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

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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 mammals 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  $5 \times 10^7$  to  $1 \times 10^8$  cfu of *Lactobacillus* per day.

### Animal sacrifice and sampling

After seven weeks with bacterial supplementation in the drinking water, animals were sacrificed by CO<sub>2</sub> inhalation. The abdomen and chest were opened and spleen, liver,

**Table 1. Dietary regime and bacterial supplements.**

| Group   | Diet <sup>1</sup> | Bacterial strains   |
|---------|-------------------|---|
| AIN-76A | AIN-76A           | -   |
| CS-ctrl | CS                | -   |
| 299v    | CS                | <i>Lactobacillus plantarum</i> 299v                             |
| LF      | CS                | <i>Lactobacillus fermentum</i> GOS47 + <i>L. fermentum</i> GOS1 |
| LP      | CS                | <i>L. fermentum</i> GOS57, <i>L. plantarum</i> GOS42            |
| LFP     | CS                | GOS47 + GOS1 + GOS57 + GOS42                                    |

<sup>1</sup> AIN-76A is a traditional, purified rodent diet; CS is a custom designed, purified open source diet where the sucrose in AIN-76A had been replaced by corn starch.

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, Hilden, Germany). 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 Naïve 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<sup>4</sup> copies of 16S rRNA genes/reaction for total bacteria, 10<sup>2</sup> copies of 16S rRNA genes/reaction for both the *C. leptum*-group and *Enterobacteriaceae*. The *B. fragilis*-group had a detection limit of 10<sup>3</sup> 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 log<sub>10</sub> 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

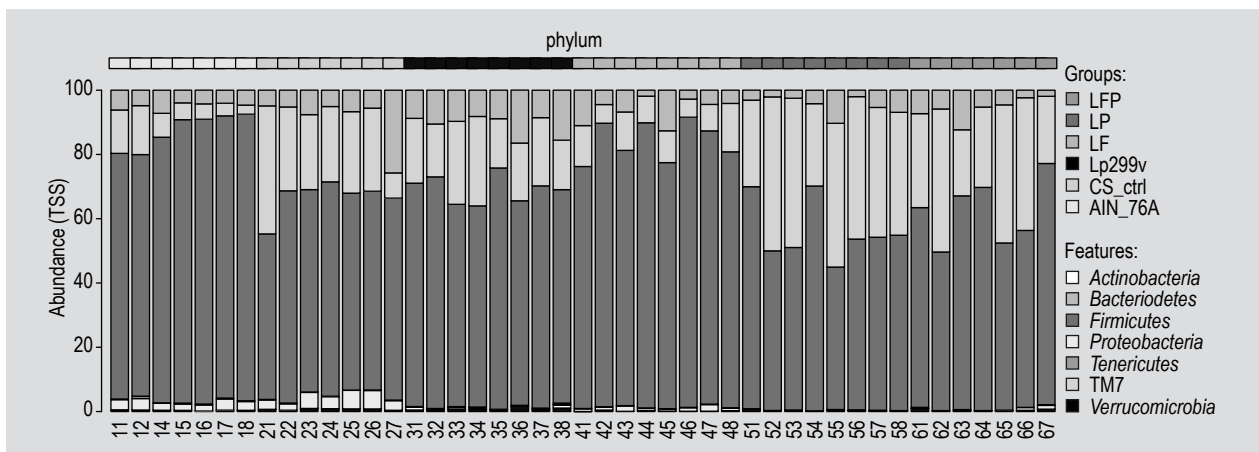
The sequencing failed in two samples, one from group AIN-76A and one from CS-ctrl, and therefore these mice were removed from the sequencing analysis. *Lactobacillus* supplementation increased the diversity of the microbiota and changed its structure: *Firmicutes* and *Bacteroidetes* were the most dominating 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 2. Median values, and interquartile ranges, of body and organ weights of mice in different test groups.<sup>1</sup>**

