The combination of mare's milk and grape polyphenol extract for treatment of dysbiosis induced by dextran sulfate sodium

SAMAT KOZHAKHMETOV[1,2,3,♥], DMITRIY BABENKO[4], MADIYAR NURGAZIYEV[1],
ALTYNAY TUYAKOVA[1], AYAULYM NURGOZHINA[1], NURISLAM MUHANBETGANOV[1],
LAURA CHULENBAYEVA[1], SHYNGGYS SERGAZY[1], ALEXANDR GULYAYEV[1],
TIMUR SALIEV[5] , ALMAGUL KUSHUGULOVA[1,2,3]

[1]Laboratory of Human Microbiome and Longevity, Center for Life Sciences, National Laboratory Astana, Nazarbayev University. Z05H0P9, Kabanbay Batyr avenue 53, block S1, Nur-Sultan, Kazakhstan. Tel. +7 717 2 706521, ♥email: skozhakhmetov@nu.edu.kz
[2]Kazakhstan Society of Researchers of Human Microbiome. Tawelsizdik 34, Z00T2C6, Nur-Sultan, Kazakhstan
[3]SaumalBioTech LLP. Z05H0K9, Kabanbay Batyr avenue 6/4, Nur-Sultan, Kazakhstan
[4]Research Center Karaganda Medical University. M01K6T3, Gogol street 40, Karagandy, Kazakhstan
[5]S.D. Asfendiyarov Kazakh National Medical University. A05H2A6, Tole Bi Street 94, Almaty, Kazakhstan

Abstract. *Kozhakhmetov S, Babenko D, Nurgaziyev M, Tuyakova A, Nurgozhina A, Muhanbetganov N, Chulenbayeva L, Sergazy S, Gulyayev A, Saliev T, Kushugulova A. 2020. The combination of mare's milk and grape polyphenol extract for treatment of dysbiosis induced by dextran sulfate sodium. Biodiversitas 21: 2275-2280.* This study showed the potential of a biological product based on mare's milk and a complex of grape polyphenols to modulate intestinal microflora after dextran sulfate sodium (DSS)-induced dysbiosis. Rat ulcerative colitis has been developed using intra-gastric administration of 10% DSS solution. To track changes in the structure of the microbiome at all stages of the study, the next-generation sequencing of the 16S rRNA gene section and LotuS conveyor were used. The results of sequencing demonstrated a decrease in biological diversity of microbiota after the induction of colitis, and recovery after 7 days of use of the (MMGPE). The product induced the structural changes of the microbiome damaged by DSS. Representatives of SCFA producing bacteria increased concentrations of *Prevotella, Alloprevotella, Lactobacillus, Ruminococcaceae,* and *Blautia.*

Keywords: DSS-induced dysbiosis, gut microbiome, microbial diversity, MMGPE, rats

INTRODUCTION

The microflora of the human intestine is a complex structured ecosystem, which includes parietal intestinal flora and transit microorganisms from food. Intestinal microorganisms are a dynamic system that depends on many factors such as food, environment, pre-existing diseases, etc. Destruction of the microbial ecosystem structure is of great importance in the pathogenesis of various diseases, for example, for the development of inflammatory bowel syndrome, Crohn's disease, and ulcerative colitis (UC). In fact, the patients with ulcerative colitis suffer from the impaired diversity and stability of the intestinal microbiome with a decrease in *Firmicutes* and an increase in the number of *Bacteroidetes* and facultative anaerobes (Shen et al. 2018). As a result of ulcerations, the intestinal wall ceases to be a normal niche for the living of microorganisms and performs a barrier function. This, in turn, can impact digestive processes in the intestine, leading to a decrease in the mucus layer, a disruption in the shape of epithelial cells, and dystrophic phenomena in the epithelium (Pei et al. 2019). UC therapy today is not satisfactory, and it does not lead to complete remission. In addition, patients often have relapses, because standard treatment is usually not effective enough for a stable remission.

