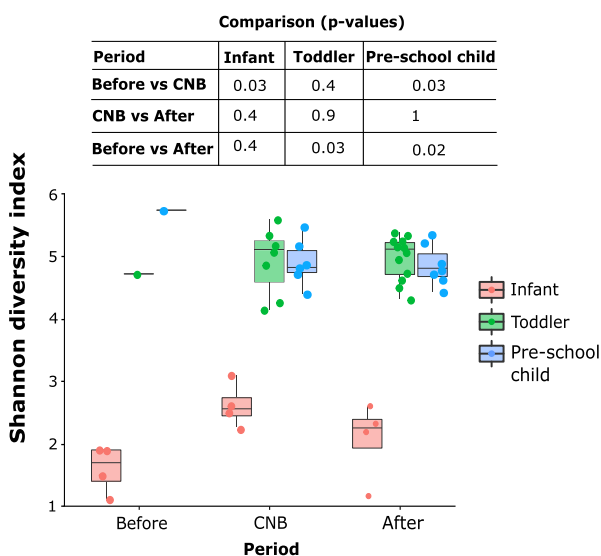| Period	Infant	Toddler	Pre-school child
Before vs CNB	0.03	0.4	0.03
CNB vs After	0.4	0.9	1
Before vs After	0.4	0.03	0.02

FIGURE 3　Influence of the Colinfant New Born (CNB) consumption on the bacterial diversity. Comparison of the Shannon diversity index 'before, during, and after' the CNB administration.

'before CNB administration' period in the toddler and pre-school child. Bacterial genera with relative abundances higher than 5% are visualised in Figure 1 (taxa with abundances lower than 5% are shown as 'Others'); all sequencing results are listed in Supplementary Table S4.

In the younger children, an increase in diversity was detected during the CNB supplementation (infant) or after the administration (toddler) (Figure 3). In the 4-year-old, consumption of probiotics led to lower microbial diversity.

Significant changes in the abundance of individual bacterial genera during or after the period of CNB pro-

biotics administration were identified only in the infant and the 2-year-old toddler stool samples (Figure 4). In the toddler samples, the only significant differences were observed during the period of CNB administration. In the infant, the effect of the CNB consumption lasted longer and resulted in an increased abundance of *Lactobacillus*, *Streptococcus*, and *Haemophilus* (q < 0.05). The influence of the CNB consumption on the overall *Escherichia* abundance was most evident also in the younger children. In the toddler, an increase of *Escherichia* (q < 0.05) was detected during the supplementation and in the infant after the consumption (q = 0.06). In the 4-year-old child, we could only detect the *Escherichia* genus before and during the CNB administration period.

4　Discussion and conclusions

The human gut microbiome develops over early childhood, and bacterial colonisation is influenced by mode of delivery, diet, antibiotics or probiotic administration, and other factors (Guittar *et al.*, 2019; Koenig *et al.*, 2011; Reyman *et al.*, 2019). Supplementation with probiotics during infancy and childhood represents an opportunity to beneficially manipulate the intestinal microbiome (Davis *et al.*, 2020).

