



Resembling breast milk: influence of polyamine-supplemented formula on neonatal BALB/cOlaHsd mouse microbiota

Carlos Gómez-Gallego^{1*}, M. Carmen Collado², Gaspar Pérez², Toni Ilo³, Ulla-Marjut Jaakkola³, María J. Bernal⁴, María J. Periago¹, Rafael Frias³, Gaspar Ros¹ and Seppo Salminen⁵

¹Department of Food Science and Nutrition, Faculty of Veterinary Sciences, University of Murcia, Campus de Espinardo, 30071, Espinardo, Murcia, Spain

²Institute of Agrochemistry and Food Science, IATA-CSIC, Spanish National Research Council, 46980, Paterna, Valencia, Spain

³Central Animal Laboratory, University of Turku, 20014 Turku, Finland

⁴Hero Group, Global Technology Centre for Infant Nutrition, 30820, Alcantarilla, Murcia, Spain

⁵Functional Foods Forum, University of Turku, 20014 Turku, Finland

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Abstract

Infant microbiota is influenced by numerous factors, such as delivery mode, environment, prematurity and diet (breast milk or formula). In addition to its nutritional value, breast milk contains bioactive substances that drive microbial colonisation and support immune system development, which are usually not present in infant formulas. Among these substances, polyamines have been described to be essential for intestinal and immune functions in newborns. However, their effect on the establishment of microbiota remains unclear. Therefore, the aim of the present study was to ascertain whether an infant formula supplemented with polyamines has an impact on microbial colonisation by modifying it to resemble that in breast-fed neonatal BALB/c mice. In a 4 d intervention, a total of sixty pups (14 d old) were randomly assigned to the following groups: (1) breast-fed group; (2) non-enriched infant formula-fed group; (3) three different groups fed an infant formula enriched with increasing concentrations of polyamines (mixture of putrescine, spermidine and spermine), following the proportions found in human milk. Microbial composition in the contents of the oral cavity, stomach and small and large intestines was analysed by quantitative PCR targeted at fourteen bacterial genera and species. Significantly different ($P < 0.05$) microbial colonisation patterns were observed in the entire gastrointestinal tract of the breast-fed and formula-fed mice. In addition, our findings demonstrate that supplementation of polyamines regulates the amounts of total bacteria, *Akkermansia muciniphila*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides-Prevotella* and *Clostridium* groups to levels found in the breast-fed group. Such an effect requires further investigation in human infants, as supplementation of an infant formula with polyamines might contribute to healthy gastrointestinal tract development.

Key words: Polyamines: Microbiota: Infant formulas: Breast-feeding

Microbiota is known to play an important role in the maturation of the immune system and the establishment of the gut barrier. It has been well established that early microbial colonisation provides the neonate with vital stimuli that guide the maturation of the immune system. It is also well known that disturbances in this process can result in the development of immune disorders, which are regarded as a failure in the development of a balanced immune response^(1,2), as well as a predisposition to diseases later in life⁽³⁾. The development of the human microbiome is a complex process that might begin during the perinatal period, when the infant is exposed to the mother's microbiota, and continues to develop over the individual's lifetime. The first microbes and the succession of microbiota provide important

stimuli for the maturation of the intestinal immune system⁽⁴⁾. Thus, the establishment of healthy gut microbiota in early life is likely to be critical for normal development, acting a key step in the development of long-term well-being. Aberrancies in early microbial colonisation have been reported to be associated with a higher risk of a variety of diseases, including allergies, gut inflammatory conditions, and, more recently, obesity and diabetes⁽⁵⁾. Breast milk delivers numerous growth factors to the infant's gut, influencing the colonisation and maturation of bacteria in the intestinal mucosa, as well as antibacterial factors that influence the colonisation process. The intestinal microbiota of healthy breast-fed infants is mainly composed of bifidobacteria^(6,7), the amounts of which can reach up to 60–90% of the total faecal microbiota⁽⁸⁾.

Abbreviations: IF, infant formula; PUT, putrescine; SPD, spermidine; SPM, spermine.

* **Corresponding author:** C. Gómez-Gallego, fax +34 868 888497, email carlosgg@um.es

The microbial profile of formula-fed infants is more complex than, but similar to, that of adults, with predominant facultative anaerobes, such as *Bacteroides* and *Clostridium*, followed by *Staphylococcus*, *Streptococcus* and bacteria of the Enterobacteriaceae family. The colonisation of bifidobacteria is delayed^(9–13). The microbial colonisation pattern is characterised by changes in the main bacterial groups: the *Bacteroides–Prevotella* group; *Bifidobacterium*, *Clostridium*, *Lactobacillus* and *Staphylococcus* genera; Enterococcaceae and Enterobacteriaceae families^(1,14). Factors such as delivery mode, birth environment, prematurity, hygiene measures, maternal vaginal and cutaneous microbiota and infant feeding type (breast milk or formula) influence the establishment of microbiota^(1,6,15). It has been demonstrated that breast-fed infants are colonised with less number of bacteria of the Enterobacteriaceae family and *Clostridium* group compared with the formula-fed infants^(1,16). Other studies have also demonstrated that human infants given an infant formula are more affected by gastrointestinal disorders than the breast-fed infants. These differences are associated with an increase in local inflammation and, thus, different microbial populations^(17,18).

