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Development, quality and safety evaluation of a probiotic whey beverage

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ABSTRACT

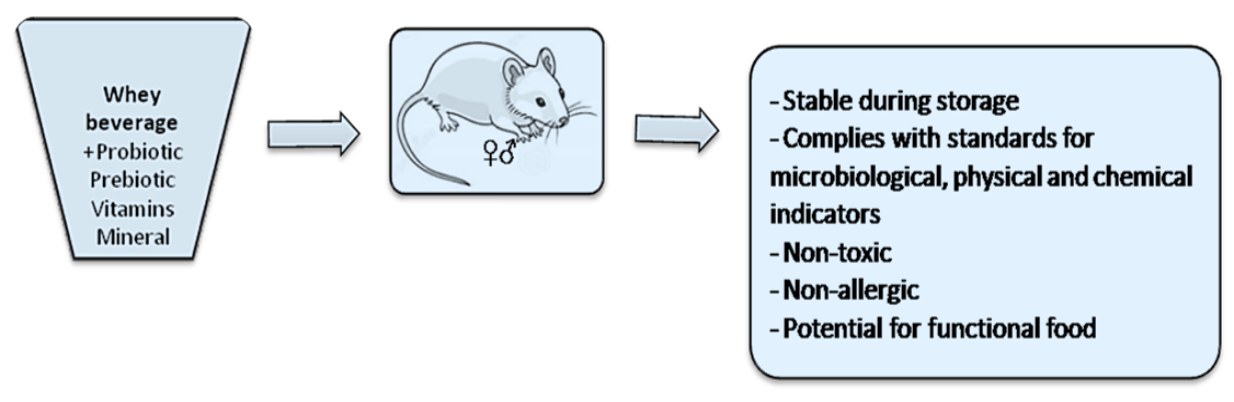
Background: This study was aimed at the development of a whey beverage enriched with a probiotic starter culture, prebiotics, vitamins, and minerals, and evaluation of microbiological, physico-chemical, and toxicological characteristics of the developed beverage.

Methods: The beverage formulation was determined based on organoleptic analysis. The assessment of microbiological and physico-chemical parameters was carried out in accordance with regulatory standards. The safety assessment of the developed drink was carried out *in vivo.*

Results: A beverage formulation based on whey enriched with probiotic bacteria *Lactobacillus casei* 1A, *Lactobacillus paracasei* 2A, *Lactobacillus brevis* 4 LB, prebiotic inulin, vitamins (A, C) and minerals (potassium iodide) was developed. The organoleptic, physico-chemical, and microbiological properties of the developed drink were determined. The quality of the beverage complied with food safety regulations, the viability of probiotic bacteria and the acidity of the beverage remained stable during storage. Acute score toxicity and allergenic properties *in vivo* did not reveal any physiological abnormalities and made it possible to classify the developed product as a low-hazard substance.

Conclusion: The optimal composition of a probiotic whey beverage has been developed, which can be considered as a potential product for functional nutrition.

Keywords: Whey beverage, Formulation, Probiotic, Functional, Safety Assessment, Toxicity, Allergenic properties, *In vivo*



INTRODUCTION

Today, the scientific community highly values advancements for preparing fermented milk products (beverage) with preventive values, enriched with minerals, vitamins, probiotics and prebiotics [1]. Their compositions vary quite a bit, are centered around utilizing milk and non-dairy substances, to provide protein, lipid, carbohydrate, vitamin and/or mineral benefit to existing products [2]. In developing functional milk drinks, it is necessary to adjust the amino acid, fatty acid, mineral and vitamin composition, as well as to give the products therapeutic and prophylactic properties by including biologically active substances and food additives of natural origin in their composition [3].

The range of functional probiotic drinks available on the Kazakhstan market is limited [4]. For the development of the dairy industry and import substitution in the milk market, expansion of products through the introduction of technologies that increase the nutritional and biological value may become a priority. The most relevant areas are the technologies that effectively process raw dairy materials, including whey, and the production of functional drinks based on it. There are about 200 dairies in the country. Every day, each of them produces up to 7 tons of waste, which are most often disposed of. Currently, whey beverages are being developed, but in fact, there are no more than two types of whey drinks on the shelves in local supermarkets, which does not satisfy the market demand [5-7].