The biology of *E. coli* in its primary niche, the human intestinal tract, is poorly explored (Lasaro *et al.*, 2014). Successful colonisation of the intestinal tract with *E. coli* depends on the competition for nutrients with a dense and diverse microbiome and on the *E. coli* genetic content (Conway and Cohen, 2015; Schierack *et al.*, 2008). In our study, we analysed for the first time how the ini-
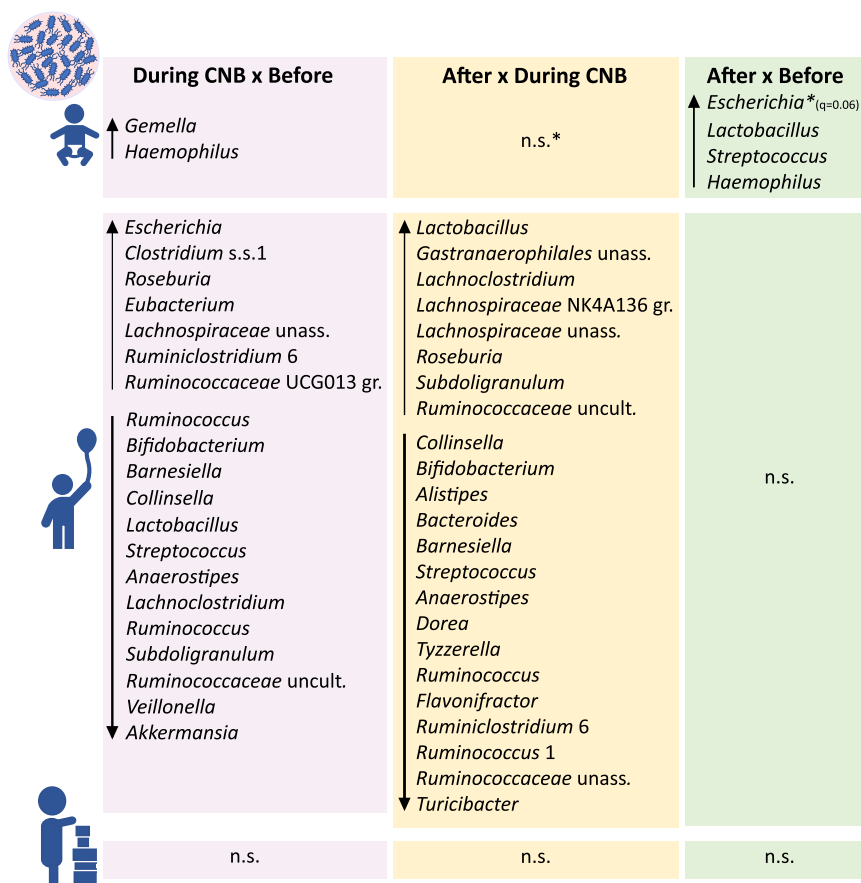
	During CNB x Before	After x During CNB	After x Before
			▲ *Escherichia**(q=0.06) *Lactobacillus* *Streptococcus* *Haemophilus*
	▲ *Gemella* *Haemophilus*	n.s.*	
	▲ *Escherichia* *Clostridium* s.s.1 *Roseburia* *Eubacterium* *Lachnospiraceae* unass. *Ruminiclostridium* 6 *Ruminococcaceae* UCG013 gr. *Ruminococcus* *Bifidobacterium* *Barnesiella* *Collinsella* *Lactobacillus* *Streptococcus* *Anaerostipes* *Lachnoclostridium* *Ruminococcus* *Subdoligranulum* *Ruminococcaceae* uncult. *Veillonella* ▼ *Akkermansia*	▲ *Lactobacillus* *Gastranaerophilales* unass. *Lachnoclostridium* *Lachnospiraceae* NK4A136 gr. *Lachnospiraceae* unass. *Roseburia* *Subdoligranulum* *Ruminococcaceae* uncult. *Collinsella* *Bifidobacterium* *Alistipes* *Bacteroides* *Barnesiella* *Streptococcus* *Anaerostipes* *Dorea* *Tyzzerella* *Ruminococcus* *Flavonifractor* *Ruminiclostridium* 6 *Ruminococcus* 1 *Ruminococcaceae* unass. ▼ *Turicibacter*	n.s.
	n.s.	n.s.	n.s.

FIGURE 4   Statistically significant changed bacterial genera after the Colinfant New Born administration. Bacterial genera with abundance (at least in three samples) minimally 0.3% of the total number of reads of the least sequenced sample were included in the statistical analysis. * Only statistically significant results are visualised. For all results, see Supplementary Table S5.

tial composition of the microbiome affects the colonisation of the intestinal tract with the probiotic *E. coli* CNB strain. Initial sample composition revealed that younger children (infant and toddler) with lower bacterial diversity and number of unique genera detected were successfully colonised with the probiotic *E. coli* strain.

We observed *E. coli* isolates with different genetic content. Ecological studies of commensal *E. coli* revealed one dominant *E. coli* strain per host, but this dominance is temporary and more competitive *E. coli* strains displace less competitive ones (Apperloo-Renkema and Van der Waaij, 1991; Souza *et al.*, 2002). In our previously published pilot study with one infant, the CNB strain, successfully colonised the intestinal tract the day after probiotic administration and established in the intestinal tract during the first year of life (Micenková *et al.*, 2020).

In this follow-up study, the resident CNB probiotic strain was again a great competitor in the infant intestinal tract and replaced the original *E. coli* detected after the delivery, which contained fewer genetic determinants (phyloB2, *fimA*, colicin Ia).

In the 2-year-old toddler, the first detected *E. coli* originated from the probiotic preparation. Also in this case, the ability to colonise was remarkably high, and the probiotic strain was cultivated from the toddler's sample collected more than two years after the CNB supplementation. No other *E. coli* strains were successfully cultivated from the infant's and 2-year-old toddler's stool samples after the CNB administration till to the end of the experiment. We hypothesise, that the probiotic strain CNB is the one providing a huge colonisation resistance to other *E. coli* strains. The mixture of various fimbriae types (genes *fimA*, *sfa*, and *pap*), factors for competition for vital elements within microbial communities in the environment body (e.g. siderophores genes *fyuA*, and *fepC*) and bacteriocins (antimicrobial agents; mH47, and mM) makes from this strain a successful competitor and resident coloniser (Micenková *et al.*, 2020). A similar property was previously described for other probiotic strain of *E. coli*. *E. coli* Nissle 1917 has multiple properties for better gut colonisation, including Curli, type 1, and F1C fimbriae, and it also successfully competes with the micro-