Breast milk is known to have a complex composition of nutrients and bioactive components that are designed to fulfil the needs of the young growing infant. Protective nutrients, such as cytokines, oligosaccharides and even microbes, in breast milk provide the newborn with the means to adapt to his or her particular environment^(19,20). Breast milk contains polyamines such as spermidine (SPD), spermine (SPM) and putrescine (PUT)^(21,22), which are gaining relevance due to their reported biological roles in eukaryotic cells⁽²³⁾. Polyamines are involved in the growth and development of the digestive tract wall and colonic mucosa in neonatal mammals⁽²⁴⁾ by helping with the maintenance of intestinal mucosal integrity^(25,26) and intestinal permeability⁽²⁷⁾. Moreover, there is evidence that polyamines participate in several processes related to the immune system, including its development and maturation^(28,29), inflammatory response^(30–32) and normal functioning⁽³³⁾. The concentration and effect of these compounds in infant formulas compared with those in human milk are of special interest, because their concentrations are lower than those in human milk⁽³⁴⁾.

In a previous study⁽³⁵⁾, a fluorescence *in situ* hybridisation analysis of samples of intestinal content has shown that an infant formula enriched with polyamines influences microbial colonisation patterns with a higher number of beneficial bacteria, such as *Bacteroides–Prevotella*, *Bifidobacterium* and *Akkermansia*-like bacteria, as well as the *Clostridium* sub-group. The *perfringens/histolyticum* and *Lactobacillus–Enterococcus* groups have been observed by fluorescence *in situ* hybridisation in samples of the large intestine from mouse pups fed breast milk, an infant formula and an infant formula enriched with polyamines. These previously reported animal model results are in accordance with the fact that the *Bifidobacterium*-dominated microbiota and *Lactobacillus* spp. are more frequently present in breast-fed human infants than in the formula-fed infants⁽¹⁾, suggesting that polyamines may interact with microbiota, driving the microbiota to a breast-feeding standard. However, further studies targeting

more bacterial groups and gastrointestinal sites are needed to determine the effect of polyamines on microbiota, mainly during early life. Thus, to expand our previous knowledge, in the present study, we aimed to compare the differences in the development of microbiota in the entire gastrointestinal tract of infants fed breast milk and a manufactured formula and to ascertain whether an infant formula enriched with different concentrations of polyamines influences early microbial colonisation in the neonatal BALB/cOlaHsd mouse model.

Materials and methods

Animals and study design

A total of sixty pups, derived from a breeding colony of BALB/cOlaHsd mice supplied by Harlan Laboratories[®], were used in the present study. The progenitor mice were 8 weeks old and were allowed to acclimatise for 30 d before breeding. All the mice were determined to be healthy on the basis of individual physical examinations and to be pathogen free based on the results of routine microbiological screening carried out in the colony in accordance with European recommendations⁽³⁶⁾.

On day 14 after birth, same-day-born litters were mixed and individually identified. Individual pups were randomly assigned to one of the four dietary groups according to dietary treatment. The study groups were breast-fed (unweaned) pups (n 12), early-weaned pups fed an infant formula (IF) (n 12) and early-weaned pups fed an IF enriched with low (n 12), intermediate (n 12) and high (n 12) concentrations of polyamines. The non-enriched formula and formula enriched with polyamines were prepared with warm water, following the manufacturer's instructions, and given to the pups twice daily (100 μ l each time) by oral administration. During the study, the unweaned pups were caged in pairs (one male and one female) with a mother. The weaned pups were caged in pairs (one male and one female) with a 28–32-d-old female mouse acting as a trainer to teach them how to eat and drink. Infant formulas, both non-enriched and enriched with polyamines, were orally administered to the control and treatment groups, respectively, twice daily. Handling was done at the same time range to avoid the influence of biological rhythms. The early-weaned pups were fed a porridge made with the IF not enriched with polyamines.

The study was carried out at the Central Animal Laboratory, University of Turku, Finland. Pilot experiments were carried out to optimise handling and treatment. The experimental protocol was approved by the National Ethics Committee for Animal Experiments in Finland (ESLH-2009-04 845/Ym-23). The mice were handled in accordance with Finnish legislation and the Council of European Convention ETS 123 on the use of vertebrate animals for scientific purposes.

Formulas and polyamines

PUT (D13208; Aldrich), SPD (2626; Sigma) and SPM (85590, Fluka) were added to the IF (3.38% PUT, 35.48% SPD and 61.14% SPM) based on the proportions found in human

milk^(21,22,34). The concentrations tested in the three polyamine groups were as follows: (1) low: 2.10 µg/d PUT, 22.05 µg/d SPD and 38.00 µg/d SPM; (2) intermediate: 4.20 µg/d PUT, 44.10 µg/d SPD and 76.00 µg/d SPM; (3) high: 8.40 µg/d PUT, 88.20 µg/d SPD and 152.00 µg/d SPM. The polyamines were prepared in water and kept refrigerated at 4°C until their addition to the IF. The polyamines were added to the IF immediately before feeding it to the mice to avoid degradation by polyamine oxidase.

The manufactured formula used in the present study was a commercial IF used for babies up to 6 months of age and fortified with nucleotides, α-lactalbumin, and *n-3* and *n-6* fatty acids, supplied by HERO España S.A. The commercial formula that was chosen contained no oligosaccharides or pre- or probiotics that could influence the microbial colonisation patterns. The non-enriched formula and formula enriched with polyamines (100 µl) were prepared with warm water, following the manufacturer's instructions, and given to the pups twice daily by oral administration.

