Study protocol

Official Title: Effect of Natural Food on Gut Microbiome and Phospholipid Spectrum of Immune Cells in COVID-19 Patients
NCT number: NCT05970861
Document date: The 1st of October, 2024

Main Investigator: Abdugani Musayev, Ph.D., M.D.
Central Contact Backup (Project Manager): Vildan Indershiyev
Study Officials:
Ainur Yeshmanova, Ph.D.
Nikolay Safonov
Zhannat Nurmakhanova, Ph.D
Zhanar Nurgaliyeva, Ph.D.

Keywords: COVID-19, gut microbiome, antiphospholipid antibodies, rehabilitation, food products

Study Description

Brief Summary:

The efficacy of natural foods such as freeze-dried mare's milk (Saumal) in post-COVID syndrome therapy has not been studied. The literature review has shown that researchers have focused more on evidence-based medications and less on natural products. Some raw foods, such as freeze-dried mare's milk, contribute to forming complete immune complexes and have antioxidant, membrane stabilizing, and antiviral effects.

The use of Saumal proved its effectiveness in patients suffering from chronic hepatitis C. After 4 weeks of using freeze-dried mare's milk, the biodiversity of the intestinal microbiome was increased. The content of bacteria secreting short-chain fatty acids also increased.

The study aims to confirm these effects at the gene level in patients who underwent COVID-19. This study will allow us to develop a highly evidence-based component of rehabilitation therapy in patients after COVID-19.

Detailed Description:

The study consists of three stages.

Stage I. Randomized division of 75 patients under study into a main group of 38 patients receiving Saumal for 4 weeks and a control group of 37 patients not receiving Saumal. All respondents have suffered from COVID-19 in the last 6 months. At the moment of the research, all patients will be staying in rehabilitation centers in Almaty City.

The study's biological material will be collected from the respondents (gut microbiome, antiphospholipid antibodies, biochemical blood analysis (glucose, ALT, AST, uric acid, TAGs, alkaline phosphatase)).

Stage II. The main group will be given Saumal for four weeks, after which the gut microbiome, antiphospholipid antibodies, and biochemical blood analysis will be collected from both groups again. This will allow us to assess the impact of freeze-dried mare's milk on the condition of the gut microbiome, antiphospholipid antibodies, and biochemical blood analysis.

Stage III. Data analysis.

1. Based on the results of gut microbiome sequencing, ELISA (antiphospholipid antibodies), and biochemical blood analysis in the main and control groups, we determine the dynamic changes of those parameters during COVID-19 rehabilitation.

2. According to the results, the effectiveness of Saumal itself regarding these 2 parameters will be evaluated.

3. Develop recommendations and an algorithm for patient management after COVID-19 to prevent complications.

Study Design

Study Type: Interventional
Primary Purpose: Treatment
Interventional Study Model: Parallel Assignment
Number of Arms: 2
Masking: None (Open Label)
Allocation: Randomized
Enrollment: **60 patients.** Originally, we planned to include 38 patients in the main group and 37 in the control group. Overall, these groups were evenly divided, with 30 patients in each group.

Arms and Interventions

Arms	Assigned Interventions
Experimental: Main Group Patients who had suffered from COVID-19 in the six months before the start of the study. Patients will receive Saumal (freeze-dried mare milk) for four weeks.	**Dietary Supplement**: Freeze-dried Mare Milk (Saumal) Freeze-dried Mare Milk (Saumal) is frequently reported for having therapeutic and dietary properties associated with specific chemical composition and certain physical properties of the product. It contains a total of about 40 biologically active components, the most important of them vitamins A, C, B1, B2, B6, and B12, amino acids, enzymes, and trace elements (low molecular weight peptides, lactalbumins, and globulins). Saumal will be administered to patients at a dose of 25 grams of dry powder per 200 ml of water, 2 times a day, 30 minutes before the meal, for 4 weeks.
No Intervention: Control Group Patients who had suffered from COVID-19 in the six months before the start of the study. There will be no intervention.	

Eligibility

Minimum Age: 18 Years
Maximum Age: 75 Years
Sex: All
Gender Based: No
Accepts Healthy Volunteers: No

Inclusion Criteria:
Age 18 years and older;
Patients in rehabilitation after COVID-19;
Signed informed consent;

Presence of patient's history of COVID-19, reliably established by PCR+ /presence of IgG/ diagnosis of coronavirus pneumonia on Computer Tomography based on discharge from hospital or outpatient records.