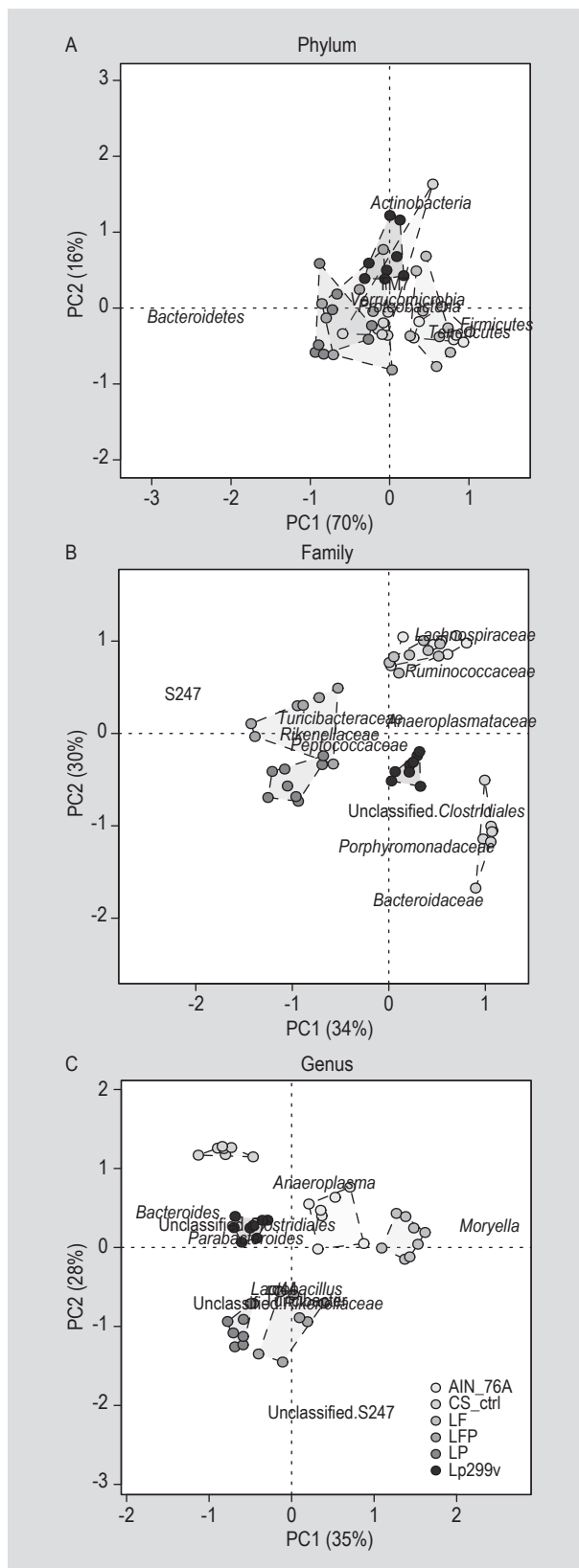
| Weight (g)       | Animal groups <sup>2</sup> |                     |                     |                     |                     |                     |
|------------------|----------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                  | AIN-76A                    | CS-ctrl             | 299v                | LF                  | LP                  | LFP                 |
| Body             | 29 (28.7-31.3)             | 27.1 (26.6-27.8)    | 28.1 (25.6-28.7)    | 28.1 (27.2-29.8)    | 27.6 (26.5-27.9)    | 26.2 (25.6-28.7)    |
| Spleen           | 0.070 (0.067-0.087)        | 0.064 (0.063-0.073) | 0.064 (0.062-0.068) | 0.066 (0.064-0.072) | 0.073 (0.064-0.084) | 0.063 (0.061-0.072) |
| Liver            | 1.4 (1.31-1.51)            | 1.27 (1.20-1.38)    | 1.19 (1.04-1.30)    | 1.21 (1.17-1.28)    | 1.19 (1.01-1.26)    | 1.12 (1.10-1.19)    |
| Epidymal fat pad | 0.757 (0.548-0.936)        | 0.628 (0.528-0.790) | 0.607 (0.494-0.754) | 0.597 (0.435-0.772) | 0.464 (0.385-0.664) | 0.463 (0.41-0.536)  |
| Small intestine  | 0.035* (0.032-0.042)       | 0.025 (0.019-0.028) | 0.024 (0.021-0.026) | 0.030 (0.024-0.033) | 0.030 (0.024-0.033) | 0.023 (0.021-0.025) |
| Caecum           | 0.239 (0.218-0.264)        | 0.264 (0.235-0.271) | 0.266 (0.231-0.285) | 0.263 (0.233-0.286) | 0.275 (0.221-0.328) | 0.275 (0.218-0.306) |

<sup>1</sup> An asterisk denotes statistically significant difference compared to CS-ctrl,  $P<0.05$ .