To date, drugs based on 5-aminosalicylic acid are used as a classic approach for UC therapy. However, there are attempts to employ various probiotics, but the benefits of their use in comparison with placebo have not been identified yet (Derwa et al. 2017). Unlike probiotics, fecal microbial transplantation leads to persistent remission of ulcerative colitis (Moayyedi et al. 2015; Costello et al. 2019; Tian et al. 2019). Another promising direction is the use of products that stimulate the growth of certain microflora, such as polyphenols and mare's milk. Numerous studies have shown how polyphenols can contribute to changes in the microbial composition of the intestine, at the same time there are no reports on their effect on the treatment of UC. It was found out that resveratrol extracted from grapes had anti-inflammatory activity along with stimulation of the growth of *Lactobacillus* and *Bifidobacterium* after DSS-induced colitis (Hu et al. 2019). Similarly, Chen et al. demonstrated that treatment with resveratrol resulted in an increase of concentrations of *Bacteroides* and *Akkermansia* (Chen et al. 2016). Another study showed that *Akkermansia* produces a significant amount of SCFA when polyphenols are added (Naito et al. 2018). Mare's milk, also being a prebiotic, stimulates the growth of certain bacteria. In addition, mare's milk has a complex of biologically active compounds including vitamins: A, B_1, B_2, B_6, B_9, B_{12}, C, trace elements Na, Ca, K, P, Fe, Mg, Cu, I, S, Si. Zn, Co, lysozyme, lactoferrin. In our study, we studied the therapeutic potential of a biologically active substance based on mare's milk and a polyphenol complex against ulcerative colitis (Rather et al. 2020).

MATERIALS AND METHODS

Ethical approval

The study was approved by the local ethics committee of the Center for Life Sciences, National Laboratory of Astana, Nazarbayev University, Nur-Sultan, Kazakhstan (approval No. 20 dated 22 September 2017).

Animals study

The study was carried out on 15 laboratory animals (male Wistar rats with an average body weight range of 250-280) in the vivarium of National Center for Biotechnology (Nur-Sultan, Kazakhstan) with a standard ration and care. Rats were placed in separate cages in a room free of pathogens and for acclimatization 7 days before the start of the experiment. During acclimatization and experiment, rats consumed a standard commercially available chow. The animals were kept and the experiments conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2011) and the ethical principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe 2006).

Colitis in male rats was reproduced using a 10% DSS solution (Dextran sodium sulfate, MW-40 kDa, Sigma Aldrich) (Chassaing et al. 2014), which was administered to the rats intragastrically using a probe for 7 days in a volume of 5 ml. A 10% DSS solution was prepared ex tempore before use. Intact rats received an equivolume amount of drinking water intragastrically using a probe (manipulation control). The studied product (MMGPE) to study the efficacy against microbial dysbiosis in the DSS-induced colitis model in rats was administered intragastrically at a dose of 500 mg/kg of animal body weight, once a day for 7 days after the end of a 7-day course of taking a 10% solution DSS. Three months old Wistar rats with an average body weight range of 250-280 g were randomly divided into 3 groups: (i) HC: healthy animals (without colitis) received drinking water intragastrically instead of 10% DSS for 7 days and then another 7 days instead of treatment (n = 5). (ii) EG: experimental group animals (with colitis) received a 10% dextran sulfate sodium (DSS) solution for 7 days, and study product intragastrically at a dose of 500 mg/kg body weight once per day for 7 days (n = 5). (iii) CG: comparison group animals (with colitis) received a 10% DSS solution for 7 days and as a treatment the 5-ASA (5-aminosalicylic acid) intragastrically at a dose of 100 mg/kg of animal body weight once for 7 days (n = 5). Rats were removed from the experiment by an overdose of carbon dioxide (Hewett et al. 1993). Fecal samples were collected before and after the experiment and tested for consistency and color. Other tested parameters including intestinal permeability and body weight.

Measurement of the rats gut microbiota

DNAs were isolated from fecal using the QIAamp DNA Mini Kit (Qiagen, 51306). The concentration of double-stranded DNA in isolated samples was determined using a Qubit 2.0 instrument and a Qubit dsDNA HS Assay kit (ThermoFisher, catalog number 32853).

