Effects of low lactose mare's milk yogurt consumption on gut microbiota function

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ABSTRACT

Background: This study shows that the intake of low-lactose yogurt with the addition of mare's milk can affect the composition, biodiversity, and functional potential of the intestinal microflora.

Purpose of the study: To study the effect of low-lactose drinking yogurt from a combination of mare's and cow's milk on the composition and functional repertoire of the fecal microbiota of rats.

Methods: The study of the effect of low-lactose drinking yogurt from a combination of mare's and cow's milk on the intestinal microflora was carried out by dietary intervention for 4 weeks. Changes in the fecal microbiota were determined using V1-V3 sequencing of the hypervariable target region of the 16S rRNA gene. Functional prediction was carried out on the basis of the taxonomic structure of the amplicon sequence.

Results: The results demonstrated a decrease in overall biodiversity both within the samples and between groups of animals. Discriminatory analysis revealed an increase in the relative content of Ruminococcaceae, Peptostreptococcaceae, indeterminate taxon at phylum level, Prevotellaceae and a decrease in Helicobacteraceae,
Eubacterium coprostanoligenes group, Muribaculaceae, Lachnospiraceae, Lactobacillaceae. In addition, changes in the microbial structure also affected the predicted functional repertoire of the gut microbiota.

**Conclusion:** These results demonstrate the consumption of low-lactose drinking yogurt from a combination of mare's and cow's milk, for a short time, can affect the composition and functional repertoire of the fecal microbiota.

**Taxon level**
- Ruminococcaceae, Peptostreptococcaceae, Prevotellaceae.
- Helicobacteraceae, Eubacterium coprostanoligenes group, Muribaculaceae, Lachnospiraceae, Lactobacillaceae.

**Functional level**
- Biosynthesis of biotin, Fatty acid biosynthesis.
- Lipopolysaccharide metabolism, Nitrogen metabolism, Carbohydrate metabolism, Pyrimidine metabolism, Nitrate assimilation, Tetracycline resistance, Multidrug resistance.

**Key words:** Gut microbiome, rats, low-lactose yogurt, microbial diversity, functional repertoire.

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**INTRODUCTION**
Dairy products have been part of the human diet for a long time. They are considered healthy foods and are part of many diets. Their nutritional properties are mainly related to the content of calcium, phosphorus, zinc, potassium, easily digestible protein and vitamins. The main carbohydrate of mammalian milk is lactose. Lactose is a disaccharide with probiotic properties. The consumption of lactose, in small amounts, can contribute to the relative enrichment of the intestinal microbiota with the taxa Bifidobacterium, Faecalibacterium and Lactobacillus [1] and reduce the amount of Bacteroides/Clostridia [2]. However, two-thirds of the adult population lose the ability to digest lactose. This is due to a decrease in the expression of the LCT gene. Consumption of dairy products causes discomfort, diarrhea, stomach pain, and bloating. The existing and supported hypothesis is that populations whose ancestors had access to milk are genetically adapted to lactose. But compared to the rest of the world, Kazakhstan and Central Asia are an exception to this rule. Although it is generally recognized that the milk of mares containing up to 6-7% milk sugar was consumed by our population as early as 5500 BC during the Botai culture. However, lactase deficiency is very common in Kazakhstan, Central Asia and Mongolia (12-30%) in comparison with Western populations and Middle Eastern populations. According to Laure Segurel et al. (2020) this may be due to the same consumption of mare's milk prone to rapid fermentability by intestinal flora [3]. Goodrich et al. (2016) suggest that lactose fermentation in the large intestine is mainly associated with bifidobacteria [4]. Fermented milk products can be a good alternative to whole milk. They can also be
enriched with probiotics and other ingredients. Also, these fermented products according to the definition of Martirosyan D. et al. [5] can be considered functional. Our studies also revealed an increase in the genus Bifidobacterium in respondents who took mare's milk for 3 months (unpublished data). They, like lactobacilli, break down lactose into organic acids with the release of energy. The aim of this study is to study the effect of low-lactose drinking yogurt from a combination of mare's and cow's milk on the composition and functional repertoire of the fecal microbiota of rats.

**METHODS**

*Preparation of the investigated product:* The studied product was fermented milk yogurt from a mixture of mare's and cow's milk, self-made with low lactose content. The product was prepared in the laboratory under conditions as close as possible to the factory technology. Own starter culture, based on strains of *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus paracasei ssp paracasei*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*. Lactase was added at a concentration of 0.002% of the final volume of the product. The final product included only sourdough and natural milk. Fermentation time is 8 hours, temperature is 38 °C. The product has a characteristic sour-milk taste and a clot without syneresis. The acidity of the product is 110 °T.

