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Lactobacillus fermentum and Lactobacillus plantarum increased gut microbiota diversity and functionality, and mitigated Enterobacteriaceae, in a mouse model

C. Linninge1,4, J. Xu2, M.I. Bahl3, S. Ahrné1 and G. Molin1*

1Department of Food Technology, Engineering and Nutrition, Lund University, 221 00 Lund, Sweden; 2Department of Clinical and Experimental Medicine, Linköping University, 582 25 Linköping, Sweden; 3National Food Institute, Technical University of Denmark, Kgs. Lyngby, Denmark; 4BioGaia AB, Mobilvägen 10, 22362 Lund, Sweden; goran.molin@food.lth.se

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Abstract

Probiotics should bring ‘balance’ to the intestinal microbiota by stimulating beneficial bacteria, whilst mitigating adverse ones. Balance can also be interpreted as high alpha-diversity. Contrary, Escherichia coli is often regarded as an adverse component of the resident intestinal microbiota. The aim of the present study was to implement a mouse model for in vivo screening of Lactobacillus-strains for ability to increase gut-microbiota diversity and to mitigate E. coli. Mice were divided into six groups, two dietary control-groups and four groups administered strains of Lactobacillus fermentum and/or Lactobacillus plantarum. All animals were pre-treated with antibiotics, and E. coli in order to equalise the microbiota from the start. After 7 weeks of Lactobacillus administration, the animals were sacrificed: DNA was extracted from caecum tissue, and the microbiota composition was analysed with terminal restriction fragment length polymorphism (T-RFLP) and 16S rRNA gene sequencing. The diversity of the caecal microbiota decreased when the dietary carbohydrate source was limited to corn starch. Conversely, the diversity was restored by Lactobacillus-supplements. The tested combinations of two Lactobacillus strains exerted different influences, not only on the taxonomic level, but also on the inferred microbiome functions. The mixture of L. fermentum GOS47 and L. fermentum GOS1 showed potential for anti-inflammatory activity and short chain fatty acid production. On the other hand, co-administration of L. fermentum GOS57 and L. plantarum GOS42 significantly decreased the viable count of Enterobacteriaceae. These results warrant further investigation of the tested strains as candidates for probiotics. Furthermore, the findings demonstrated that the current experimental animal model is suitable for in vivo studies of the effect of bacterial supplements on the gut-microbiota.

Keywords: probiotics, screening, microbiota, microbiome-functions

1. Introduction

A traditional view on probiotics is that the administrated, live microorganism shall bring ‘balance’ to the intestinal microbiota and mitigate adverse bacteria (Fuller, 1989; Parker, 1974). Balance can also be interpreted in terms of high alpha-diversity. In contrast, examples of adverse components of the intestinal microbiota, that can cause overgrowth and, hence, severely decreased diversity are genera of the family Enterobacteriaceae, and in mammalians especially the species Escherichia coli. Pro-inflammatory lipopolysaccharides (LPS) are associated to the cell surface of E. coli (Poxton and Edmond, 1995). Furthermore, specific strains of E. coli can also be invasive, enterotoxin producing, and carrier of multiple antibiotic resistance genes. One example of an adverse effect of commonplace E. coli, is that early colonisation in infants increases the risk for atopic eczema later in life (Penders et al., 2007), and the same effect has low diversity, early in life (Abrahamsson et al., 2012; Ismail et al., 2012; Wang et al., 2008). It has also been observed that Enterobacteriaceae are abundant in the colonic mucosa of patients suffering from diverticular disease (Linninge et al., 2018). A clinically problem of considerable magnitude involving E. coli is urinary tract infections (UTIs) where it has been seen that uropathogenic E. coli often dominates the faecal microbiota of the
patients (Yamamoto et al., 1997). Uropathogenic E. coli has a capacity to migrate from the intestinal tract to the urinary tract, and E. coli is the infecting agent in about 80% of all UTIs, (Hooton and Stamm, 1997; Svanborg and Godaly, 1997). Uropathogenic strains of E. coli with multiple antibiotic resistance is of special public concern, because such infection can’t be efficiently treated with antibiotics. Mouse-models are frequently used in preclinical investigations. For example, colitis in mouse strongly alter the colonic microbiota (Håkansson et al., 2015) and increases the abundance of Enterobacteriaceae (Constante et al., 2017). Another mouse model was used for in vivo verification of a potential probiotic strain selected by in vitro tests (Silva et al., 2016), but the promising achievements failed to deliver in vivo.