Exclusion Criteria:
Chronic inflammatory bowel disease;
Gut microbiota transplantation;
Chronic pancreatic disease, period of exacerbation;
Liver cirrhosis, Metavir stage 3-4;
Any disease in the decompensation stage;
Neuralgic and psychological disorders that interfere with the study;
Cancer;
Non-transportable patients;
Patients who do not reside in Almaty;
Patients who have refused to participate in the study.

Examined for eligibility - 76 people;
Confirmed eligible - 60 people. According to our strict exclusion criteria, we had to remove 16 people [8 – oncological patients, 5 – moved house, 3 patients refused to participate];
Included in the study - 60 patients;
Completing follow-up and analysed - 60 patients.

Statistical Analysis Plan

Gut microbiota:

IBM SPSS ver. 23.0 (Chicago, IL, USA) was used to determine the statistical significance of the obtained data. All the indicators were verified for compliance with the law on normal distribution. If the variables obeyed this law, the Student's paired t-test was applied to determine statistical significance between the values before and after milk intake. Otherwise, the Wilcoxon sign rank criterion was used.

A single-factor analysis of variance was employed to verify the effect of administering freeze-dried mare's milk, which could potentially affect the indicators of gut microbiota.

Antiphospolipid antibodies:

The 95% confidence interval for aPL value was calculated for both median and mean. The confidence interval for the latter one was calculated in the descriptive statistics menu in IBM SPSS ver. 23.0 (Chicago, IL, USA).

It is necessary to find the value of the confidence coefficient (Student's criterion (t)) in the table of critical t-values, considering the total number of respondents (n) to find out a confidence interval for the median with a given confidence probability (p). After that, the calculation of the natural integer number (C) is needed for such a probability value: $Cp = (n/2 - t(p) \sqrt{n} /2)$. The lower confidence limit for the median is X (Cp), where X is the ordinal number of the value in the ranked variation series corresponding to the lower boundary of the confidence interval. The upper confidence limit for the median is X (n + 1 - Cp), where X is the ordinal number of the value in

the ranked variation series corresponding to the upper boundary of the confidence interval.
If the normal distribution law is followed, the Student's paired t-test was used to determine statistical significance between values before and after freeze-dried mare's milk. If not, the Wilcoxon sign rank criterion was used for intergroup comparison. One-way analysis of variance (ANOVA) was used to determine the relationship between the mare's milk prescription factor and aPL levels in both groups.
Quality of life:

IBM SPSS ver. 23.0 (Chicago, IL, USA) was used to determine the statistical significance of the obtained data. All the indicators were verified for compliance with the law on normal distribution. If the variables obeyed this law, the Student's paired t-test was applied to determine statistical significance between the values before and after milk intake. Otherwise, the Wilcoxon sign rank criterion was used.

A single-factor analysis of variance was employed to verify the effect of administering freeze-dried mare's milk, which could potentially affect the quality-of-life scores.

Results section

Gut microbiota analysis.

In the main group, after taking freeze-dried mare's milk, there was a decrease in the content of bacteria phyla Bacteroidetes, Proteobacteria, Verrucomicrobia and Chloroflexi (by 3.03%, 2.83%, 0.47% and 0.16, respectively), and an increase in the content of bacteria phyla Nitrospirae, Tenericutes, Cyanobacteria, Spirochaetes, Euryarchaeota, Actinobacteria and Firmicutes (by 0.02 %, 0.05%, 0,15%, 0,17%, 0,24%, 1,42% and 7.62%, respectively).

The Fusobacteria phylum was absent according to the results of mare's milk intake in all respondents. The Elusimicrobia phylum, on the contrary, was present in some respondents as a result of therapy.

Attention is also drawn to the presence of the Synergistetes phylum, which was absent in the control group both before and after therapy. Moreover, the difference between the Firmicutes, Proteobacteria, Chloroflexi, Cyanobacteria and Synergistetes phyla was statistically significant (Table 1).

Table 1. The percentage terms of all phyla of the gut microbiota in the main group before and after taking freeze-dried mare's milk (CI - confidence interval).