Sample collection and DNA extraction

After the 4 d dietary intervention, the pups were anaesthetised with isoflurane and killed by cervical dislocation, following which the entire intestinal tract was removed. Samples of oral mucosa were collected using a sterile swab, and the contents of the stomach, small intestine and large intestine, including the caecum, were collected for further analysis. DNA was extracted using a modified QIAGEN stool DNA extraction kit (QIAGEN) with a previous bead-beating step.

Microbial composition analysis by quantitative PCR

PCR primers used for the characterisation of microbiota in the present study included those specific for total bacteria; *Bifidobacterium* genus and species, including *B. longum*, *B. breve*, *B. bifidum*, *B. animalis-lactis* and *B. catenulatum*;

Bacteroides-Prevotella group; *Clostridium coccoides*; *Clostridium leptum* subgroup; *Akkermansia muciniphila*; *Lactobacillus* group; Enterobacteriaceae family; *Enterococcus* group; and *Staphylococcus* and *Streptococcus* group (Table 1). These oligonucleotides were purchased from Isogen (Isogen Life Science). Quantitative PCR were carried out as described previously⁽⁴⁹⁾. Quantitative PCR amplification and detection were carried out using the LightCycler[®] 480 Real-Time PCR System (Roche). Each 10 µl reaction mixture contained SYBR[®] Green PCR Master Mix (Roche), 0.5 µl of each of the specific primers, at a concentration of 0.25 µM, and 1 µl of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was carried out after amplification to distinguish the targeted PCR products from the non-targeted PCR products. The concentration of bacteria in each sample was calculated by comparing the *C_t* values obtained from the standard curves. These were constructed using serial 10-fold dilutions of pure culture-specific DNA fragments corresponding to 10²–10⁸ gene copies/ml.

Statistical analysis

The SPSS 15.0 software (IBM) was used for statistical analysis. Due to non-normal distribution, a non-parametric test was used, and microbial data are expressed as medians with interquartile ranges. Comparisons among the data of more than two groups of pups were made by applying the Kruskal–Wallis test, and comparisons between data of two groups were made by applying the Mann–Whitney *U* test. The Bonferroni adjustment test was also used to correct the significance of multiple test comparisons among the three groups, which has the advantage of reducing type I errors and the disadvantage of increasing type II errors. The χ^2 test was used to establish differences in bacterial prevalence among the studied groups. The possible correlation between variables was studied by applying Spearman's rank correlation coefficient. Differences were considered significant at $P \leq 0.05$.

Table 1. Sequences of primers used in the study*

Probes	Target	Primer (5'–3')		Annealing temperature (°C)	Reference
		Forward	Reverse		
Universal	16S	AGAGTTTGATCCTGGCTCAG	GGCTGCTGGCACGTAGTTAG	50	Kullen <i>et al.</i> ⁽³⁷⁾
<i>Akkermansia muciniphila</i>	16S	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGT	60	Collado <i>et al.</i> ⁽³⁸⁾
<i>Bacteroides-Prevotella- Porphyromonas</i>	16S	GGTGTCGGCTTAAGTGCCAT	CGGAYGTAAGGGCCGTGC	64	Rinttilä <i>et al.</i> ⁽³⁹⁾
<i>Bifidobacterium</i> genus	16S	GATTCTGGCTCAGGATGAACGC	CTGATAGGACGCGACCCCAT	60	Gueimonde <i>et al.</i> ⁽⁴⁰⁾
<i>B. longum</i> group	16S	TTCCAGTTGATCGCATGGTCTTCT	GGCTACCCGTCAGCCACG	65	Gueimonde <i>et al.</i> ⁽⁴⁰⁾
<i>B. catenulatum</i> group	16S	GCCGGATGCTCCGACTCCT	ACCCGAAGGCTTGCTCCCGAT	64	Gueimonde <i>et al.</i> ⁽⁴⁰⁾
<i>B. bifidum</i>	16S	TGACCGACCTGCCCATGCT	CCCATCCCACGCGATAGAAT	61	Gueimonde <i>et al.</i> ⁽⁴⁰⁾
<i>B. breve</i>	16S	AATGCCGGATGCTCCATCACAC	GCCTTGCTCCCTAACAAAAGAGG	62	Gueimonde <i>et al.</i> ⁽⁴⁰⁾
<i>B. animalis-lactis</i>	16S	TCACGACAAGTGGGTTGCCA	GTTGATCGGCAGCTTGCCG	60	Sheu <i>et al.</i> ⁽⁴¹⁾
<i>Clostridium coccoides</i> group	16S	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCGAA	53	Matsuki <i>et al.</i> ^(42,43)
<i>Clostridium leptum</i> subgroup	16S	GCACAAGCAGTGGAGT	CTTCTCCGTTTTGTCAA	60	Matsuki <i>et al.</i> ^(42,43)
<i>Lactobacillus</i> group	16S	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	60	Walter <i>et al.</i> ⁽⁴⁴⁾ and Heilig <i>et al.</i> ⁽⁴⁵⁾
Enterobacteriaceae family	16S	CATTGACGTTACCCGAGAAAGC	CTCTACGAGACTCAAGCTTGC	63	Bartosch <i>et al.</i> ⁽⁴⁶⁾
<i>Enterococcus</i> group	16S	CCCTTATTGTTAGTTGCCATCATT	ACTCGTTGTAAGTCCATTGT	61	Rinttilä <i>et al.</i> ⁽³⁹⁾
<i>Staphylococcus</i> group	TUF	GGCCGTGTTGAACGTGGTCAAATCA	TIACCATTTCAGTACCTTCTGGTAA	60	Martineau <i>et al.</i> ⁽⁴⁷⁾
<i>Streptococcus</i> group	TUF	GTACAGTTGCTTCAGGACGTATC	ACGTTTCGATTTCATCACGTT	61	Picard <i>et al.</i> ⁽⁴⁸⁾

*Y represents a (C/T) wobble nucleotide.