The aim of the present study was to implement a mouse model for in vivo screening of different Lactobacillus-strains with probiotic potential, and search for strains able to increase microbiota diversity and to reduce the relative abundance of Enterobacteriaceae in vivo. The applied mouse model has previously been developed for this purpose (Linninge et al., 2015). The gut microbiota between different animals is in the model initially equalised by a short antibiotic pre-treatment followed by E. coli administration. Three novel strains of Lactobacillus fermentum and one of Lactobacillus plantarum were tested in combinations, in order to minimise the number of animals. The test-strains were compared with the scientifically established, and commercially available probiotic strain L. plantarum 299v (Molin, 2008). The caecal microbiota composition and diversity of the mice was investigated by 16S rRNA gene sequencing and terminal restriction fragment length polymorphism (T-RFLP).

2. Material and methods

Experimental set-up

Male C57BL/6NCrl mice, 8 weeks old with body weight 18.9 to 22.5 g, were purchased from Charles River laboratories (Sulzfeld, Germany), and randomly divided into six groups (eight animals/group; one cage/group). The animals were maintained in a temperature-controlled environment on a 12-h light/dark cycle. All experiments followed the national guidelines for the care and use of animals, and the study was approved by the Malmö/Lund regional ethics committee for laboratory animals (ethical permission, M209-11). All animals were pre-treated according to Linninge et al. (2015) in order to equalise the microbiota before the beginning of the test-series. Briefly, an antibiotic cocktail of ampicillin, metronidazole and clindamycin was given daily for three days in the drinking water (Linninge et al., 2015). The antibiotics were consumed at clinical relevant doses. Two non-pathogenic strains of E. coli were then given in the drinking water, for two days (Linninge et al., 2015). Water consumption was determined daily, and an average dose of 10^8 cfu of each E. coli strain was consumed by each mouse.

Diet and bacterial supplementation

The widely used and well-known, purified rodent diet AIN-76A was given to one group, while five groups were given a custom designed, purified open source, corn starch diet (CS-ctrl diet); with the intention to stimulate Enterobacteriaceae in the gut. The general composition of the CS-diet is based on AIN-76A. Both diets had an energy composition of 20.8 E% protein, 67.7 E% carbohydrates and 11.5 E% fat, and corn oil is the source of fat in both diets. The carbohydrates are provided by corn starch and sucrose in AIN-76A, while the sucrose was replaced by additional corn starch in the CS-diet; the total amount of carbohydrates was the same in both diets. Both diets were produced by Research Diets (New Brunswick, NJ, USA). The CS-ctrl diet was previously composed by Linninge et al. (2015) and was called ‘research diet’ (RD). Feed was administered ad libitum. After the five days pre-treatment, animals were given different Lactobacillus-strains in the drinking water for seven weeks according to Table 1.

The novel strains of L. fermentum GOS47, L. fermentum GOS1, L. fermentum GOS57 and L. plantarum GOS42 (= DSM 32131) have all been isolated from human saliva of healthy young adults. L. plantarum 299v was originally isolated from healthy human intestinal mucosa (Molin et al., 1993). The Lactobacillus-strains were supplemented in the drinking water. Drinking water with bacterial supplement was changed daily; each animal consumed 5×10⁷ to 1×10⁸ cfu of Lactobacillus per day.

Animal sacrifice and sampling

After seven weeks with bacterial supplementation in the drinking water, animals were sacrificed by CO₂ inhalation. The abdomen and chest were opened and spleen, liver,
small intestine (SI), caecum and epididymal fat pads were carefully dissected and weighed. Caecum tissue (rinsed in sterile PBS) and caecum content (stored in glycerol buffer; Karlsson et al., 2011b) were immediately frozen on dry ice, and then stored at -80 °C until analysis.

DNA extraction

DNA from caecum tissue was extracted according to Sand et al. (2015) with the modification that 100 µl Phosphate Buffered Saline (PBS, Oxoid, Basingstoke, UK) was added to 100 µl supernatant before extraction in the EZ1 Advanced XL and eluted in 200 µl.

Terminal restriction fragment length polymorphism

Endpoint PCR was performed in a reaction of 25 µl, including 1-3 µl template, for 25 cycles according to Sand et al. (2015). Terminal restriction fragment length polymorphism (T-RFLP) analysis including digestion with the restriction endonuclease MspI was performed as previously described (Sand et al., 2015). Thresholds for internal standard and terminal restriction fragments (T-RFs) were set to 20 and 40 fluorescence units, respectively.