<sup>2</sup> 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.**



**Figure 2. Principal component analysis (PCA) of the relative abundance of mice caecal microbiota at different hierarchical levels. One dot represent one animal and different diet groups are shown with different greyscales. The location of the taxa shows how those bacteria drive the separation of the groups.**

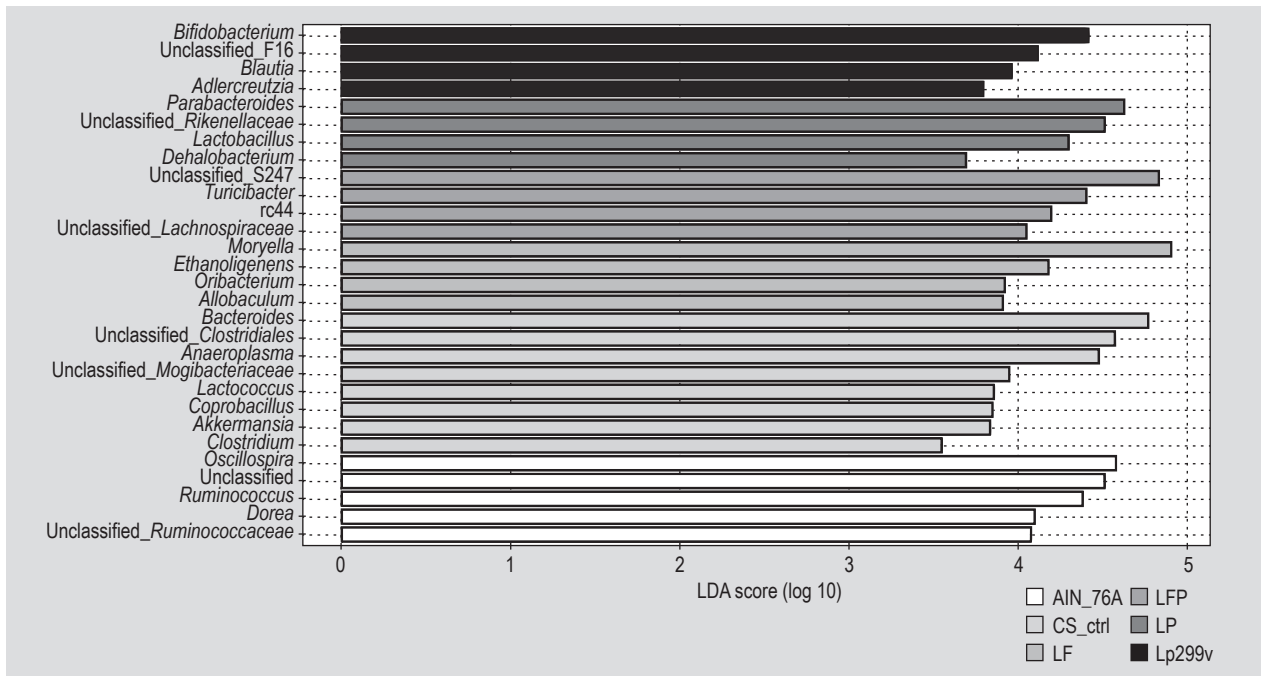
The dietary change in carbohydrate source, where sucrose was replaced by corn starch, decreased the microbiota diversity in the CS-ctrl group, while *Lactobacillus* supplementation restored the diversity (Table 3). There was also a significant difference in the beta diversity between the six treatment 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.



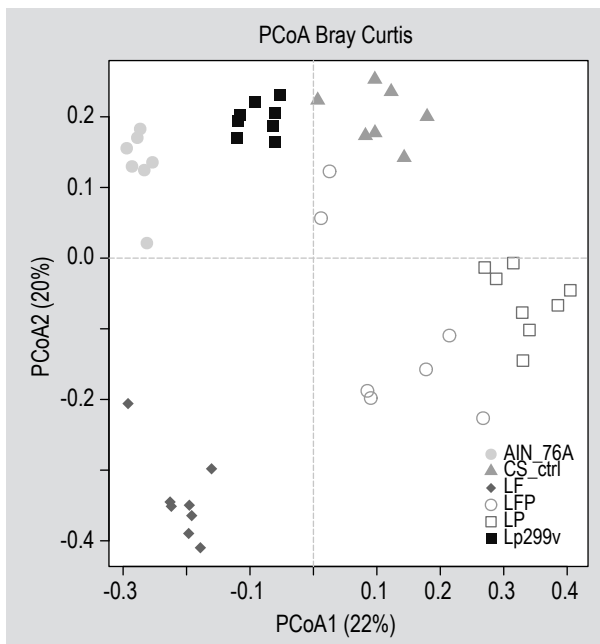
**Figure 3.** Significantly enriched genera among the tested six groups revealed by linear discriminant analysis coupled with effect size (LEFSe). Statistical significance was set to  $P < 0.05$  and  $LDA > 3.0$ .