Results

Shifts in microbial colonisation patterns in neonatal gastrointestinal tract according to diet

The colonisation patterns of total bacteria and *Lactobacillus*, *Bifidobacterium*, *A. muciniphila* and *Streptococcus* groups in the breast-fed neonatal BALB/c mice were different from those in the formula-fed mice (Fig. 1). The colonisation patterns of total bacteria were significantly different throughout the gastrointestinal tract (from the oral cavity to the large intestine) of both the breast-fed ($P=0.0001$) and formula-fed ($P=0.0001$) mice, with the large intestine showing the highest number of bacteria. In the breast-fed mice, no differences were detected in the colonisation patterns of total bacteria in the stomach and small intestine ($P=0.248$), while in the formula-fed mice, the colonisation patterns in the small intestine and oral cavity were similar ($P=0.366$). The colonisation patterns of the *Lactobacillus* and *Streptococcus* groups in the gastrointestinal tract of the breast-fed mice were significantly different from those in the formula-fed mice. The colonisation patterns of *Bifidobacterium* from the stomach to the large intestine in the breast-fed group were significantly different from those in the formula-fed group, in which no differences were observed between the pattern in the stomach and that in the small intestine ($P=0.564$). No significant differences in the colonisation pattern of *A. muciniphila* were observed in the breast-fed group ($P=0.069$); however, in the formula-fed

group, significantly higher amounts of *A. muciniphila* were observed in the large intestine than in the stomach and small intestine. There were no differences in the colonisation patterns in the stomach and small intestine ($P=0.132$).

Interestingly, the numbers of bacteria of all the groups that were analysed were found to be increased throughout the intestinal tract, with the large intestine showing the highest numbers. However, this tendency was not observed for the *Streptococcus* group, the amounts of which were increased throughout the intestinal tract from the mouth to the small intestine, but were decreased in the large intestine (Fig. 1).

Microbial composition of the breast-fed and formula-fed neonatal BALB/c mice

Microbial composition in the oral cavity and stomach.

Microbial composition in the oral cavity and stomach of the formula-fed BALB/c mice was different from that in the breast-fed mice. Higher DNA concentrations of total bacteria ($P<0.001$) were observed in the oral cavity of the formula-fed mice than in that of the breast-fed mice. There were significantly higher amounts of total bacteria ($P=0.0001$), bacteria of the *Lactobacillus* group ($P=0.001$) and those of the *Streptococcus* group ($P=0.008$) in the stomach of the breast-fed mice than in that of the formula-fed mice.

Microbial composition in the small intestine. Higher amounts of total bacteria ($P=0.000$), *A. muciniphila* ($P=0.005$),