Next generation sequencing

Next generation sequencing of the gut microbiota was performed using ion torrent sequencing with the Ion Torrent PGM platform (Life Technologies, Carlsbad, CA, USA) at the Technical University of Denmark (DTU) core facility (Lyngby, Denmark), in accordance with Sand et al. (2015). The primers were the same as used by Tulstrup et al. (2015), which were targeting V3 region of the 16S rRNA gene. The PCR reactions were conducted with 4 µl HF-buffer, 0.4 µl dNTP (10 mM of each base), 1 µM forward primer, 1 µM reverse primer, 5 ng template DNA, and 0.2 µl Phusion High-Fidelity DNA polymerase (F-530S, Thermo Scientific, Waltham, MA, USA) in a total reaction volume of 20 µl.

Sequences were demultiplexed and primers were removed using CLC bio genomic workbench (Qiagen). Further quality trimming was performed using default settings (quality score = 0.05, trim ambiguous nucleotides = 2) only reads with a final length between 110-180 bp were kept for downstream analysis. The number of good quality reads used for taxonomical assignment ranged from 19,225 to 98,494 per sample. The filtered sequences were then processed with an open source bioinformatics tool QIIME2 (https://qiime2.org/) (Caporaso et al., 2010). The sequences were further denoised with Qiime 2 plugin dada2 (Callahan et al., 2016). The resulting amplicon sequence variants (ASVs) were used for diversity analyses (alpha and beta) and taxonomic classification. The Naive Bayes classifier was trained on V3 region sequences of Greengenes 13_8 release (99% operational taxonomic units (OTUs)) (DeSantis et al., 2006) using QIIME2 plugin feature-classifier. Subsequent multivariate data analyses were performed at Calypso online web-server (Zakrzewski, 2017). Besides the taxonomic profiling, inferred metagenomes were predicted using PICRUSt (version 1.1.2) (Langille et al., 2013). The predicted metagenome was further analysed with HUMANN 0.99 (Abubucker et al., 2012) and statistical analyses were performed using STAMP v2.1.3 (Parks et al., 2014).

Quantitative PCR

Bacterial abundance of: total bacteria, the Clostridium leptum-group, the Bacteroides fragilis-group and the family Enterobacteriaceae were estimated in caecal tissue using separate quantitative PCR assays as previously described (Karlsson et al., 2011a; Linninge et al., 2015). Detection limit was 10^4 copies of 16S rRNA genes/reaction for total bacteria, 10^2 copies of 16S rRNA genes/reaction for both the C. leptum-group and Enterobacteriaceae. The B. fragilis-group had a detection limit of 10^3 copies/reaction. Samples below detection limit were set to the lowest detection limit for each specific assay. For standard curves, 10-fold dilution series of the target DNA were made in EB-buffer (Qiagen). Bacterial numbers were expressed as log10 16S rRNA genes/g caecum tissue.

Viable count of Enterobacteriaceae and lactobacilli

Caecum content was thawed and homogenised in glycerol buffer (Karlsson et al., 2011b) containing freezing medium, diluted and plated on Rogosa agar (Oxoid Ltd.) and incubated anaerobically at 37 °C for 72 h for lactobacilli count, and plated on violet red bile dextrose agar (VRBD), and incubated aerobically at 37 °C for 24 h for Enterobacteriaceae count (Merck, Darmstadt, Germany).

Calculations

Bacterial diversity based on T-RFLP was estimated by calculation of richness (number of T-RFs) and Shannon and Simpson diversity indices as previously described (Sand et al., 2015). Statistical analyses were performed in GraphPad Prism 7 (La Jolla, CA, USA) using Kruskal-Wallis test and Dunn’s multiple comparisons test to reveal differences compared to group CS-ctrl. P<0.05 was considered statistically significant. Statistical analyses on the 16S amplicon data and the inferred metagenome data obtained by PICRUSt (Langille et al., 2013) were performed at Calypso online web-server (Zakrzewski, 2017).
3. Results

Animal status

The pre-treatment with antibiotics and *E. coli* were well tolerated by all animals. No adverse effects were observed, and no signs of disease occurred during the experimental period. In group LFP, one animal was injured, probably by other animals; the injured animal was removed from the cage and excluded from the study. No difference in feed consumption was observed between the groups. Animals of the control group with the CS-diet, and without any *Lactobacillus* supplementation (CS-ctrl), had a significantly (*P*<0.05) lighter small intestine than animals of the AIN-76 diet. No other statistically significant differences in animal constitution were seen between the different test groups (Table 2).