Library for Next-generation sequencing (NGS) generated with NEXTflex® 16S V1-V3 Amplicon-Seq Kit (PerkinElmer, catalog number NOVA-4202-04), according to the manufacturer's instructions. 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). Amplicons were sequenced on the MiSeq instrument (Illumina Systems).

Demultiplexing, filtering, denoise, chimeric sequences, and determining OTU and taxonomic identification were performed using LotuS pipeline (Hildebrand et al. 2014).

Analysis of alpha diversity to assess the abundance of the community, the calculation of alpha biodiversity (Shannon indices), beta biodiversity as well as the construction of taxonomic distribution at the phylum and genus level were performed using vegan (Oksanen et al. 2019) and phyloseq R packages (v.1.24.2) (McMurdie and Holmes 2013) and graphs were generated using web-based platform for comprehensive analysis - MicrobiomeAnalyst (Chong et al. 2020).

Statistical analysis

Non-parametric Mann-Whitney (MW) and Kruskal-Wallis (KW) tests were used for comparing two or more groups, respectively. The raw read counts were normalized by the total number of reads. A metagenomic biomarker discovery approach, Linear discriminant analysis Effect Size (LEfSe), was used to identify the microbial components whose sequences were statistically different between groups. For LEfSe, Kruskal-Wallis and pairwise Wilcoxon tests were performed, followed by Linear discriminant analysis (LDA) to assess the effect size of each differentially abundant taxon. Bacteria with markedly increased numbers were defined as those with an LDA score (log10) of over 2.

RESULTS AND DISCUSSION

In this study, a biological product based on grape polyphenols (Kazakhstan breeding Cabernet Sauvignon) and mare's milk was tested for the ability to restore the intestinal microflora of rats after DSS-induced colitis. The results showed that the dynamics of body weight of the EG group did not change compared to HC group animals.

Disease Activity Index (DAI) was calculated on a scale of 0 to 4; weight loss (0: no; 1: 0-5%; 2: 5-10%; 3: 10-20%; 4, > 20%); stool consistency (0: normal; 2: loose stools; 4: watery diarrhea); bleeding (0: no; 1: traces; 2: weak hidden blood; 3: obvious hidden blood; 4: severe bleeding). After 1 week of taking DSS, the stool was mainly soft, and in some cases pasty, while the HC group animals have stools. No bleeding was observed. DAI showed no difference from CG taking 5-aminosalicylic acid. To determine the ability of the developed product to modulate the microflora, rats stool samples were collected before treatment and after 7 days of drug administration. In total, 30 stool specimens were collected from 15 male rats.

Rat feces samples were collected in sterile centrifuge tubes and immediately frozen at-80 °C.

High throughput sequencing yielded an average of 72,541 read operations per sample. Analysis of the gut rat microbiome revealed a high taxonomic diversity of bacteria. LotuS based microbial community analysis yielded a total of 3341 operational taxonomic units (OTUs). Based on phylogenetic analysis using the 16S rRNA gene SILVA database more reads were classified on level of phylum as *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Tenericutes, Spirochaetes, Candidatus saccharibacteria* (Figure 1).

To determine the diversity of intestinal microbial communities in the various studied groups, the α-diversity metric implemented in R. was used. The most representative bacterial genera in the group before using the biological product were: *Lactobacillus, Prevotella, Romboutsia, Helicobacter, Bacteroides, Ruminococcus, Treponema Butyricicoccus, Parasutterella*. However, the use of 10% DSS led to a decrease in biodiversity and α-diversity in the EG group (Figure 2.A), whilst the Shanon index was increased in the group taking 5-ASA (5-aminosalicylic acid) (Figure 2.B), and in the control group it remained at the same level (Figure 2.C).