biota for trace metals. For example, it protects mice from infection with pathogenic *Salmonella* in an iron-dependent manner (Sorbara and Pamer, 2019). In the 4-year-old child, the CNB strain has passed through the gastrointestinal tract without colonising the intestinal tract. Despite the genetic relatedness and the same household of toddler and pre-school child, the effect of CNB administration was different, probably due to the differences in age and initial microbial composition before supplementation. The microbial ecosystem develops early in life until about 3-5 years of age, when a relatively stable adult-like microbiota is established (Rodríguez *et al.*, 2019). Before this state is reached, the gut microbiota is more susceptible to modulation by external factors (Laursen, 2021; Laursen *et al.*, 2017; Milani *et al.*, 2017) and may be more suitable for successful colonisation by CNB. In our study, the most striking difference between the children's initial microbiome composition (the last samples collected before the CNB administration) was the increasing number of different genera from *Clostridia* class from infant to the pre-school child. The gut microbiome of the pre-school child was characterised by specific microbiota communities enriched with taxa belonging to the family *Lachnospiraceae*, *Ruminococcaceae*, genus *Eubacterium*, and other members of the class *Clostridia*. These bacteria typically settle in the mucosal layer of the intestine (Belzer *et al.*, 2017; Chen *et al.*, 2020). Also, experiments with the probiotic *E. coli* Nissle 1917 strain showed that it strongly adheres to human and porcine intestinal epithelial cells (Barth *et al.*, 2009). These observations probably point to the same intestinal ecological niche of strongly adherent probiotic *E. coli* strains and different genera from *Clostridia*. An intestinal environment colonised by a diverse population of *Clostridia* together with the presence of another *E. coli* strain (phylo B2; *fimA*) before the CNB supplementation probably led to colonisation resistance in the 4-year-old pre-school child.

While human clinical studies have analysed the effects of *Bifidobacterium* and *Lactobacillus* administration on the composition of gut microbiome, the impact of *E. coli* probiotic strains supplementation is unknown (Davis *et al.*, 2020; Derrien and Van Hylckama Vlieg, 2015). In our study, three different age-related effects were observed. In the infant, the effect of increased diversity and uniformity during the CNB consumption was followed by a significant increase of *Lactobacillus*, *Haemophilus*, and *Streptococcus* after the consumption. In the toddler, the opposite effect was observed with changes in microbiome composition during CNB

supplementation, followed by an increase in diversity and uniformity after the consumption. In pre-school child, lower diversity was found after consumption of CNB with no statistically significant effect on microbial composition. Comparable results were observed in the experiments with newborns and children up to 18 years of age (Davis *et al.*, 2020). Some probiotic strains of *Lactobacillus* and *Bifidobacterium* affected the abundance of different bacterial taxa, while others did not (Davis *et al.*, 2020). These conflicts may also be related to children's age, duration of the administration, and other factors (Garcia Rodenas *et al.*, 2016).

Supplementation with CNB *E. coli* A0 34/86 probiotics affected the composition of gut bacteria, particularly in infant and toddler, and we hypothesise that this was probably due to lower diversity of the initial microbiome compared to the older pre-school child. In our study with three children of different ages, we demonstrated for the first time that supplementation of the microbiome at an early age during infancy and toddlerhood with CNB probiotics might be associated with high colonisation capacity of *E. coli* strain A0 34/86. It seems that the administration of CNB in its recommended duration is efficient only very early in childhood. We could only speculate whether prolonging the administration period would help the colonisation capacity of *E. coli* A0 34/86 in the older children. We are aware of the small sample size of our pilot study, and more extensive longitudinal studies are needed to confirm our observations.

Supplementary material

Supplementary material is available online at:
https://doi.org/10.6084/m9.figshare.24999404

Table S1. Complete list of DNA samples isolated from stools of three children and detected *E. coli* strains.

Table S2. List of primers and the length of PCR products.

Table S3. Samples types analysed by 16S rRNA gene sequencing and the number of reads after quality filtering and chimeras removing.

Table S4. Diversity and relative abundance of the bacterial population analysed from the sequencing of 16S rRNA gene in DNA samples isolated from children at the genus level.

Table S5. Influence of the Colinfant New Born administration on the bacterial population in children. Statistical significance and the mean relative values during the studied periods.

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Authors' contribution

Conceptualization: LM, EB; methodology: KB, JB, DŠ; analysis: PA, SS; writing and editing: LM, EB, SS, JB, DŠ. All authors have read and agreed to the published version of the mansucript.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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