Microbiota

The sequencing failed in two samples, one from group AIN-76A and one from CS-ctrl, and therefore these mice were removed from the sequencing analysis. *Lactobacillus* supplementation increased the diversity of the microbiota and changed its structure: *Firmicutes* and *Bacteroidetes* were the most dominating phyla followed by *Actinobacteria* in all groups, while *Tenericutes* were more abundant in the two dietary control groups without *Lactobacillus* supplementation (AIN-76 and CS-ctrl, respectively; Figure 1). Abundant taxa differed between the groups: At the hierarchical levels of family and of genus distinctive clusters were formed (Figure 2). Different bacterial genera were found statistically enriched with large effect size, between the animal groups (Figure 3). For example, *Bifidobacterium* was favoured in the group 299v, and *Lactobacillus* abundance was highest in the LP group (Figure 3).

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Animal groups2</th>
<th>AIN-76A</th>
<th>CS-ctrl</th>
<th>299v</th>
<th>LF</th>
<th>LP</th>
<th>LFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body</td>
<td>29 (28.7-31.3)</td>
<td>27.1 (26.6-27.8)</td>
<td>28.1 (25.6-28.7)</td>
<td>28.1 (27.2-29.8)</td>
<td>27.6 (26.527.9)</td>
<td>26.2 (25.6-28.7)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.070 (0.067-0.087)</td>
<td>0.064 (0.063-0.073)</td>
<td>0.064 (0.062-0.068)</td>
<td>0.066 (0.064-0.072)</td>
<td>0.073 (0.064-0.084)</td>
<td>0.063 (0.061-0.072)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.4 (1.31-1.51)</td>
<td>1.27 (1.20-1.38)</td>
<td>1.19 (1.04-1.30)</td>
<td>1.21 (1.17-1.28)</td>
<td>1.19 (1.01-1.26)</td>
<td>1.12 (1.10-1.19)</td>
<td></td>
</tr>
<tr>
<td>Epidymal fat pad</td>
<td>0.757 (0.548-0.936)</td>
<td>0.628 (0.528-0.790)</td>
<td>0.607 (0.494-0.754)</td>
<td>0.597 (0.435-0.772)</td>
<td>0.464 (0.385-0.664)</td>
<td>0.463 (0.41-0.536)</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.035* (0.032-0.042)</td>
<td>0.025 (0.019-0.028)</td>
<td>0.024 (0.021-0.026)</td>
<td>0.030 (0.024-0.033)</td>
<td>0.030 (0.024-0.033)</td>
<td>0.023 (0.021-0.025)</td>
<td></td>
</tr>
<tr>
<td>Caecum</td>
<td>0.239 (0.218-0.264)</td>
<td>0.264 (0.235-0.271)</td>
<td>0.266 (0.231-0.285)</td>
<td>0.263 (0.233-0.286)</td>
<td>0.275 (0.221-0.328)</td>
<td>0.275 (0.218-0.306)</td>
<td></td>
</tr>
</tbody>
</table>

1 An asterisk denotes statistically significant difference compared to CS-ctrl, *P*<0.05.
2 Animal diet groups are explained in Table 1.

Figure 1. Bar plot of relative abundance of the mice caecal microbiota at phylum level. Horizontal greyscale code bars at the top indicate the different treatment groups. One bar corresponds to one mouse and numerical numbers are the randomisation identities of the animals.
The dietary change in carbohydrate source, where sucrose was replaced by corn starch, decreased the microbiota diversity in the CS-ctrl group, while Lactobacillus supplementation restored the diversity (Table 3). There was also a significant difference in the beta diversity between the six treatment groups ($P<0.001$, PERMANOVA on Bray Curtis distances, Figure 4).