**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.<sup>1</sup>

|  | Animal groups <sup>2</sup> |                     |                      |                      |                        |                     |
|--|----------------------------|---------------------|----------------------|----------------------|------------------------|---------------------|
|  | AIN-76A                    | CS-ctrl             | 299v                 | LF                   | LP                     | LFP                 |
| Diversity                                    |                            |                     |                      |                      |                        |                     |
| Richness (T-RFLP)                            | 10.5 (5.25-15.3)           | 9 (8-15)            | 23* (13-26)          | 17 (15-20)           | 20.5** (17.8-23.5)     | 14 (12-17)          |
| Cho 1 (NGS)                                  | 97 (89-103)                | 83 (75-91)          | 94 (87.8-94.8)       | 105* (97.1-108)      | 101 (85.5-108)         | 118** (96-121)      |
| Shannon diversity index (T-RFLP)             | 2.08 (1.32-2.35)           | 1.87 (1.61-2.30)    | 2.34 (2.16-2.87)     | 2.43* (2.37-2.58)    | 2.68*** (2.54-2.75)    | 2.34 (1.92-2.41)    |
| Shannon diversity Index (NGS)                | 5.23** (5.17-5.38)         | 4.73 (4.62-4.82)    | 5.19* (5.09-5.34)    | 4.71 (4.49-4.99)     | 4.95 (4.86-5.09)       | 5.54** (4.93-5.84)  |
| Simpson diversity index (T-RFLP)             | 0.826 (0.667-0.866)        | 0.792 (0.714-0.842) | 0.858 (0.826-0.923)  | 0.884* (0.863-0.905) | 0.903*** (0.891-0.918) | 0.848 (0.772-0.892) |
| Simpson diversity index (NGS)                | 0.96* (0.95-0.96)          | 0.94 (0.92-0.94)    | 0.95 (0.94-0.96)     | 0.89 (0.87-0.93)     | 0.94 (0.94-0.95)       | 0.97* (0.94-0.97)   |
| Quantification of 16S rDNA (copies/g tissue) |                            |                     |                      |                      |                        |                     |
| Total bacteria                               | 8.68 (8.41-8.79)           | 8.95 (8.76-9.04)    | 8.70 (8.56-9.01)     | 9.15 (9.00-9.36)     | 9.05 (8.94-9.21)       | 8.92 (8.80-9.05)    |
| <i>Clostridium leptum</i>                    | 7.12 (6.81-7.27)           | 7.03 (6.97-7.19)    | 6.92 (6.68-7.04)     | 9.15 (9.00-9.36)     | 9.05 (8.94-9.21)       | 8.92 (8.80-9.05)    |
| Viable count (log cfu/g caecum content)      |                            |                     |                      |                      |                        |                     |
| Lactobacilli                                 | 5.72* (5.18-5.98)          | 0 (0-0)             | 6.48**** (6.37-6.59) | 5.59* (4.99-6.14)    | 6.18*** (5.90-6.57)    | 4.93 (3.55-5.64)    |
| <i>Enterobacteriaceae</i>                    | 2.95 (1.97-3.64)           | 3.62 (3.29-4.09)    | 3.73 (3.37-4.17)     | 3.26 (2.80-3.54)     | 2.63* (2.37-2.89)      | 2.3 (0-3.50)        |

<sup>1</sup> 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 \*\*\*\*  $P < 0.0001$ .

<sup>2</sup> Animal diet groups are explained in Table 1.