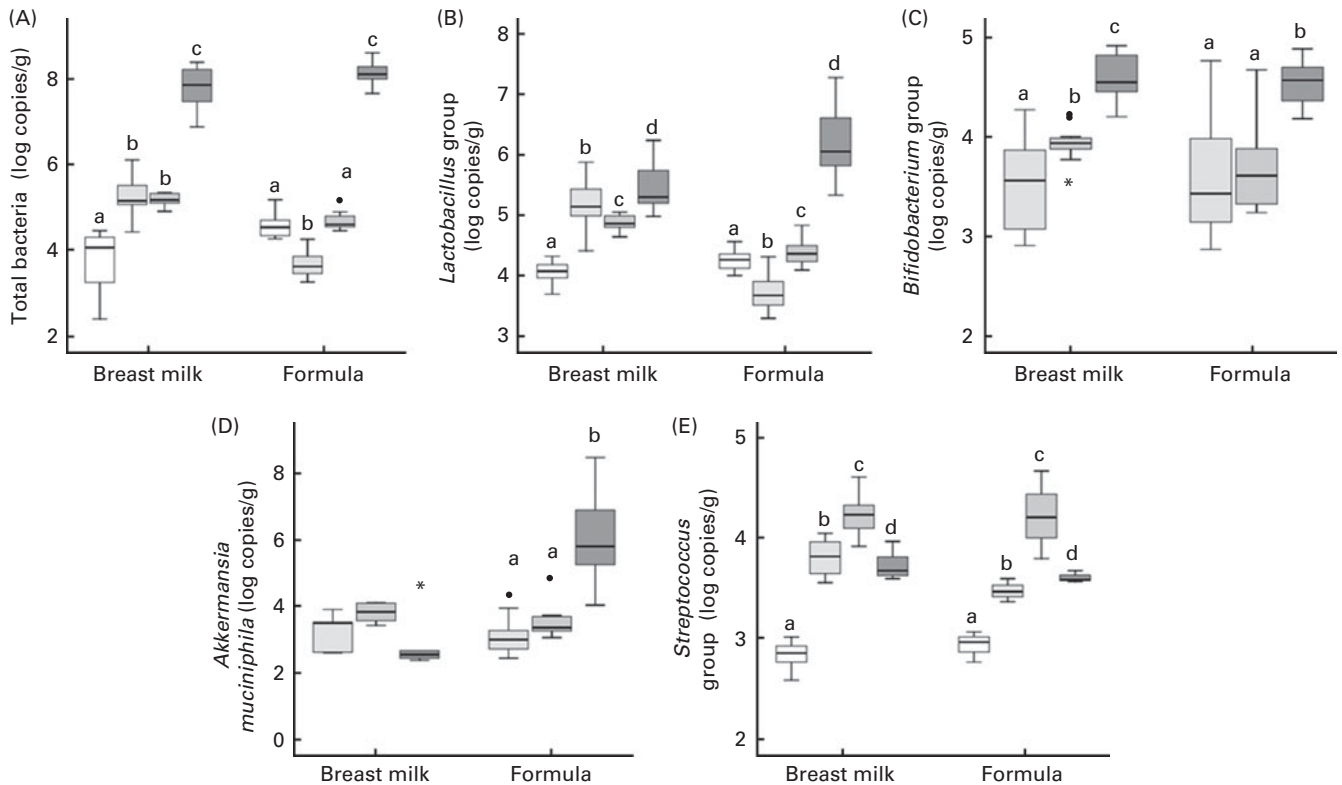


Fig. 1. Microbial colonisation in the gastrointestinal tract of BALB/c neonatal mice on different diets: breast milk and formula. (A) Total bacteria; (B) *Lactobacillus* group; (C) *Bifidobacterium* group; (D) *Akkermansia muciniphila*; (E) *Streptococcus* group. Differences among the sites were calculated using the Friedman test for related samples. ^{a,b,c,d} Values with unlike letters were significantly different among the areas for the same diet group ($P<0.05$). □, Oral cavity; □, stomach; □, small intestine; ■, large intestine.

Bifidobacterium ($P=0.012$), *Lactobacillus* ($P=0.000$), bacteria of the *Bacteroides–Prevotella* group ($P=0.007$) and *Streptococcus* ($P=0.001$) were found in the breast-fed BALB/c neonatal mice than in the formula-fed mice. The prevalence of the bacteria of the *Enterococcus* group was higher in the formula-fed mice than in the breast-fed mice ($P=0.005$), while *C. leptum*, *C. coccoides* and *Staphylococcus* were more common in the breast-fed mice ($P=0.035$, $P=0.041$ and $P=0.004$, respectively).

Microbial composition in the large intestine. Higher amounts of *A. muciniphila* ($P=0.000$), *Lactobacillus* ($P=0.005$), bacteria of the *Bacteroides–Prevotella* group ($P=0.000$), *Enterococcus* ($P=0.007$), bacteria of the Enterobacteriaceae family ($P=0.000$) and *C. leptum* ($P=0.024$) were found in the formula-fed mice than in the breast-fed mice during early life. In general, a higher prevalence of bacterial groups was found in the breast-fed group.

Impact of polyamine supplementation on microbiota composition

The formulas enriched with different concentrations of polyamines had an impact on microbial composition based on the polyamine concentration used and gastrointestinal site. On comparing the impact of different concentrations of polyamines throughout the gastrointestinal tract – oral cavity and stomach (Table 1S, available online), small intestine (Table 2S, available online) and large intestine (Table 3S, available online) – it was found that the microbial composition of the formula-fed mice was modified by the polyamines and that it became similar to that of the breast-fed mice in the majority of cases.

Oral cavity and stomach. The amounts of total bacteria and *Lactobacillus* in the oral cavity of the low polyamine group were significantly different from those present in the oral cavity of the intermediate and high polyamine groups (Fig. 2). However, higher concentrations of polyamines in the formula were found to be correlated with lower amounts of total bacteria and bacteria of the *Lactobacillus* and *Streptococcus* groups in the oral cavity and with higher amounts of total bacteria and *Lactobacillus* and bacteria of the *Bacteroides–Prevotella* group in the stomach.

Small intestine. The amounts of bacteria of the *Lactobacillus* group in the intermediate polyamine group were significantly different from those in the other polyamine groups ($P=0.001$) (Table 2S, available online). No differences were observed in the composition of other bacterial groups in the three polyamine groups. *B. animalis* was the most common *Bifidobacterium* species found in the intermediate and high polyamine groups, compared with *B. longum*, which was more prevalent in the formula-fed mice.

Increasing concentrations of polyamines in the formula were found to be correlated with higher amounts of total bacteria and *A. muciniphila* and bacteria of the *Bifidobacterium* group, *Lactobacillus* group, *Bacteroides–Prevotella* group and Enterobacteriaceae family in the small intestine (Fig. 3).