Lactobacillus supplementation increased the lactobacilli population and suppressed Enterobacteriaceae, but the total amount of bacterial 16S rRNA genes in caecum tissue, and the number of genes in the C. leptum group did not differ depending on dietary regime or bacterial supplement (Table 3). 16S rRNA genes of Bacteroides fragilis were not detectable in any of the groups. No 16S rRNA genes of Enterobacteriaceae were found in groups AIN-76A, 299v, LF, LP and LFP. 16S rRNA genes of Enterobacteriaceae above the detection limit of PCR, could only be quantified in the CS-ctrl group. The amount corresponded to log 6.81 16S rRNA gene copies/gram caecum tissue, with an interquartile range of 6.24-7.3. In contrast, Enterobacteriaceae enumerated by viable count were found in all groups, indicating a certain effect of the pre-treatment procedure. Viable count had a lower detection level than PCR in the present settings. The supplementation of L. fermentum GOS57 + L. plantarum GOS42 in group LP resulted in a significant decrease in the number of Enterobacteriaceae, compared to CS-ctrl group (Table 3).

Supplementation of Lactobacillus in groups 299v, LF and LP significantly increased the viable count of lactobacilli in caecum content (Table 3). Lactobacilli of non-probiotic origin were found in the AIN-76A group, while the CS-ctrl group lacked detectable lactobacilli, which also was one purpose of the pre-treatment procedure of the animals.

**Microbiome**

Inferred microbiome functions differed between groups: Inferred metagenomic profiles were different between the two dietary control groups, but also between groups supplemented with different Lactobacillus strains (Figure 5). Ten KEGG pathways and six KEGG modules were significantly different between AIN-76 and CS-ctrl (Figure 6). After observing the considerable differences between the two control groups, the Lactobacillus supplemented groups were compared with CS-ctrl. Abundance of 15 KEGG pathways and 16 KEGG modules were significantly different with large effect size (Table 4). For instance, pathways involved in the short chain fatty acids (SCFAs) production (Ko00620, ko00640) were enriched in the LF and 299v groups compared to CS-ctrl. On the other hand, multiple sugar transport system (M00207) was most abundant in the CS-ctrl group.
Figure 3. Significantly enriched genera among the tested six groups revealed by linear discriminant analysis coupled with effect size (LEFSe). Statistical significance was set to $P<0.05$ and LDA>3.0.

Table 3. Diversity based on next generation sequencing (NGS) and terminal restriction fragment length polymorphism (T-RFLP), quantification of 16S rRNA genes and viable count in different test groups.1

<table>
<thead>
<tr>
<th>Animal groups2</th>
<th>AIN-76A</th>
<th>CS-ctrl</th>
<th>299v</th>
<th>LF</th>
<th>LP</th>
<th>LFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diversity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Richness</td>
<td>10.5 (5.25-15.3)</td>
<td>9 (8-15)</td>
<td>23* (13-26)</td>
<td>17 (15-20)</td>
<td>20.5** (17.8-23.5)</td>
<td>14 (12-17)</td>
</tr>
<tr>
<td>Cho 1 (NGS)</td>
<td>97 (89-103)</td>
<td>83 (75-91)</td>
<td>94 (87.8-94.8)</td>
<td>105* (97.1-108)</td>
<td>101 (85.5-108)</td>
<td>118** (96-121)</td>
</tr>
<tr>
<td>Shannon index</td>
<td>2.08 (1.32-2.35)</td>
<td>1.87 (1.61-2.30)</td>
<td>2.34 (2.16-2.87)</td>
<td>2.43* (2.37-2.58)</td>
<td>2.68** (2.54-2.75)</td>
<td>2.34 (1.92-2.41)</td>
</tr>
<tr>
<td>Shannon index</td>
<td>5.23** (5.17-5.38)</td>
<td>4.73 (4.62-4.82)</td>
<td>5.19* (5.09-5.34)</td>
<td>4.71 (4.49-4.99)</td>
<td>4.95 (4.86-5.09)</td>
<td>5.54** (4.93-5.84)</td>
</tr>
<tr>
<td>Simpson index</td>
<td>0.826 (0.667-0.866)</td>
<td>0.792 (0.714-0.842)</td>
<td>0.858 (0.826-0.923)</td>
<td>0.884* (0.863-0.905)</td>
<td>0.903*** (0.891-0.918)</td>
<td>0.848 (0.772-0.892)</td>
</tr>
<tr>
<td>Simpson index</td>
<td>0.96* (0.95-0.96)</td>
<td>0.94 (0.92-0.94)</td>
<td>0.95 (0.94-0.96)</td>
<td>0.89 (0.87-0.93)</td>
<td>0.94 (0.94-0.95)</td>
<td>0.97* (0.94-0.97)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quantification of 16S rDNA (copies/g tissue)</th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>8.68 (8.41-8.79)</td>
<td>8.95 (8.76-9.04)</td>
<td>8.70 (8.56-9.01)</td>
<td>9.15 (9.00-9.36)</td>
<td>9.05 (8.94-9.21)</td>
<td>8.92 (8.80-9.05)</td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td>7.12 (6.81-7.27)</td>
<td>7.03 (6.97-7.19)</td>
<td>6.92 (6.68-7.04)</td>
<td>9.15 (9.00-9.36)</td>
<td>9.05 (8.94-9.21)</td>
<td>8.92 (8.80-9.05)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Viable count (log cfu/g caecum content)</th>
<th></th>
<th></th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Lactobacilli</td>
<td>5.72* (5.18-5.98)</td>
<td>0 (0-0)</td>
<td>6.48*** (6.37-6.59)</td>
<td>5.59* (4.99-6.14)</td>
<td>6.18*** (5.90-6.57)</td>
<td>4.93 (3.55-6.64)</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>2.95 (1.97-3.64)</td>
<td>3.62 (3.29-4.09)</td>
<td>3.73 (3.37-4.17)</td>
<td>3.26 (2.80-3.54)</td>
<td>2.63* (2.37-2.89)</td>
<td>2.3 (0-3.50)</td>
</tr>
</tbody>
</table>