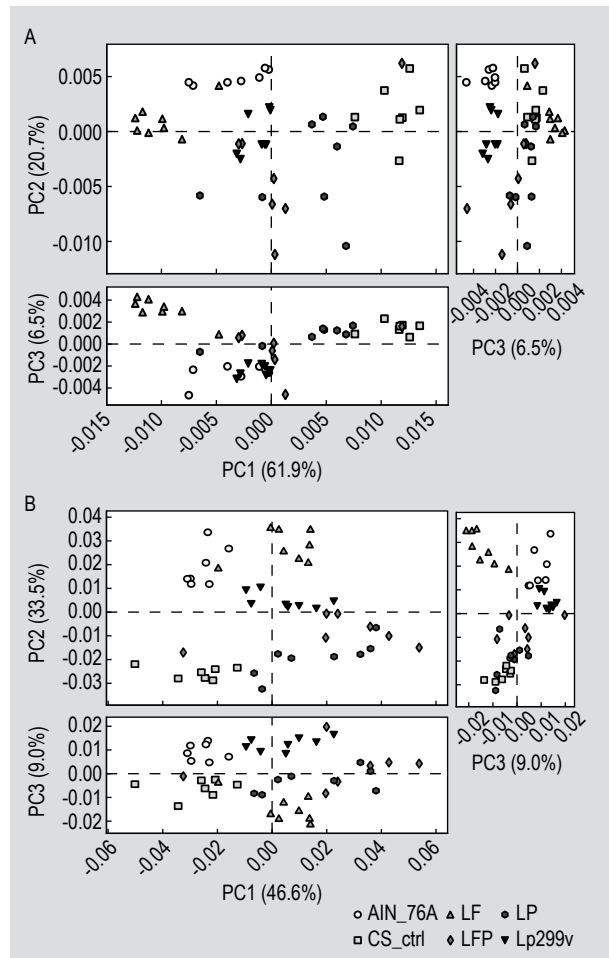


**Figure 4.** Principal coordinates analysis (PCoA) of beta diversity using Bray-Curtis distances shows clear separation between the tested six groups. Each point represents one mouse and the tested six groups are marked with different shapes and greyscale colours.