Phylum name	Before	After	95% CI (t-student)	p (t-student)
Bacteroidetes	32,32%	29,43%	-0,01; 0,07	0,178
Firmicutes	45,31%	51,87%	-0,09; -0,02	0,001
Proteobacteria	9,25%	6,52%	0,1; 0,28	<0,001
Actinobacteria	3,75%	4,9%	-0,15; 0,14	0,951
Euryaychaeota	1,28%	1,52%	-0,42; 0,1	0,204
Verrucomicrobia	1,29%	0,83%	-0,09; 0,32	0,223
Chloroflexi	0,23%	0,07%	0,04; 0,28	0,011
Cyanobacteria	0,16%	0,31%	-1,95; -0,17	0,028
Spirochaetes	0,08%	0,25%	-0,51; 0,17	0,324
Nitrospirae	0,01%	0,03%	-0,07; 0,02	0,235
Tenericutes	0,04%	0,09%	-0,2; 0,01	0,491
Fusobacteria	0,07%	0,00%	-0,05; 0,2	0,247
Elusimicrobia	0,00%	0,11%	-0,24; 0,02	0,109

Synergistetes	0,01%	0,09%	-0,14; -0,02	0,009

In the control group, after taking freeze-dried mare's milk, there was a decrease in the content of bacteria phyla Bacteroidetes, Firmicutes, Chloroflexi and Cyanobacteria (by 2.5%, 0.4%, 0.07% and 0.07%, respectively), and an increase in the content of bacteria phyla Spirochaetes, Tenericutes, Fusobacteria, Verrucomicrobia, Proteobacteria, Euryarchaeota and Actinobacteria (by 0.005%, 0.01%, 0,04%, 0,24%, 0,26%, 0,48% and 3.56%, respectively).

The Nitrospirae and Elusimicrobia phyla were absent as a result of taking mare's milk in all respondents of the control group. At the beginning of therapy, some respondents were found to have bacteria of the Caldithrix phylum, which were absent during the repeated measurement. The Thermotogae phylum was found in several members of the control group during two measurements.

Moreover, the difference between the Actinobacteria and Chloroflexi phyla in the control group was statistically significant (Table 2).

Table 2. The percentage terms of all phyla of the gut microbiota in the control group after 2 stool tests (CI - confidence interval).

Phylum name	**First test**	**Second test**	**95% CI (t-student)**	**p (t-student)**
Bacteroidetes	32,27%	29,77%	-0,06; 0,08	0,754
Firmicutes	46,5%	46,1%	-0,06; 0,07	0,899
Proteobacteria	7,65%	7,91%	-0,1; 0,12	0,823
Actinobacteria	5,8%	9,36%	-0,46; -0,1	0,003
Euryaychaeota	0,39%	0,87%	-0,74; 0,24	0,209
Verrucomicrobia	1,72%	1,96%	-0,97; 0,85	0,871
Chloroflexi	0,10%	0,03%	0,002; 0,15	0,044
Cyanobacteria	0,41%	0,34%	-1,42; 1,89	0,728
Spirochaetes	0,007%	0,012%	-0,01; 0,004	0,324
Nitrospirae	0,01%	0,00%	-0,01; 0,02	0,324
Tenericutes	0,04%	0,05%	-0,12; 0,1	0,857
Fusobacteria	0,01%	0,05%	-0,13; 0,05	0,357
Elusimicrobia	0,03%	0,00%	-0,01; 0,07	0,090
Caldithrix	0,02%	0,00%	-0,01; 0,03	0,160
Thermotogae	0,02%	0,01%	-0,03; 0,06	0,454

The proportion of Bacteroidetes and Firmicutes in the gut microbiota is most significant. Summarizing their values in the main and control groups, we can see that their content increased in the former and decreased in the latter. Statistical analysis revealed a significant difference in the content of these phyla between the main group compared to the control group (Table 3).

Table 3. The percentage terms of Bacteroidetes and Firmicutes phylum in the main and control groups.

Main group			Control group		
Bacteroidetes+Firmicutes (before)	Bacteroidetes +Firmicutes (after)	p (Wilcoxon)	Bacteroidetes+Firmicutes (first test)	Bacteroidetes +Firmicutes (second test)	p (Wilcoxon)
77,63%	81,3%	0,026	78,76%	75,87%	0,213

To determine the effect of freeze-dried mare's milk on the gut microbiota at the phylum level, a one-factor analysis of variance was performed. A statistically significant relationship between such a factor as the administration of freeze-dried mare's milk was revealed in the Firmicutes, Elusimicrobia and Proteobacteria phyla (Table 4).

Table 4. The relationship between the use of prescribed freeze-dried mare's milk and indicators of gut microbiota at the phylum level.

Phylum name	**p (One-way ANOVA)**
Bacteroidetes	0,180
Firmicutes	0,048
Proteobacteria	0,043
Actinobacteria	0,186
Euryaychaeota	0,403
Verrucomicrobia	0,583
Chloroflexi	0,220
Cyanobacteria	0,280
Spirochaetes	0,340
Nitrospirae	0,143
Tenericutes	0,657
Fusobacteria	0,140
Elusimicrobia	0,044
Bacteroidetes + Firmicutes	0,037

Overall, the intake of freeze-dried mare's milk by post-COVID patients primarily contributed to an increase in the content of the sum of two major phyla of gut microbiota — Bacteroides and Firmicutes, with the latter being particularly significant. There is also a notable decrease in the number of bacteria from the Proteobacteria phylum in the main group, which includes representatives of pathogenic microflora.