Whey contains various nutrients like vitamins, lactose, essential amino acids, among others [8]. In addition, previous studies have reported that whey proteins are isolated as sources of bioactive peptides and can exhibit a number of physiological functions in the human body, affecting the immune, cardiovascular, nervous systems, and gastrointestinal tract [9].

Enrichment of whey-based products with probiotic bacteria increases its functionality. The development of beverages containing probiotic bacteria is a topical area of food production, and the maintenance of stabilized viable bacteria during shelf life is usually a technological problem [10]. In addition, in the process of developing the optimal functional composition of preventive products containing probiotics, it is necessary to assess the quality and safety for further production of products, taking into account the requirements of regulations in the field of development and production of products for functional nutrition.

Thus, the goal of the study is to assess the optimal composition, quality, and safety of a whey beverage, taking into account local raw materials, probiotic bacteria, minerals, vitamins, and prebiotics.

METHODS

Microbial cultures: Experiments were carried out with probiotic starter cultures Lactobacillus casei 1A, Lactobacillus paracasei 2A, Lactobacillus brevis 4LB. MRS media manufactured by HiMedia Laboratories (India) was used to grow starter cultures.

Storage of cultures of microorganisms: Microorganisms are grown under optimal conditions until the beginning of the stationary phase of growth or the end of the formation of resting forms. To protect against the damaging effect of low temperatures, the cells were preliminarily suspended in MRS medium containing 25% glycerol [11].

Preliminary study: This study was conducted at the Republican Collection of Microorganisms (RCM) in Astana, Kazakhstan. At the initial stage, preliminary tests were carried out on the selection of a probiotic culture, supplements in the form of a prebiotic, vitamins and minerals, and process parameters [12]. Probiotic cultures were stored at the RCM National Depository. We assess the viability of microorganism cultures using the Vanderzant method [13].

Physical and chemical analyzes: Physico-chemical parameters were measured in accordance with the requirements of regulatory documentation [14]. Titratable acidity was determined by a 0.1 mol/L NaOH milk titration and expressed as % Lactic acid. A pH-meter was utilized to measure pH [15].

Testing with the Compact DryTest: Compact Dry (Nissui Pharmaceutical Co., Japan) is a lyophilized medium in a cup applied to a fabric (mesh) base. The test sample, 1 ml of solution, is applied to the cup and diffusely distributed over the surface of the cup. After incubation, the results are obtained in the form of colonies grown on the surface of the medium [16].

Technological process of beverage production: Whey was a byproduct of the production of cottage cheese, produced by JSC "Astana-Onim", located in the Akmola region, Kazakhstan. Whey was collected immediately after draining the cottage cheese, then it was pasteurized at 85°C for 10 minutes. The technological process of obtaining a whey beverage is carried out in the following sequence:

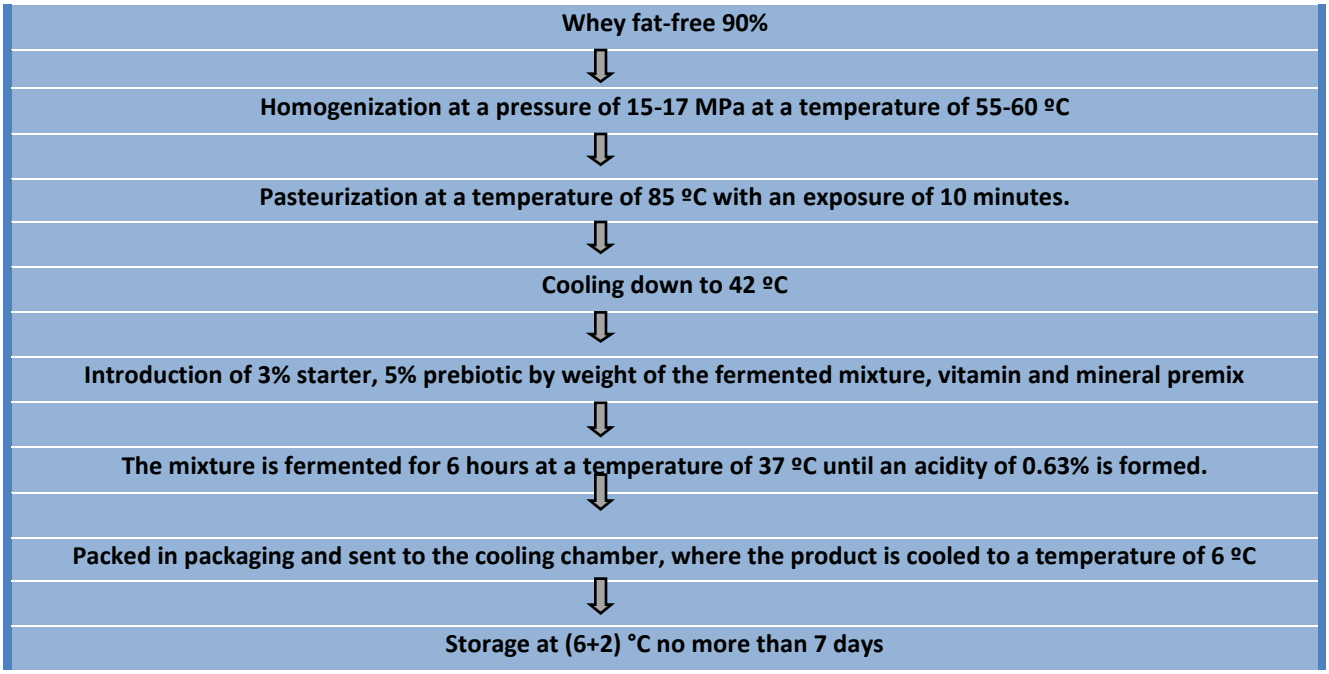


Figure 1: Sequence of technological process of obtaining a whey beverage.

Organoleptic assessment: The sensory commission consisted of nine employees of the Republican Collection of Microorganisms. Sensory experts were chosen based on their knowledge of the organoleptic properties of whey drinks. The beverage sample was transferred into 50 mL polyethylene containers and stored in a refrigerator (at 6 ± 2°C) overnight. After being shook before consuming, samples were judged on appearance, texture, taste, smell and color.

Ethical aspects: Research on animals was performed in compliance with ethical guidelines for the treatment of animals, based on normal operating procedures that comply with the guidelines established by the European Convention for the Protection of Vertebrate Animals used for other scientific studies [17]. The experiment protocol was agreed upon on January 19, 2021 by the Local Ethical Commission "National Center for Biotechnology", where relevant studies were carried out.

SAFETY RATING IN VIVO

Assessment of acute toxicity of the beverage: To assess the acute toxicity of the developed drink, the studied objects were delivered to CD -1 mice once at the greatest technically achievable dose (50 mL per kg of animal body weight) at the amount of $1x10^7$CFU/mL probiotic cultures.

The study whey beverage (WB) for acute toxicity studies were administered in such a way as to determine the minimum dose at which 100% of the animals would die and the maximum dose at which 100% of the animals would remain alive. The control group of animals were given drinking water at the volume (2.0 mL) and following the same scheme (once) as a manipulation control as the studied prophylactic drugs.

It was planned to register the terms of development of potential death in animals as a result of intoxication. Following the experiment, the internal organs of the selected animals were examined. The mean lethal concentration (LD_{50}) in case of death of laboratory rodents was determined using the Kerber method [18].

Assessment of the allergenic properties of the whey beverage

Conjunctival test on rabbits: To set up the sample, 1 drop of the WB was injected into the rabbits under the top eyelid, and a drop of sterile saline was administered to the second eye (control). Responses were taken into account 15 minutes later (rapid reaction) and 24-48 hours later (delayed hypersensitivity) and assessed on a scale of (in points): 0, absence of reaction; 1 - little redness in the lacrimal duct; 2 - redness in the lacrimal duct and sclera to the cornea; 3 - reddening of the whole conjunctiva and sclera. If the reaction also caused in itching, purulent ophthalmitis may result. Experimental Animals: Rabbits New Zealand males, weighing 3.0-3.5 kg, 3 heads in each group.