Large intestine. The supplementation of IF with polyamines had a significant impact on microbial composition throughout the gastrointestinal tract of the BALB/c mice after 4 d of intervention (Table 3S, available online). Formula supplemented with intermediate concentrations of polyamines showed lower amounts of total bacteria ($P=0.018$) and higher amounts of *Bifidobacterium* ($P=0.0001$) and *Lactobacillus*

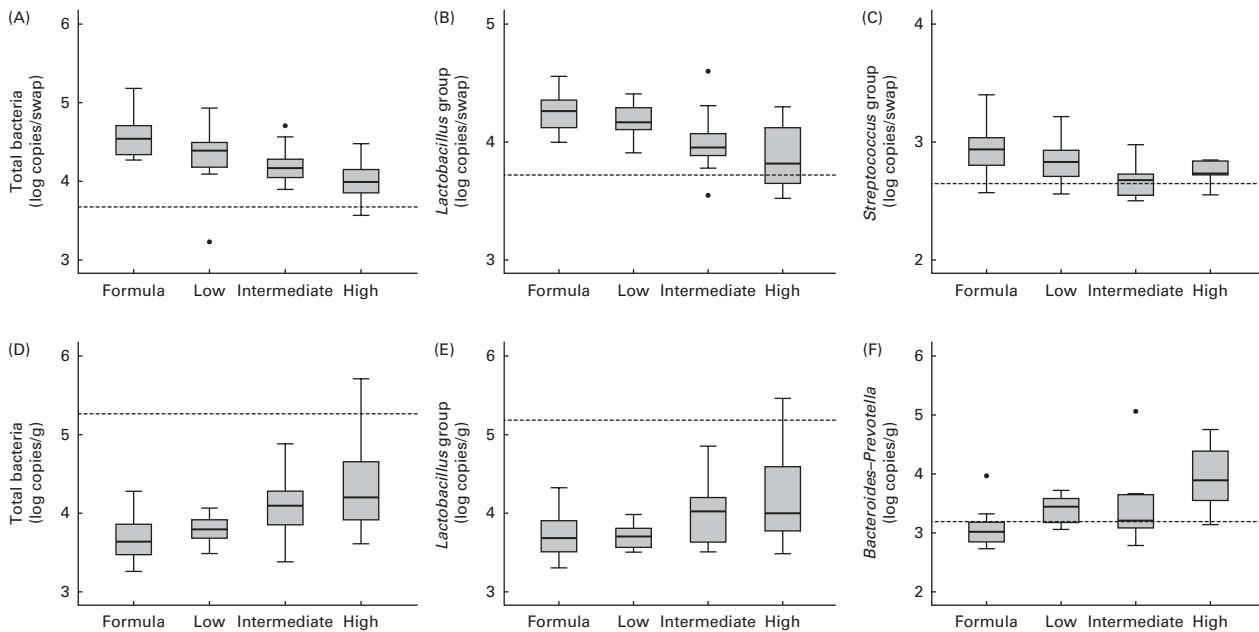


Fig. 2. Box-and-whisker diagrams and Spearman’s rank test correlations among the microbial compositions in the samples of the oral cavity and stomach of BALB/c mice, from 0 to increasing quantities of polyamine mixture added to the infant formula. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3) and largest observation. The correlation coefficient and significance level are expressed as θ . Oral cavity: (A) total bacteria ($\theta = -0.66$, $P=0.0001$); (B) *Lactobacillus* group ($\theta = -0.55$, $P=0.0001$); (C) *Streptococcus* group ($\theta = -0.45$, $P=0.002$). Stomach: (D) total bacteria ($\theta = 0.51$, $P=0.001$); (E) *Lactobacillus* group ($\theta = 0.42$, $P=0.003$); (F) *Bacteroides–Prevotella* ($\theta = 0.50$, $P=0.003$). Dotted line represents the mean value for the breast-fed group.

group ($P=0.004$) than the other groups of low and high concentrations of polyamines. We also analysed the *Bifidobacterium* species in the large intestine. *B. animalis* was the most common *Bifidobacterium* species found in all the groups included in the study, followed by *B. breve* and *B. catenulatum*. However, *B. bifidum* was detected in only one sample.

Higher concentrations of polyamines in the formula were found to be correlated with higher amounts of total bacteria, *Bifidobacterium* and *C. coccoides* and lower amounts of *A. muciniphila* (Fig. 4).

Discussion

The present study assessed the effect of infant feeding type on microbial colonisation patterns in the entire intestinal tract. The most predominant groups found in the intestinal contents of mice in the present study were similar to those found in human infants⁽¹⁴⁾. The bacterial populations in the breast-fed group could be considered to be present at normal levels during lactation in the BALB/cOlaHsd mice, with a non-altered mucus layer and without pathology. Significant differences in the predominant microbial groups between the breast-fed and IF-fed mice were observed, including in sites that have not been focused upon in previous research, such as the oral cavity and stomach. In general, we found significantly higher amounts of bacteria in the stomach and small intestine of the breast-fed mice than in those of the formula-fed mice. In particular, we found high amounts of total bacteria, bacteria of the *Lactobacillus* and *Bifidobacterium* groups, and *A. muciniphila*. An opposite scenario was found in the large intestine, with higher amounts of bacteria being found in the

formula-fed group, mainly due to high amounts of *A. muciniphila* and bacteria of the Enterobacteriaceae family. These differences could be correlated with an altered intestinal microbial colonisation pattern. Due to general similarities in the mammalian diet during early life and the development of the gastrointestinal tract, something similar may occur in humans.

The present study provides novel data on the impact of polyamines on microbial composition. To our knowledge, this is the first study to show how an IF enriched with polyamines has a significant impact on early microbial composition in the entire gastrointestinal tract of neonatal BALB/c mice. In general, the addition of polyamines to the manufactured formula regulated microbial populations to amounts found in the breast-fed group.