#### 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

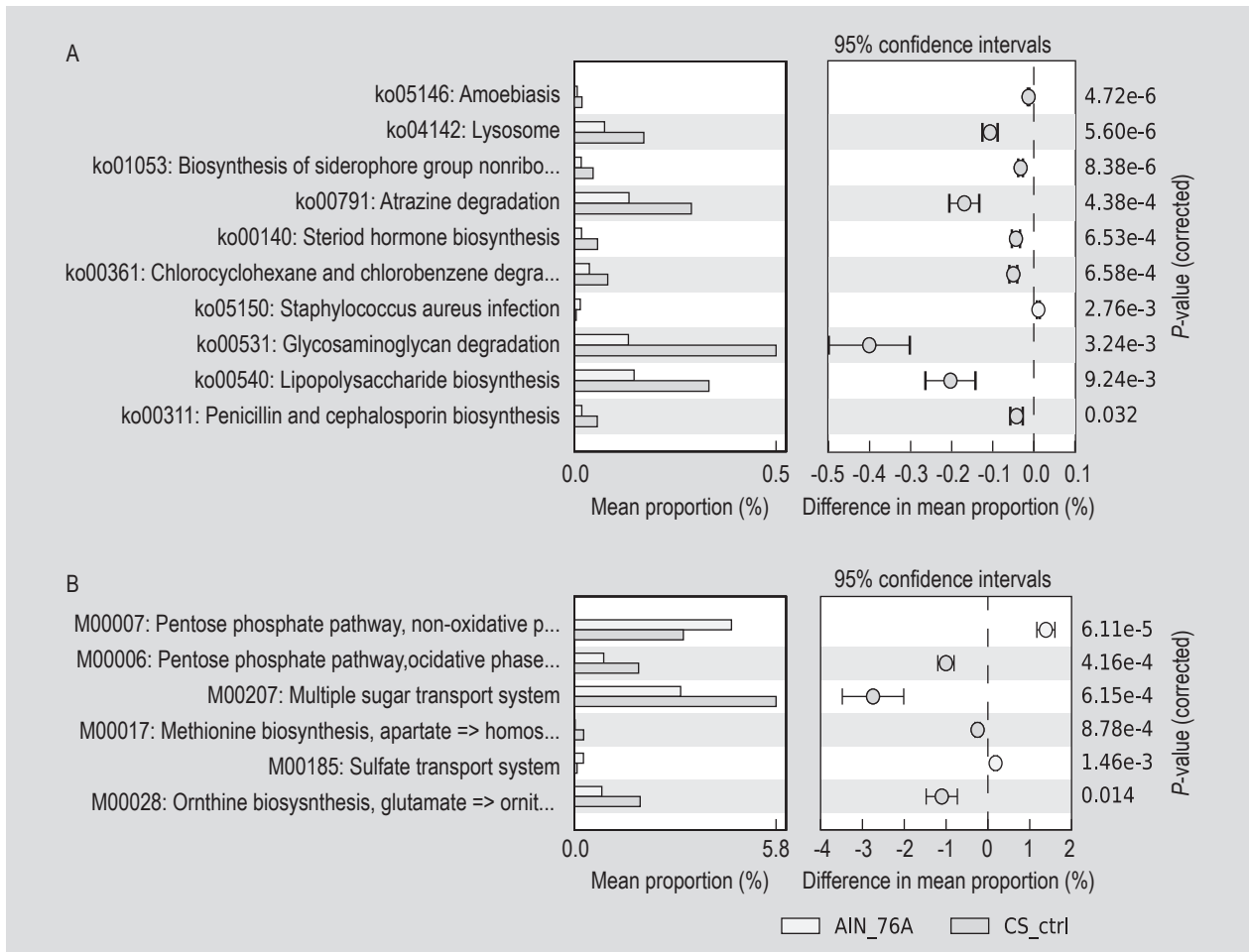


**Figure 5.** Principal component analysis (PCA) of the relative abundance of inferred KEGG pathways (A) and modules (B) shows considerable difference in the microbiome functions between the tested six groups. Each point represents one mouse and the groups are marked with different shapes and colours.

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





**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.**

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

**Table 4. Significantly enriched KEGG pathways and modules with large effect size ( $\eta^2 > 0.80$ ). P-values were corrected with Bonferroni correction.<sup>1</sup>**