*Research on rats:* The study of the effect of fermented dairy products on the microflora and functional repertoire of the intestine was carried out on 15 mongrel rats of both sexes with an average initial body weight of 212 ± 34.4 g. Before the experiment, the animals were kept in vivarium conditions, on normal nutrition for 7 days. The animals were fed 5 ml of yogurt daily. To study the effect of low-lactose yogurt on intestinal microflora, samples of rat feces were collected before and after feeding with a fermented milk product. The primary collection – the first control point of fecal samples was carried out after 7 days of the usual diet. After that, a fermented milk probiotic active product was introduced into the diet of the animals for 4 weeks. The second control point for collecting stool samples was carried out after 28 days of the introduction of the probiotic product into the diet.

The study was approved by the Local Ethical Committee of the Center for Life Sciences of the National Laboratory of Astana Nazarbayev University (resolution № 01-2021 of 18.01.2021) (Nur-Sultan, Kazakhstan).

*DNA extraction:* Fecal samples were collected in a DNA/RNA Shield-Fecal Collection Tube (Zymo Research, R1101). Genomic DNA from fecal samples were extracted using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, D4300). Qualitative control of DNA isolation was performed by electrophoresis in 1% agarose gel. The concentration and purity of each DNA sample will be determined using an Invitrogen Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, California, United States). Sterile water served as a negative control.

*Library preparation:* Preparation of DNA libraries was performed in accordance with the 16S Metagenomic Sequencing Library Preparation guide (part no. 15044223 rev. B, 2013) as follows: DNA amplification of the V1-V3 hypervariable target region of the 16S rRNA gene. Purification of the reaction mixture was carried out using Agencourt AMPure PCR purification kit (Beckman Coulter Inc. Beverly, Massachusetts, USA). Dual indices and Illumina sequencing adapters the Nextera XT Index Kit will be used. The library quality was quantified by Qubit dsDNA HS Assay Kit with the Qubit 2.0 fluorometer system (Invitrogen, Life Technologies, Grand Island, NY, USA). Library validation will be conducted using Agilent DNA 1000 Kit and Agilent Technologies 2100 Bioanalyzer. For cluster generation and sequencing, libraries will be
pooled denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing.

**Processing of sequencing data:** The LotuS2 (Less OTU Scripts 2) is used to process 16S amplicon sequencing data from raw reads into taxon density tables. Demultiplexing, quality filtering, and dereplication of reads are implemented using a simple demultiplexer (sdm). Chimeras will remove using algorithms for detecting chimeric sequences UCHIME. Taxonomic post-processing of amplicon sequences in LCA with sequence clustering UPARSE performed using SILVA, 16S rRNA gene database.

**Functional capabilities of the gut microbiome:** Functional metagenomes were predicted based on the 16S rRNA sequencing data of the gut microbiome using PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states) v2.5.0 with default parameters [6]. Briefly, the ASVs were placed into a reference tree (NSTI cutoff value of 2) containing 20,000 full 16S rRNA sequences from prokaryotic genomes, which were then used to predict individual gene family copy numbers for each ASV. The predictions are based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO). The produced KEGG orthologs (KOs) were mapped to the KEGG module annotation downloaded on 01.04.2022 from the KEGG BRITE database [7].

**Statistical analysis:** Analysis of alpha diversity to assess the abundance of the community and the calculation of biodiversity Shannon, Simpson, Chao1, and Ace indexes, as well as the construction of taxonomic distribution at the phylum and genus level was performed using phyloseq package (v.1.24.2) [8]. All graphs were generated using ggplot2 (v.3.0.0) [9].

Non-parametric Mann-Whitney (MW) and Kruskal-Wallis (KW) tests were used when comparing two or more Shannon index comparison groups respectively. For ordination plots of beta diversity metrics, sampling counts were first transformed with the Hellinger standardization transformation method. Then weighted Unifrac distance was calculated, and the graphs of the Principal Coordinate Analysis (PCoA) were generated from a distance. ANOSIM and PERMANOVA tests with 9999 permutations compared the after group vs. before group [10].

**RESULTS**

In this research, we studied the effect of a fermented lactic acid product with a low lactose content on the composition and functional profile of the intestinal microbiota of rats.