Antiphospholipid antibody analysis.

29 out of 30 main group patients (96.67%) had normal IgM and IgG levels before taking freeze-dried mare's milk. In the case of the control group, aPL levels were normal in all respondents. The aPL levels were normal in patients of both groups after the second ELISA (Table 5).

Table 5. Antiphospholipid antibody scores of each patient in the main and control groups after two assays (aPL -antiphospholipid antibodies).

Main group				**Control group**			
aPL (before)		aPL (after)		aPL (first assay)		aPL (second assay)	
IgM (U/ml)	IgG (U/ml)	IgM (U/ml)	IgG (U/ml)	IgM (U/ml)	IgG (U/ml)	IgM (U/ml)	IgG (U/ml)
8,97	1,55	6,22	1,34	1,33	1,2	1,91	1,8
2,7	0,943	2,45	1,1	4,3	4,1	4,11	3,87
0,936	0,535	0,936	0,535	1,32	1,46	1,3	1,35
1,12	0,841	1,12	0,841	4,5	4,3	3,2	2,1
3,17	0,943	2,61	1,01	5,1	4,5	3,33	3,51
3,56	0,433	3,21	0,877	5,89	5,67	5,64	6,01

27,2	1,45	3,98	1,2	3,1	2,89	2,67	2,1
7,77	2,25	4,01	2,34	5,22	6,01	3,77	4,13
1,46	5,47	0,56	0,98	8,97	9,34	6,54	7,8
1,53	***22,4***	4,01	2,67	6,32	6,54	4,78	5,33
1,43	1,1	3,1	2,56	6,89	7,2	5,89	6,6
3,46	1,21	3,5	1,11	6,4	5,6	6,1	5,1
4,42	1	4,21	1,12	5,12	4	4,85	3,66
4,42	1	4,21	0,66	8,9	9,8	7,91	8,76
1,35	1,22	1,33	1,56	0,654	0,533	0,632	0,612
2,96	2,06	2,9	2	3,57	3	3,4	2,76
2,26	1,55	2,12	1,41	2,67	2,45	2,3	1,98
3,14	1,67	3,14	1,67	8,43	8,12	7,3	7,6
0,78	3,25	0,98	2,65	9,52	9,9	9,21	9,43
4,37	1,58	4,01	1,76	5,99	5,76	5,77	5,4
1,27	1,88	1,34	1,7	5,99	5,76	5,54	5,32
1,48	1,67	1,44	1,66	4,06	4	3,8	3,4
1,27	0,82	0,93	0,7	5,6	6,7	5,8	6,5
3,69	1,85	3,41	2,1	5,78	6,3	5,65	6,2
0,88	1,06	1,32	1,01	6,8	7,4	6,32	6,54
5,2	1,15	4,34	1,45	4,89	5,1	4,76	4,7
8,5	1,58	7,21	1,33	7,2	7,8	6,5	7,1
1,48	0,85	1,56	1,01	0,64	0,77	0,77	0,87
2,67	1,18	0,78	0,98	2,31	2,1	2,22	1,98
0,98	1,61	1,12	1,45	1,2	2,3	1,5	2,6

There were no significant differences between the aPL values of the main and control groups (Table 6).

Table 6. Statistical analysis of antiphospholipid antibody levels of patients in the main and control groups after two assays (CI - confidence interval).

	95% CI (first assay)	95% CI (second assay)
Main group, IgM, mean	1,97; 5,66	2,12; 3,36
Main group, IgG, mean	0,73; 3,67	1,20; 1,65
Control group, IgM, mean	4,02; 5,89	3,63; 5,26
Control group, IgG, mean	4,03; 6,01	3,60; 5,41
Main group, IgM, median	1,43; 3,69	1,33; 3,98
Main group, IgG, median	1; 1,67	1,01; 1,67
Control group, IgM, median	3,57; 6,32	3,2; 5,8
Control group, IgG, median	3; 6,54	2,6; 6,2

The next calculations were done to stock parameters, before bringing them to a normal distribution. A statistically significant difference was found between IgM values before and after taking freeze-dried mare's milk. There was the statistically significant difference in both IgM and IgG values in the control group (Table 7).

Table 7. Statistical analysis of stock antiphospholipid antibody levels.