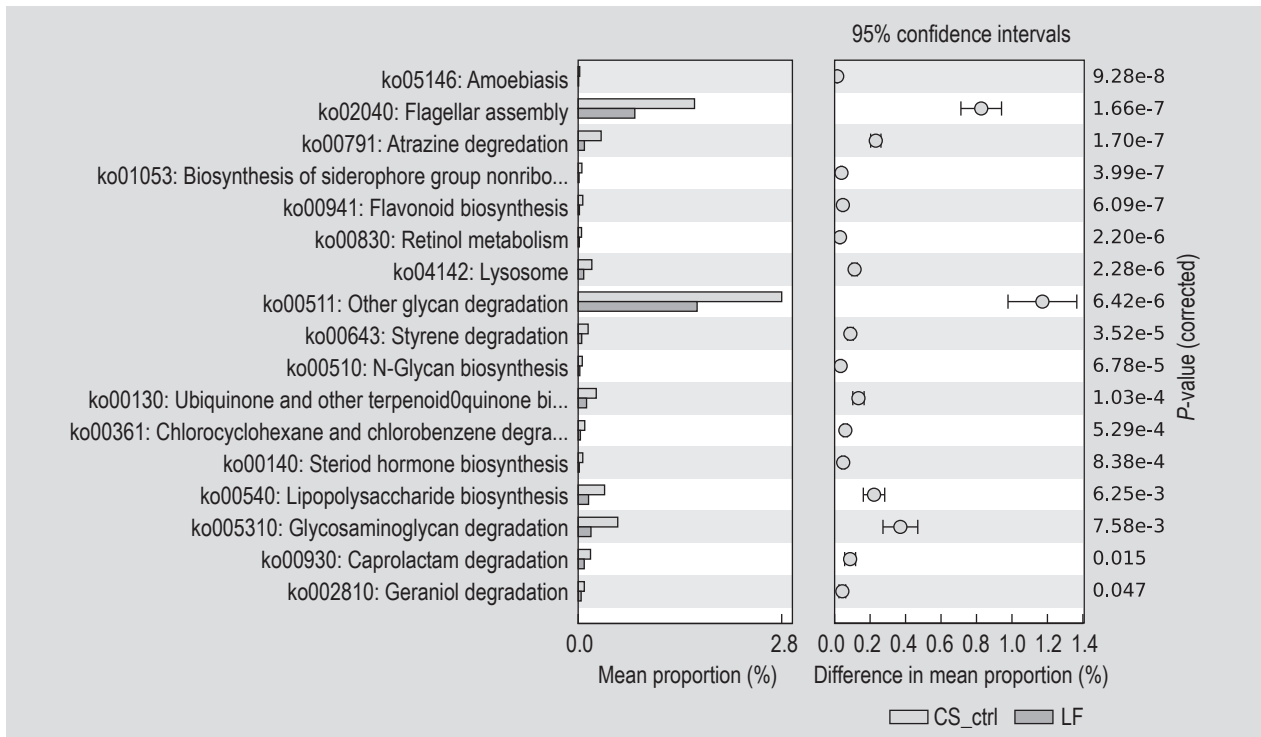
| KEGG pathways  | P-values<br>(corrected) | Effect<br>size | Animal groups <sup>2</sup> |             |             |             |             |
|--|-------------------------|----------------|----------------------------|-------------|-------------|-------------|-------------|
|  |                         |                | CS-ctrl                    | LF          | LFP         | LP          | Lp299v      |
| ko00660: C5-Branched dibasic acid metabolism                                     | 2.55E-15                | 092            | 2.43 (0.03)                | 2.71 (0.03) | 2.35 (0.06) | 2.41 (0.04) | 2.39 (0.02) |
| ko00730: Thiamine metabolism   | 1.14E-13                | 090            | 1.7 (0.02)                 | 2.11 (0.06) | 1.9 (0.06)  | 1.82 (0.05) | 1.94 (0.03) |
| ko00253: Tetracycline biosynthesis   | 1.45E-13                | 090            | 0.52 (0.02)                | 0.91 (0.05) | 0.63 (0.06) | 0.6 (0.06)  | 0.71 (0.02) |
| ko00061: Fatty acid biosynthesis   | 1.29E-12                | 088            | 1.36 (0.02)                | 1.8 (0.07)  | 1.49 (0.07) | 1.47 (0.06) | 1.58 (0.03) |
| ko00290: Valine, leucine and isoleucine biosynthesis                             | 1.31E-12                | 088            | 2.65 (0.03)                | 2.83 (0.02) | 2.62 (0.04) | 2.67 (0.02) | 2.68 (0.01) |
| ko00550: Peptidoglycan biosynthesis  | 3.62E-12                | 087            | 1.65 (0.02)                | 1.86 (0.04) | 1.76 (0.03) | 1.72 (0.02) | 1.76 (0.02) |
| ko00640: Propanoate metabolism   | 7.65E-12                | 087            | 0.6 (0.01)                 | 0.69 (0.02) | 0.6 (0.02)  | 0.58 (0.02) | 0.65 (0.01) |
| ko00620: Pyruvate metabolism   | 5.85E-11                | 085            | 1.2 (0.01)                 | 1.27 (0.01) | 1.21 (0.02) | 1.21 (0.01) | 1.26 (0.01) |
| ko00270: Cysteine and methionine metabolism                                      | 5.26E-10                | 083            | 1.18 (0.01)                | 1.31 (0.01) | 1.26 (0.03) | 1.23 (0.03) | 1.3 (0.02)  |
| ko00400: Phenylalanine, tyrosine and tryptophan biosynthesis                     | 7.28E-10                | 083            | 1.11 (0.02)                | 1.27 (0.02) | 1.17 (0.06) | 1.11 (0.02) | 1.24 (0.01) |
| ko00195: Photosynthesis  | 1.44E-09                | 082            | 0.45 (0.02)                | 0.52 (0.01) | 0.47 (0.02) | 0.44 (0.02) | 0.49 (0.01) |
| ko00900: Terpenoid backbone biosynthesis   | 2.63E-09                | 081            | 1.1 (0.02)                 | 1.47 (0.06) | 1.3 (0.09)  | 1.26 (0.07) | 1.33 (0.02) |
| ko00785: Lipoic acid metabolism  | 3.49E-09                | 081            | 0.73 (0.07)                | 0.37 (0.05) | 0.69 (0.06) | 0.74 (0.1)  | 0.6 (0.04)  |
| ko00471: D-Glutamine and D-glutamate metabolism                                  | 5.81E-09                | 080            | 1.62 (0.02)                | 1.97 (0.05) | 1.84 (0.09) | 1.82 (0.07) | 1.87 (0.02) |
| ko00760: Nicotinate and nicotinamide metabolism                                  | 6.90E-09                | 080            | 0.83 (0.01)                | 1.03 (0.02) | 0.92 (0.05) | 0.89 (0.04) | 0.9 (0.02)  |
| <b>KEGG modules</b>  |                         |                |                            |             |             |             |             |
| M00222: Phosphate transport system   | 5.09E-13                | 089            | 2.72 (0.02)                | 3.31 (0.09) | 2.73 (0.14) | 2.58 (0.07) | 2.57 (0.13) |