Figure 1. Bacterial Phylum abundance in rat's fecal before and after 7 days of treatment CG1, EG1 HC1-group animals before treatment; CG2, EG2, HC2-group animals after treatment
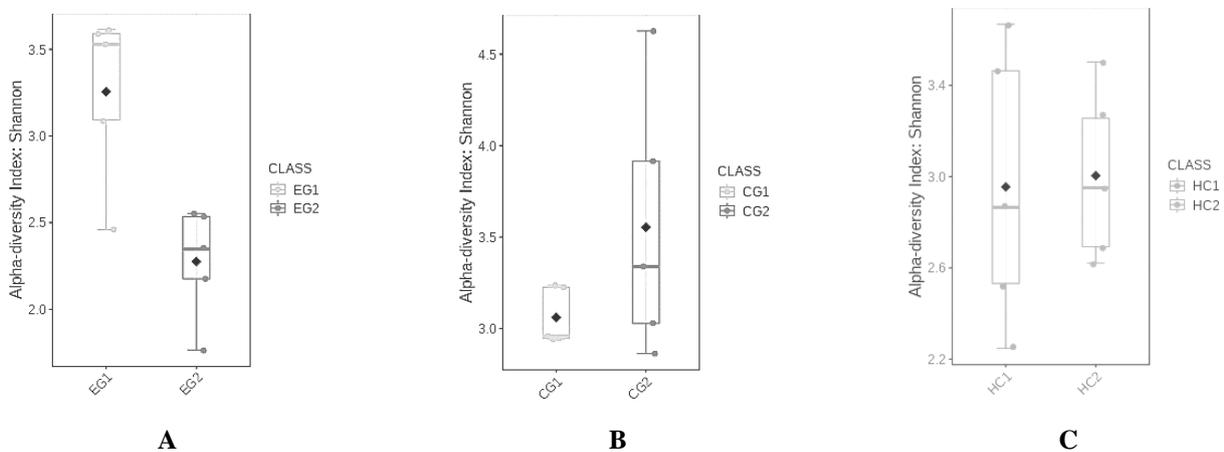

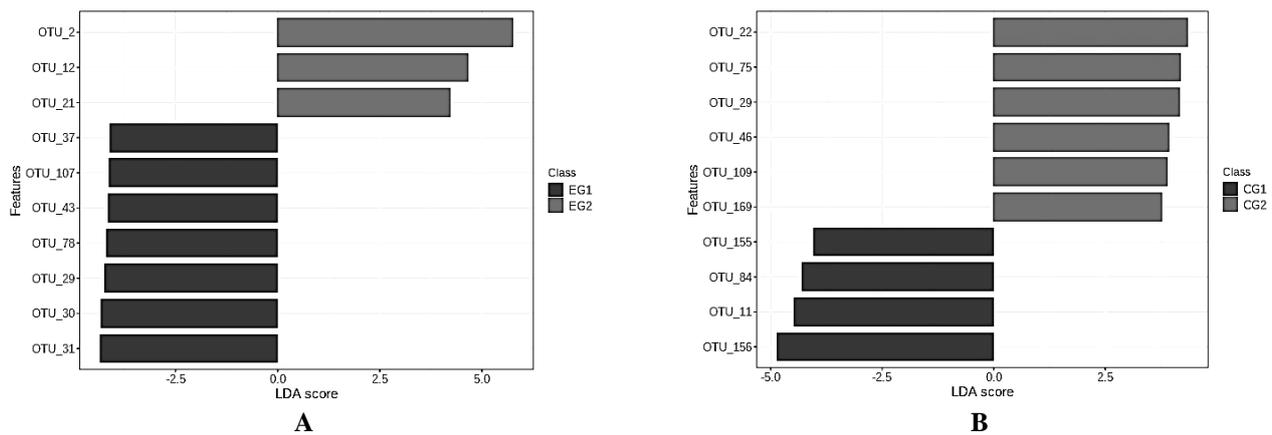**Figure 2.** α-diversity fecal bacteria in EG, HC and CG (p<0.05)