![Figure 1](image_url)

**Figure 1.** Relative abundance of a microbial community before and after consumption of a fermented dairy product. a. relative abundance for family level; b. relative abundance for genus level.
The depth of coverage was at least 36,000 readings per sample. All sequences were compared with the SILVA database. A comparison of the compositional structure of the fecal microbiota of rats before and after taking low-lactose yogurt revealed a change in the microbial composition. Figure 1 shows the differences in the 10 most common taxa at different taxonomic levels. So, figure 1a shows that after taking a fermented milk product, at the phylum level, the taxa Helicobacteraceae, Eubacterium coprostanoligenes group, Muribaculaceae, Lachnospiraceae, Lactobacillaceae depleted, in contrast, the relative number of Ruminococcaceae, Prevotellaceae, Peptostreptococcaceae, indeterminate taxon at phylum level increased. Analysis of the taxonomic structure of the genus level showed an increase in the relative abundance of Eubacterium ruminantium group, Ruminococcus, Romboutsia, indeterminate taxon at the genus level, Prevotella. At the same time, the number of Lactobacillus, a taxon identified as human gut metagenome, Lachnospiraceae NK4A136 group, and Helicobacter decreased. Figure 1 demonstrates that in the general structure, after taking the studied product, there was enrichment with phylum Firmicutes taxa depletion with Bacteroidota taxa.

Figure 2. Structural changes in fecal microflora after consumption of a low lactose yogurt. a - evaluation of the alpha diversity of the intestinal microbiota before and after consumption of low lactose yogurt. Boxplots display the median value, the first (25%) and third (75%) quartiles with whiskers from 1.5 IQR (interquartile range) minimum to maximum; b - Principal coordinate analysis (PCoA) plot in different axis PCoA1 (Axis 1) and PCoA2 (Axis 2) respectively explained 74.19 and 8.55% of the variance of the abundance of gut microbiota at the genus level.

The biodiversity within the groups was not statistically different, Shannon (p=0.486) and Simpson (p=0.539), but tended to merge. Thus, the alpha diversity of the fecal microbiota of rats before the introduction of a fermented dairy product into the diet had a slightly greater intra-group diversity (figure 2a), as shown by the Shannon and Simpson biodiversity indices. The principal coordinate analysis also showed no statistical differences in bacterial diversity between the groups (figure 2b). The microbial flora of most samples from both groups
showed similar diversity, as shown by the grouping (purple ellipse). The red ellipse indicating the microbiota of the samples after ingestion of the fermented dairy product shows a large area formed by samples with a different variety. So in this group, three samples after ingestion of the dairy product had altered microbial biodiversity.

Subsequently, to determine the differences between the groups before and after taking a lactic acid fermented product with a low lactose content, we conducted a linear discriminate analysis that revealed differences at the level of operational taxonomic units (figure 3).

**Figure 3.** Linear discriminate analysis effect size (LDA) scores (LEfse plot). An LDA score (log 10) > two indicates significantly different enrichment of bacteria taxa in the before group (purple) compared to the after group (red). Note: k – kingdom; p – phylum; c – class; o – order; f – family; g – genus.
Plot linear discriminate analysis shows that before taking the combined fermented milk product, the microflora of rats was enriched with groups of microorganisms in the bulk belonging to 2 families of Lachnospiraceae OTU (431, 375, 396, 149, 170, and 164) and Muribaculaceae OTU (351, 277, 278, 216, 184, 141, 24, 22). Whereas, against the background of taking a fermented milk product for 4 weeks, the abundance of the genus Prevotella (OTU 106) family Eggerthellaceae (OTU 64), class Clostridia (OTU 322, OTU 501), and uncultured rumen bacteria WPS-2 increased but decreased Lactobacillus genus, proteobacteria, and Negativicutes. In the next step, we tried to predict and compare the functionality of the fecal microbial flora of rats before and after taking the fermented milk product. The prediction of functional profiles was based on the taxonomic structure of the amplicon sequence.

**Figure 4.** Functional changes in the rat fecal microbial community profile. Boxplots display the median value, the first (25%) and third (75%) quartiles with whiskers from 1.5 IQR (interquartile range) minimum to maximum. The x-axis is the frequency of the presence of functional modules; the y-axis is the codes of functional modules.