The present study also expands our previous knowledge on the impact of polyamines on microbial composition in the intestine⁽³⁵⁾. We had shown previously, using flow cytometry-fluorescence *in situ* hybridisation, that the amounts of bacteria of the *Bifidobacterium* group were significantly greater in the formula-fed mice following supplementation with polyamines ($P<0.01$). We confirmed these results by quantitative PCR in the present study, and found high amounts of bacteria of the *Bifidobacterium* group in the small and large intestines of pups fed the IF enriched with polyamines. The high amounts of bacteria of the *Bifidobacterium* group found in the small and large intestines of pups fed the IF enriched with polyamines could be a biological index of the health status of the intestinal tract⁽⁵⁰⁾. Recent studies have reported that high numbers of bifidobacteria may correlate positively with the normalisation of inflammatory status,

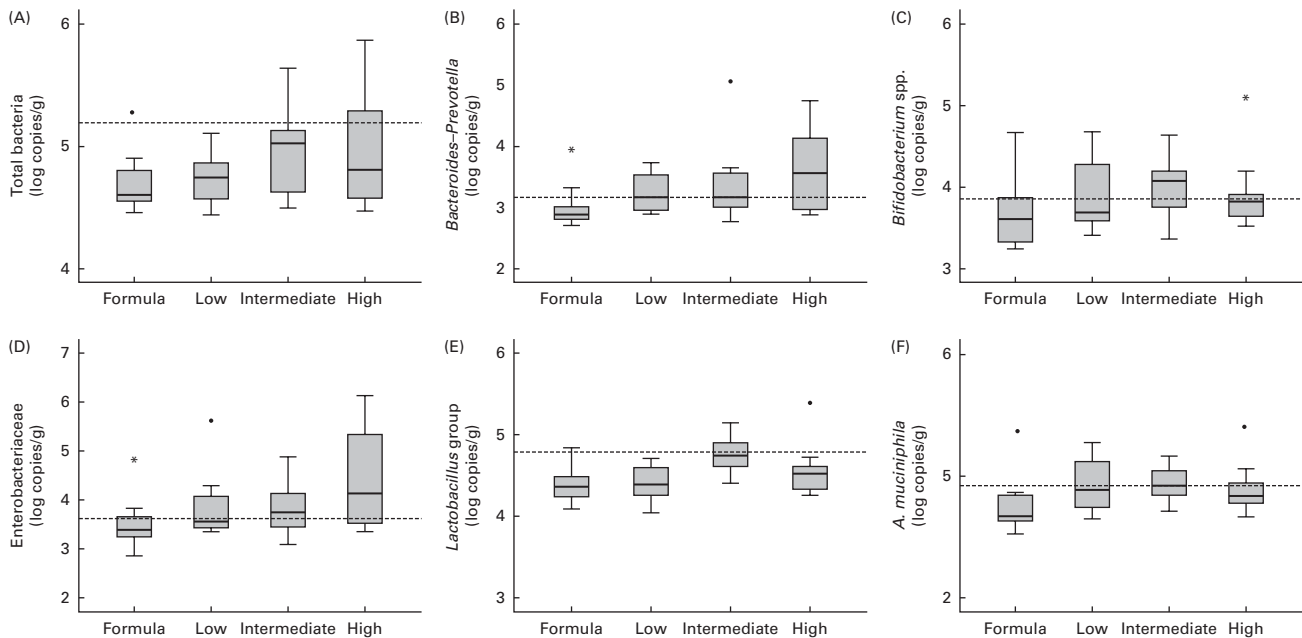


Fig. 3. Box-and-whisker diagram and Spearman's rank test correlations among the microbial compositions in the samples of the small intestine of BALB/c mice, from 0 to increasing quantities of polyamine mixture added to the infant formula. Each bar represents the smallest observation, lower quartile (Q1), median, upper quartile (Q3) and largest observation. The correlation coefficient and significance level are expressed as θ . Small intestine: (A) total bacteria ($P=0.046$); (B) *Bacteroides-Prevotella* group ($P=0.004$); (C) *Bifidobacterium* spp. ($\theta = 0.27$, $P=0.06$); (D) Enterobacteriaceae ($\theta = 0.40$, $P=0.004$); (E) *Lactobacillus* group ($P=0.007$); (F) *Akkermansia muciniphila* ($\theta = 0.31$, $P=0.035$). The dotted line represents the mean value for the breast-fed group.