1 Median values, and interquartile ranges within parenthesis are presented. Asterisks denote significantly different compared to the CS-ctrl group, * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$.

2 Animal diet groups are explained in Table 1.
L. fermentum and L. plantarum increased gut microbiota diversity

4. Discussion

The mouse model used in the present study was developed with the primary goal to build an in vivo, screening model for the evaluation of potentially probiotic strains of Lactobacillus (Linninge et al., 2015). Requirements for the model included (1) the natural microbiota of the individual animals should be as uniform as possible, (2) the spontaneously occurring Lactobacillaceae should be minimised, and (3) all animals should from the start harbour substantial amounts of Enterobacteriaceae. All these demands have been fulfilled by the model: The individual differences in microbiota within each group were considerably smaller than that between the different groups (Figure 2 and 4). No lactobacilli, but a measurable amount of Enterobacteriaceae, were detected by viable count in the CS-ctrl diet control group (Table 3). Furthermore, in contrast to the conventional AIN-76A diet, the CS-ctrl diet favoured other components of the microbiota, e.g. the gram-negative families Bacteroidaceae and Porphyromonadaceae (data not shown).

A limitation of the present model-design is that animals given different bacterial supplements cannot be maintained in the same cage, i.e. a certain ‘cage effect’ cannot be ruled out in the present study design. However, the microbiota of individual mice living in the same cage, under more conventional circumstances can differ widely in spite of the same cage-environment. At the same time, the condition of the present model strongly equalises the microbiota from the start, which should minimise individual differences (Linninge et al., 2015). Thus, the risk of a cage-effect is somewhat decreased by the selective power of the model. The fact that the animals in groups CS-ctrl and 299v cluster so closely together, while the other groups are so different, points in the same direction, i.e. that the eventual cage-effect seems to be of secondary importance (Figure 4).

A highly interesting feature for probiotics is the ability to induce increased diversity of the gut-microbiota. Despite the difference in resolution capacity between the T-RFLP and NGS, the diversity estimation of the caecal microbiota points in the same direction. All the Lactobacillus-supplemented groups showed, statistically significant increase in alpha diversity in either T-RFLP, NGS or both analysis (Table 3). Regarding the beta-diversity (Figure 4), it was observed that the effect from probiotic
strain *L. plantarum* 299v differed largely from the other *Lactobacillus* supplemented groups and was more similar to the two control groups. This indicates that just a smaller change in the dominant bacterial taxa was induced by *L. plantarum* 299v. In contrast, the Shannon diversity index was significantly increased (Table 3), which indicates that the number of rare taxa had increased in the 299v group. The 299v-supplement favoured bifidobacteria to highest degree (Figure 3) but showed no effect against Enterobacteriaceae (Table 3).