| M00157: F-type ATPase, bacteria  | 5.70E-13                | 089            | 2.81 (0.07)                | 3.43 (0.09) | 2.85 (0.15) | 2.61 (0.11) | 3.14 (0.09) |
| M00164: ATP synthase   | 5.70E-13                | 089            | 2.81 (0.07)                | 3.43 (0.09) | 2.85 (0.15) | 2.61 (0.11) | 3.14 (0.09) |
| M00001: Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate                | 1.39E-12                | 088            | 3.85 (0.07)                | 4.47 (0.1)  | 3.87 (0.12) | 3.84 (0.1)  | 4.17 (0.06) |
| M00022: Shikimate pathway, phosphoenolpyruvate + erythrose-4P => chorismate      | 2.65E-12                | 088            | 2.17 (0.04)                | 3.1 (0.12)  | 2.54 (0.16) | 2.34 (0.18) | 2.88 (0.07) |
| M00002: Glycolysis, core module involving three-carbon compounds                 | 1.00E-11                | 087            | 3.33 (0.08)                | 3.87 (0.09) | 3.4 (0.09)  | 3.34 (0.1)  | 3.64 (0.05) |
| M00359: Aminoacyl-tRNA biosynthesis, eukaryotes                                  | 1.18E-11                | 087            | 2.78 (0.06)                | 3.36 (0.1)  | 2.87 (0.11) | 2.81 (0.11) | 3.14 (0.04) |
| M00360: Aminoacyl-tRNA biosynthesis, prokaryotes                                 | 1.48E-11                | 086            | 2.81 (0.06)                | 3.37 (0.1)  | 2.9 (0.1)   | 2.84 (0.1)  | 3.17 (0.05) |
| M00007: Pentose phosphate pathway, non-oxidative phase, fructose 6P => ribose 5P | 2.39E-11                | 086            | 3.14 (0.09)                | 4.21 (0.13) | 3.4 (0.2)   | 3.26 (0.24) | 3.82 (0.08) |
| M00115: NAD biosynthesis, aspartate => NAD                                       | 1.34E-10                | 084            | 1.51 (0.1)                 | 2.84 (0.14) | 2.06 (0.27) | 1.87 (0.26) | 2.03 (0.1)  |
| M00178: Ribosome, bacteria   | 2.80E-10                | 084            | 2.56 (0.08)                | 3.2 (0.11)  | 2.74 (0.11) | 2.67 (0.12) | 2.92 (0.04) |
| M00006: Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P    | 4.79E-10                | 083            | 1.86 (0.08)                | 0.6 (0.12)  | 1.07 (0.26) | 1.41 (0.28) | 1.04 (0.09) |
| M00026: Histidine biosynthesis, PRPP => histidine                                | 7.18E-10                | 083            | 2.66 (0.06)                | 3.33 (0.1)  | 2.73 (0.17) | 2.71 (0.13) | 2.61 (0.13) |
| M00004: Pentose phosphate pathway (Pentose phosphate cycle)                      | 1.19E-09                | 082            | 2.33 (0.07)                | 1.05 (0.18) | 1.6 (0.26)  | 1.94 (0.27) | 1.63 (0.12) |
| M00207: Multiple sugar transport system  | 2.74E-09                | 081            | 5.81 (0.57)                | 1.75 (0.46) | 2.86 (0.92) | 3.95 (0.89) | 2.42 (0.31) |
| M00003: Gluconeogenesis, oxaloacetate => fructose-6P                             | 4.02E-09                | 081            | 3.22 (0.09)                | 3.68 (0.1)  | 3.03 (0.21) | 3.01 (0.1)  | 3.39 (0.08) |

<sup>1</sup> Data presented as mean ( $\pm$  standard deviation) of relative abundance.

<sup>2</sup> Animal diet groups are explained in Table 1.

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,



**Figure 7. Extended error bar plot showing significantly different abundant KEGG pathways between CS-ctrl and LF. P-values are corrected with Bonferroni correction.**

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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## 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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