The prediction of the functional capabilities of bacterial organisms has become possible due mainly to the conservative content of genes in the procaryotic genetic apparatus, based on close phylogenetic kinship. In our study, we used the PICRUSt 2 method and the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KO) database to predict metagenomic functions. In general, 457 modules were predicted, of which 408 belonged to Pathway modules (gensets in metabolic pathways), and 49 to Signature modules (gene sets that characterize phenotypic features). The main metabolic pathways were common for both groups of samples. Figure 4 shows only significantly different metabolic modules. Thus, for the bacterial community of the group after ingestion of combined low-lactose yogurt, increased biosynthesis of biotin (M00572) and fatty acid biosynthesis (M00083) was predicted. Whereas, lipopolysaccharide metabolism (M00923, M00867), nitrogen metabolism (M00529), carbohydrate metabolism (M00309), pyrimidine metabolism (M00046), nitrate assimilation (M00615), tetracycline resistance (M00704), multidrug resistance (M00700)
decreased, *Helicobacter pylori* pathogenicity signature, CagA pathogenicity island (M00564).

**DISCUSSION**

This research studied the effect of a combined low-lactose fermented milk product on the diversity and functional profile of the microbiota of feces of laboratory mongrel rats. The use of healthy laboratory rats allowed us to control the factors of dietary intervention, and the environment, which are very difficult to control in human studies. The study of microbiota diversity revealed taxonomic differences that occurred after four weeks of taking the studied product. Thus, the relative content of *Ruminococcaceae*, *Peptostreptococcaceae*, indeterminate taxon at the phylum level, *Prevotellaceae* increased after dietary intervention, while the content of *Helicobacteraceae*, *Eubacterium coprostanoligenes* group, *Muribaculaceae*, *Lachnospiraceae*, *Lactobacillaceae* decreased. Similar data were obtained in a study by Kathryn J. Burton et al., [11] showing that taking acidified milk for 2 weeks led to an increase in the relative abundance of *Ruminococcaceae*. In a study by Yufang Liu et al., [12], the relative abundance of *Prevotellaceae* and *Ruminococcaceae* increased after consuming different types of milk for four weeks, while the relative amount of *Lactobacillaceae* and *Tannerellaceae* decreased significantly. Intragroup biodiversity did not differ statistically, Shannon (p=0.486) and Simpson (p=0.539), but tended to deplete, which is consistent with most studies of the effect of fermented dairy products on the intestinal microflora, most of which show either reduced or unchanged diversity [13-17]. Also, the intergroup comparison of biodiversity (PCoA) did not show any fundamental differences. Nevertheless, linear discriminate analysis effect size with a score >2 revealed taxonomic differences between the groups before and after taking the fermented milk product. Thus, a significant decrease in representatives of the family *Lachnospiraceae* and *Muribaculaceae*, *Lactobacillus* genus, proteobacteria, and *Negativicutes* was revealed, in contrast, the relative content of *Prevotella*, family *Eggerthellaceae* increased. The analysis of gene and genome orthologs (KEGG) pathways showed that the levels of vitamin H biosynthesis, biosynthesis, and elongation of bacterial fatty acids were enriched in the model group of animals after dietary intervention. At the same time, lipopolysaccharide metabolism decreased. The combination of these two factors suggests that the consumption of fermented milk products enhances the absorption of the vitamin in the lower colon since it is known, that lipopolysaccharides inhibit the absorption of biotin by the colon due to a decrease in the membrane expression of the sodium-dependent multivitamin transporter [18]. Also, there was revealed a decrease in the modules of antibiotic resistance and pathogenetic signature.

**CONCLUSION**

To conclude, our study indicates that dietary intervention by consuming low-lactose drinking yogurt from a combination of mare’s and cow’s milk can affect the composition and functional repertoire of the fecal microbiota. Thus, the incorporation of the studied product into the diet led to a decrease in the overall biodiversity of the microflora, the number of Lactobacillus, Lachnospiraceae, and Helicobacter decreases while the taxa Eubacterium, Ruminococcus, Romboutsia, Prevotella increased. Although, we expected to see more significant structural variations in the fecal microbiota after taking a fermented milk product. At the same time, the functional repertoire of the fecal microbiota has also changed. Interestingly, the introduction of the product increased bacterial biosynthesis of biotin. The stimulating effect of combined fermented milk products deserves further study.

**List Of Abbreviations:** 16S rRNA gene: 16S ribosomal ribonucleic acid, DNA: deoxyribonucleic acid, PCR: polymerase chain reaction, LotuS2: Less OTU Scripts 2, ASV: amplicon sequences variants, NSTI: nearest
sequence taxon index, LCA: least common ancestor, KEGG: Kyoto Encyclopedia of Genes and Genomes, KO KEGG orthologs, PCoA: principal coordinate analysis, LDA: linear discriminate analysis, LEfse: linear discriminate analysis effect size, IQR: interquartile range, PICRUSt2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.

**Competing Interests:** The authors declared no conflict of interest.

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