Figure 3. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) OTU level, p-value<0.05
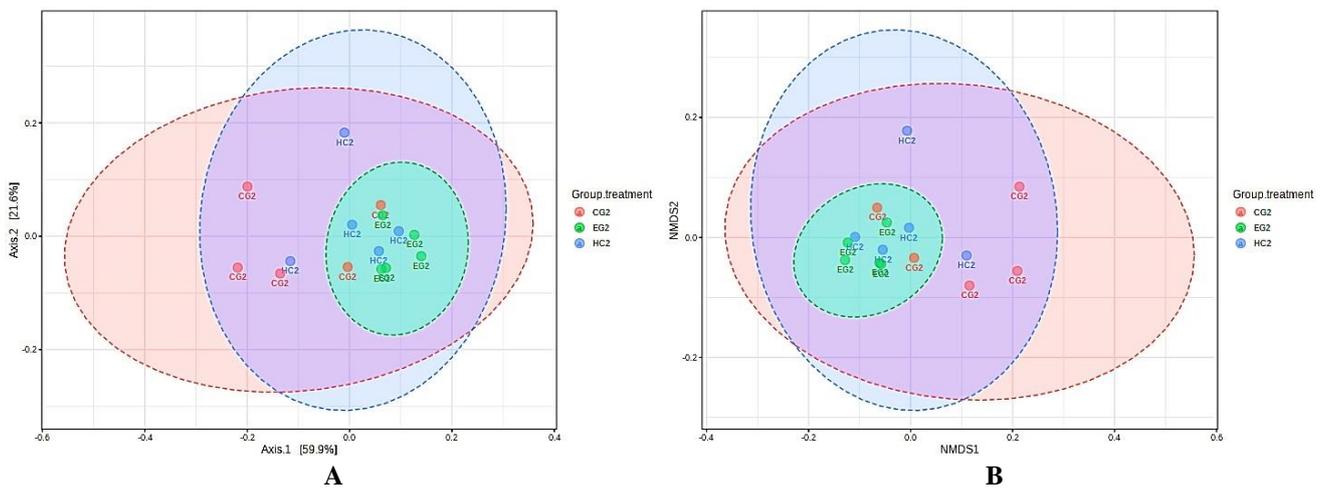

Figure 4. Effect of administration DSS on rat's intestinal microbiome. A. PCoA analyze [PERMANOVA] F-value: 5.8788; R-squared: 0.42358; p-value < 0.019. B. NMDS analyze [PERMANOVA] F-value: 3.2954; R-squared: 0.35452; p-value < 0.011

LDA in EG revealed an increase in the genus *Prevotella* (OTU_2) (Figure 3.A), *Bacteroidales* (OTU_12), and genus *Lactobacillus* (OTU_21) after treatment with a biological product. While family Lachnospiraceae (OTU_37, OTU_107), genus *Helicobacter* (OTU_43), unknown bacteria of phylum *Bacteroidetes* (OTU_29, OTU_30, OTU_31, OTU_78) decreased. In comparison group CG after treatment with 5-ASA led to an increase in order *Clostridiales* (OTU_22), *Alphaproteobacteria* (OTU_169), and decreased *Gammaproteobacteria* (OTU_11), *Actinobacteria* genus *Rothia* (OTU_156) (Figure 3.B). In addition, Differential Abundance Analysis Methods (DeSeq2) in the group receiving the biological product revealed an increase in the genera *Prevotella* and *Alloprevotella*.

The decrease in the biodiversity of the intestinal bacterial flora can be explained by ulcerations (induced by DSS) and local activation of neutrophils and macrophages. PCoA and NMDS analyzes showed the difference between microbial communities after treatment by CG2 with 5-aminosalicylic acid, EG2 biological product based on mare's milk and grape polyphenol concentrate (Figure 4). The data obtained demonstrated an increase in the growth of bacteria *Ruminococcaceae* (OTU_174) and *Bacteroidales* (OTU_223).

In the group received the (MMGPE), a decrease in the genera *Parabacteroides* (OTU_295) and *Turicibacter* (OTU_118) (Figure 2) and an increase in proteolytic and *Proteobacteria* including from family *Moraxellaceae* were observed.

Discussion

Ulcerative colitis is a chronic disease of the intestinal mucosa that requires long-term medication and regular check-ups. The disease is often accompanied by microbial dysbiosis (Xie et al. 2019). Microbial dysbiosis in UC is characterized by an increase of bacteria *Proteobacteria* and *Bacteroidetes* and a decrease of bacteria *Firmicutes* phylum and *Euryarchaeota* phylum. In addition, a decrease in the index of alpha diversity, the relative representation of

bacteria producing butyrate, hydrogen; increase in relative representation of *Ruminococcus* was also reported (Danilova et al. 2019).