Generally, the three tested combinations of novel strain-supplementations influenced the microbiota in a way different from *L. plantarum* 299v (Figure 4), indicating strain specific effects. The observed differences in microbiota between animals in group LPF seem to be imposed by another parameter than the supplementation of the mixture of all four strains (group LFP). The heterogeneity within the LPF-group may be a result of stress as one of the animals was found injured and had to be taken away; maybe the stress was due to aggression between certain animals in this particular group. No significant changes in the viable count of lactobacilli and *Enterobacteriaceae* could be seen in this disturbed group (group LPF) compared to group CS-ctrl (Table 3).

The strain specific effects were not limited to the influence on the bacterial members of the microbiota, but also to the inferred microbiome functions (Figure 5). Interestingly, a change in the sort of dietary carbohydrate in CS-ctrl led to a substantial change in microbiome functions (Figure 6). Bacterial functions involved in glycosaminoglycan degradation (ko00531) and LPS biosynthesis (ko00540) were enriched when the diet carbohydrate source was only corn starch. On the contrary, LF suppressed these two pathways (Figure 7). Bacterial glycosaminoglycan degradation has been linked to inflammatory bowel disease in both animals and humans (Lee et al., 2009; Murch et al., 1993). LPS is well known to induce production of proinflammatory mediators. Furthermore, LF had significantly enriched pathways involved in the SCFA production. Thus *L. fermentum* GOS47 and *L. fermentum* GOS1 should be considered for further investigations as candidate probiotics. On the other hand, the viable count

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Figure 6. Extended error bar plot showing significantly different abundant KEGG pathways (A) and modules (B) between AIN-76A and CS-ctrl. *P*-values are corrected with Bonferroni correction.
of Enterobacteriaceae was significantly decreased only in group LP, which can be a highly beneficial trait for a potential probiotic strain. The question remains whether the observed effect requires the presence of both strains,
or if one of them is sufficient? This is a question that can be solved by applying the present screening model on animals given singular strains and then compare them with animals given the two strains together. However, this is outside the scope of the present study, but would be an important follow up study.

Several probiotic strains of the species *L. fermentum* and *L. plantarum* are commercially available. Two such examples are *L. plantarum* 299v and *L. fermentum* ME-3, respectively. The literature about the former are vast, and the strain 299v has, for example, been proved efficient to mitigate irritable bowel syndrome (Ducrotté et al., 2012; Nobaek et al., 2000). Strain 299v was also able to increase the diversity of the abundant microbiota on colonic mucosa in humans, after a four weeks intervention period (Karlsson et al., 2010).

*L. fermentum* ME-3 has been shown to possess antimicrobial activity against intestinal pathogens and to have an outstanding high antioxidative activity (Mikelsaar and Zilmer, 2009). Generally speaking, the species *L. plantarum* and *L. fermentum* often occupy similar ecological niches in nature, they can both frequently be isolated from lactic acid fermented foods, but also from the gastro-intestinal tract of humans and animals. But, the two species are quite different, both when it comes to phenotype and genotype, so they can be expected to have different physiological and immunological effects in the gastro-intestinal tract. And perhaps, there could be advantages in combining strains of the two species in a probiotic mixture. Nevertheless, the LP-supplementation affected not only the viable count of lactobacilli (seen as an increase), and of *Enterobacteriaceae* (seen as a decrease), but resulted in a substantial increase in the proportion of lactobacilli in comparison with the total microbiota (Figure 3). No such effect was seen in the other *Lactobacillus*-supplemented groups.

### 5. Conclusions

The experimental animal model with equalised microbiota was proved suitable for *in vivo* studies on the effect of bacterial supplements on the microbiota. Supplementation with *L. fermentum* GOS47 and *L. fermentum* GOS1 and the mixture of *L. fermentum* GOS57 + *L. plantarum* GOS42 strongly affected the gut microbiota and increased diversity. Furthermore, the former combination may induce anti-inflammatory activity and SCFA production in the microbiota (inferred from 16S rRNA-gene compositional data), and the latter combination decreased the numbers of *Enterobacteriaceae*, and improved the abundance of lactobacilli. These two mixtures seem to have probiotic potential that call for further investigations.

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**Figure 7.** Extended error bar plot showing significantly different abundant KEGG pathways between CS-ctrl and LF. *P*-values are corrected with Bonferroni correction.
L. fermentum and L. plantarum increased gut microbiota diversity

Conflicts of interest

Göran Molin and Siv Ahrné are minor, minority stockholders in the public company, Probi AB. Caroline Linninge has taken up a position in the public company BioGaia AB, after the experimental part of the project was finalised, and the manuscript draft had been prepared.

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