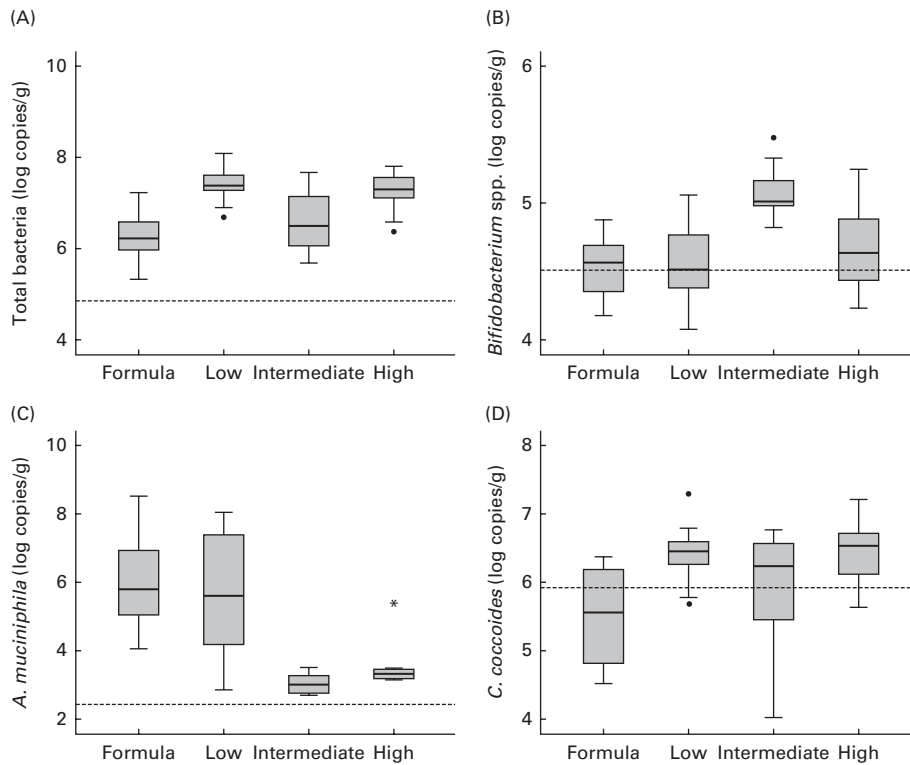


Fig. 4. Box-and-whisker diagram and Spearman's rank test correlations among the microbial compositions in the samples of the large intestine of BALB/c mice, from 0 to increasing quantities of polyamine mixture added to the infant formula. Each bar represents the smallest observation, lower quartile (Q1), median quartile, upper quartile (Q3) and largest observation. The correlation coefficient and significance level are expressed as θ . Large intestine: (A) total bacteria ($\theta = 0.35$, $P = 0.015$); (B) *Bifidobacterium* spp. group ($\theta = 0.35$, $P = 0.045$); (C) *Akkermansia muciniphila* ($\theta = -0.656$, $P = 0.0001$); (D) *Clostridium coccooides* ($\theta = 0.39$, $P = 0.006$). The dotted line represents the mean value for the breast-fed group.

improved glucose tolerance and glucose-induced insulin secretion, and reduced prevalence of atopic dermatitis^(50,51). Therefore, these results suggest that polyamine supplementation increases the number of bacteria of the *Bifidobacterium* species in the intestine and promotes healthy mucosal status, as bifidobacteria are the predominant microbiota of healthy breast-fed infants and are considered to be a hallmark of a healthy breast-fed infant.

This is the first study to identify populations of *A. muciniphila* in the oral cavity and stomach of the studied animals. The presence of *A. muciniphila* in the mouth and stomach could have two origins: (1) direct transfer through mouse milk and/or (2) exposure of the pups to maternal faeces (the study was started on day 14 and the pups began eating small amounts of solid foods on day 12). In addition, the differences between normal lactation and formula feeding in the presence of *A. muciniphila* populations in the large intestine appear to be reversed in the groups fed high-polyamine-content formula. Other researchers have reported that polyamines have a proliferative effect on enterocytes⁽²⁷⁾, which can decrease permeability to macromolecules and modulate the development and differentiation of the immune system⁽⁵²⁾. The combination of both these effects could be correlated with low local inflammation, and the amounts of *A. muciniphila* could be an indicator of healthy mucosal status. Epidemiological evidence⁽⁵³⁾ strongly suggests that the modulation of immune response mechanisms by

A. muciniphila in the gut can directly affect the development of allergic disease mechanisms in early life. However, the mechanisms by which intestinal immune responses translate into systemic anti-inflammatory or immunosuppressive effects remain to be established.

Moreover, we found reduced amounts of bacteria of the *Bacteroides-Prevotella* and *Lactobacillus* groups in the formula-fed neonatal BALB/c mice compared with the breast-fed and polyamine-enriched formula-fed mice. Together with the results obtained for bifidobacteria and *Akkermansia*, this could be an indicator of healthy mucosal status. The non-enriched formula-fed mice had significantly lower amounts of bacteria of the *C. coccooides* group than the polyamine-enriched formula-fed mice; however, no differences were found when compared with the breast-fed mice. The high effect of polyamine supplementation on bacterial populations in the small intestine could be due to the absorption of polyamines mainly in the duodenum and jejunum⁽⁵⁴⁾, and this fact allows for the postulation of a direct local impact of polyamines on the microbiota of the proximal small intestine, which continues in the large intestine in some bacterial populations.

The modulation of microbial colonisation patterns by polyamines can be explained as follows: polyamines, which are reported to be modulators of cell growth^(33,55), specifically increase the proliferation of beneficial bacterial groups; polyamines have an inhibitory effect on other bacterial groups,

making greater proliferation of beneficial bacterial groups possible; and/or the stimulation of the immune system by polyamines allows a greater spread of these beneficial host microbes.

In summary, our findings demonstrate a difference in microbial colonisation patterns between breast-fed and formula-fed BALB/cOlaHsd mice and indicate a potential effect of polyamines in minimising differences in the colonisation patterns of the main bacterial groups, which may have an impact on health. Dietary polyamine content during lactation might play a critical role in the succession and development of microbiota, maintaining a healthy environment in the gastrointestinal tract by increasing the barrier function, and modulation of immune system development, which, in turn, could be reflected in the amounts of the bacteria of *A. muciniphila*–*Bifidobacterium* spp. in the BALB/cOlaHsd mouse model. Such an effect should be further studied in human infants.

Supplementary material

To view supplementary material for this article, please visit <http://dx.doi.org/10.1017/S0007114513003565>

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