In this study, in the healthy group of animals, the top 10 taxa included the following bacterial genera: *Lactobacillus, Prevotella, Romboutsia, Helicobacter, Mycoplasma, Rikenella, Parabacteroides, Bacteroides, Parasutterella, Ruminococcus*. After the formation of the DSS-induced colitis in top 10 taxa model, *Treponema, Butyricicoccus, Turicibacter* was introduced. At the same time, the number of *Mycoplasma, Rikenella,* and *Parabacteroides* decreased. The results are consistent with published data (Gao et al. 2018).

Prebiotics are known for their ability to selectively stimulate the activity of intestinal bacteria strains associated with health such as *Bifidobacteria* and *Lactobacilli*, which are considered beneficial to human health. In addition, prebiotics can enhance the guts barrier function and host immunity, SCFA production, and suppress the populations of potentially pathogenic bacteria (Slavin 2013). We demonstrated that the use of a (MMGPE) of Kazakhstan selection led to a shift in the balance of intestinal microflora. These findings can be potentially employed for the treatment of ulcerative colitis. The genus included in the top 10 taxa: *Lactobacillus, Prevotella, Romboutsia, Helicobacter, Rikenella, Bacteroides, Parasutterella, Ruminococcus, Treponema,* and *Blautia*.

Particularly, we detected an increase in the representatives of *Ruminococcaceae* family (OTU_174), which are mucolytic bacteria (*Ruminococcus gnavus* and *Ruminococcus torques*). These strains are the main components of mucus-mucins of the intestinal mucosa, and they play a critical role in gut permeability (Hall et al. 2017). In our work, we showed an increase in the growth of *Ruminococcaceae* (Figure 2.A) caused by ulcerative colitis (induced by the administration of 10% DSS). The obtained results once again demonstrated the correlation between an increase in the group of mucolytic bacteria and signs of inflammatory bowel diseases (Png et al. 2010). The results indicate that an increase in mucosa-associated bacteria is associated with ulcerations and a decrease in the host resistance. The detected increase of *Bacteroidales* (Figure 1.B) in the EG group can be a result of antimicrobial effect of grape polyphenols. In fact, polyphenols can inhibit IBD and modulate the intestinal microflora (Snopek et al. 2018). In our study, we observed low biodiversity after the application of the (MMGPE) Shannon index 2.5 (Figure 1.A) in comparison with the group that was treated with 5-ASA, Shannon index 3.5 (Figure 1.B).

LefSe linear discriminant analysis (LDA) was used to determine significant differences in groups by genus (p <0.05). The LEfSe taxonomic cladogram (Figure 3) showed critical bacterial changes. At the generic level, an increase in the relative abundance of *Prevotella* (OTU_2), unknown order *Bacteroidales* (OTU_12) and *Lactobacillus* (OTU_21) were revealed. At the same time, the number of populations *Lachnospiraceae* (OTU_37, OTU_107), *Helicobacter* (OTU_43) was decreased. In addition, a high frequency of bacteria of the genus *Alloprevotella*

(producers of SCFA) was detected by using DeSeq2 technique. The increased number of *Prevotellaceae* in the microbiota of rats treated by a biological product can be explained by the activity of polyphenol component (Etxeberria et al. 2015). The detected increase of *Lactobacillus* was due to the stimulating effect of mare's milk (Fotschki et al. 2016).

In fact, the microbiota's shift can directly affect the vital functions of the organism (Järbrink-Sehgal and Andreasson 2020; Wan et al. 2020; Wang et al. 2020). In this study, we observed an increase in the strains producing SCFA, including *Prevotella, Alloprevotella, Lactobacillus, Ruminococcaceae, Blautia* as a result of the application of the (MMGPE). Moreover, a decrease in the number of *Helicobacter* and other representatives of pathogenic gut flora was also detected. Our findings suggest that the natural biologically active (MMGPE) possess therapeutic potential for the treatment of ulcerative colitis. However, more studies are required in order to validate its efficacy and clinical relevance.

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