Main group			**Control group**		
	IgM	IgG		IgM	IgG
p-value (Wilcoxon)	0,021	0,471	p-value (Wilcoxon)	0,0001	0,0001

After bringing the variables to the normal distribution, the statistical significance was found in the control group only (Table 8).

Table 8. Statistical analysis of normally distributed antiphospholipid antibody levels.

	95% CI	**p-value (t-student)**
Main group, IgM	-0,03; 0,11	0,185
Main group, IgG	-0,07; 0,11	0,726
Control group, IgM	0,04; 0,14	0,001
Control group, IgG	0,05; 0,15	0,0001

ANOVA was performed to determine the direct effect of the natural food product on the aPL values (Table 9). ANOVA did not reveal any statistical significances.

Table 9. Finding the effect of the freeze-dried mare's milk on the antiphospholipid antibody values (ANOVA - analysis of variance).

Antibody name	p (One-way ANOVA)
IgM	0,541
IgG	0,938

aPL values in almost all patients of both groups were ranged normally. The intersection of all confidence intervals of medians and means indicates the absence of statistical significance between these indicators. The results before and after bringing the aPL values to normal distribution differ greatly, especially for the IgM level in the main group. One-factor analysis of variance also showed the lack of significance of the studied factor. These several theses can explain the given results. First, initial aPL values could have been normalized over 6-9 months after COVID-19. Second, there were screening kits in the study. Therefore, the obtained results do not show the levels of specific phospholipids (phosphatidylcholine, phosphatidylethanolamine, aCL, anti-β2-GP1, etc.). Third, the severity of COVID-19 was not considered as an inclusion criterion.

Quality of life analysis.

The quality of life of post-COVID patients in the main group statistically significantly improved in both physical and mental health components after using mare's milk. In the control group, there was no statistically significant change in quality-of-life indicators for both physical and mental health components. Even though the average score for the physical component in the control group is higher than in the main group, the level of statistical significance and the confidence interval of both groups allow us to conclude that freeze-dried mare's milk can have a positive effect on this indicator of the quality of life of post-COVID patients (Table 10).

Table 10. The average quality-of-life score in patients of the main and control groups.

	Quality-of-life components							
	Physical				Mental			
	Before	After	95% CI (t-student)	p (t-student)	Before	After	95% CI (t-student)	p (t-student)
Main group	43,515	47,965	-7,34; -1,56	0,004	45,06	49,524	-7,02; -1,91	0,001

Control group	50,451	50,797	-0,14; 0,09	0,647	47,46	49,393	-0,33; 0,03	0,091

The impact of directly administering a natural food product on the quality of life of post-COVID patients was verified using a one-way analysis of variance. The analysis showed that the relationship between mare's milk and quality of life indicators for the physical component is statistically significant. However, no statistical significance was observed for the mental component (Table 11).

Table 11. The relationship between the prescribed freeze-dried mare's milk and the quality-of-life scores.

Quality-of-life components	p (One-way ANOVA)
Physical	0,014
Mental	0,146

References

1. Zuo T, Zhang F, Lui GCY, et al. Alterations in gut microbiota of patients with COVID-19 during time of hospitalization. *Gastroenterology.* 2020;159:944–55.
2. Yeoh YK, Zuo T, Lui GC, et al. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut.* 2021;70(4):698-706. doi:10.1136/gutjnl-2020-323020
3. Romaniuk K., Majszyk-Świątek M., Kryszak K., Danielewicz A., Andraszek K. Alternative use of mare milk. *Folia Pomer. Univ. Technol. Stetin.* 2019;348:121–130. doi: 10.21005/AAPZ2019.49.1.13.
4. Fantuz F., Salimei E., Papademas P. Macro- and micronutrients in non-cow milk and products and their impact on human health. In: Tsakalidou E., Papadimitriou K., editors. Non-Bovine Milk and Milk Products. 1st ed. Elsevier Academic Press; London, UK: 2016. pp. 209–261.
5. Kushugulova A., Kozhakhmetov S., Sattybayeva R., Nurgozhina A., Ziyat A., Yadav H., Marotta F. Mare’s milk as a prospective functional product. *Funct. Food Health Dis.* 2018;8:537–543. doi: 10.31989/ffhd.v8i11.528.
6. Musayev, A., Yeshmanova, A., Pakhomenko, Y., Kozhakhmetov, S.S., Kushugulova, A.K. Effects of Environmental Pollutants on Intestinal Microbiome Under the Influence Of Mare's Milk In Patients with Hepatitis C. *Procedia Environmental Science, Engineering and Management* 2020;7(4):605–611.