Method of skin applications on guinea pigs: The method of skin applications was performed on guinea pigs who weigh 550–600 g, with 5 animals per group. On the clipped area of the skin on the body of the albino guinea pigs, 3 drops of the WB were administered for 2 weeks, about 5 times weekly. The reaction was taken into account daily according to the skin test rating scale.

Statistical processing methods: Microsoft program Excel 97 was utilized for statistical processing of results. Distributions were described by mean and error of the mean. The nonparametric Mann - Whitney test. U –test had been utilized to assess intergroup differences.

RESULTS AND DISCUSSION

Table 1 shows the whey beverage formulation. Vitamin A (PHR1236, Sigma), Vitamin C (A92902, Sigma), potassium iodide (207969, Sigma) and prebiotic inulin from chicory root (45710, Thermo scientific) were used for testing.

Table 1. Beverage formulation.

Compound	Normal [19]	Formulation
Starter cultures *L.* casei1A, *L. paracasei* 2A, *L.brevis* 4LB, %	3	3
Inulin, %		Up to 5
Vitamin A, mg/L	0.5-1.0	0.5
Vitamin C, mg/L	50-120	50
Potassium iodide, mg/L	0.14±0.03	0.14
Whey, %	Up to 100	Up to 100
Acidity, % LA	0.18-0.675	0.63±0.03
pH	3.7-6.3	4.5±0.03
Fermentation time, (h)		6
Organoleptic properties	White to yellow homogenous liquid, sour taste	Milky color, homogenous liquid, sour taste

Mean ± SD, $p \leq 0.05$.

Organoleptic properties, fermentation activity, pH, acidity of WB, and the optimal formulation was determined, including probiotics *Lactobacillus casei* Y1*, Lactobacillus brevis* 4 LB*, Lactobacillus paracasei* Y2 at a concentration of 10^7 CFU/ml, vitamins (A, C), inulin, and potassium iodide. These probiotics contain organoleptic properties such as milky white color, sour taste, liquid consistency, pH 4.5, acidity 0.63±0.03%. Organoleptic indicators of drinks depend on the quality of raw materials, technology, type and quality starter cultures, additives and storage. As a prebiotic component of the drink, we included inulin at a concentration of up to 5% by weight of the mixture. The dairy sector is one area where inulin is widely used due to its prebiotic properties as well as the texture it imparts to products [20]. Guimaraes et al. showed the effect of inulin in the production of stable prebiotic whey beverage [21]. No recommended dosage has been identified for inulin supplementation. In some studies, participants consumed foods containing 1 to 10% inulin [22].

A monotonous or unbalanced diet can lead to a lack of one or another vitamin, which can subsequently lead to metabolic disorders and the occurrence of various diseases [23-24]. Therefore, it is important to know the average values required for intake with food or in the form of bioactive additives that ensure the ideal execution of the physiological and biochemical activities fixed in the human genotype [25-26]. In our study, the addition of a vitamin-mineral premix was calculated in accordance with the regulatory standard [19].

It should be noted that the daily intake of vitamins is approximate and conditional, designed for an average person. Based on the fact that the physiological need (for adults) per kg of body weight for vitamin C is not more than 50-100 mg vit[27]. Vitamin A is not more than 1.0-1.6 mg kg, and iodine-containing products are not more than 290 µg, the minimum dose of the drink is determined no more than 1 peliter per day [28].

To assess the food safety of WB, selective chromogenic media produced by Nissui Pharmaceutical Co (Japan) were used. Seeding was carried out from freshly prepared WB on dishes containing test strains CompactDry: Enterococci (ETC), *Staphylococcus aureus* (X-SA), coliforms (CF), *Salmonella* (SL), *Escherichia coli* (EC), *Listeria* (LS), yeasts, and fungi (YM). The study of food safety assessment showed the absence of growth of opportunistic microflora on diagnostic media - enterococci, staphylococci, coliforms, salmonella, listeria, yeast and fungi. The method has been used in previous studies to detect coliforms and staphylococci in food samples [29-31] and uropathogens [32] and showed high sensitivity of the test [33].

Physico - chemical and microbiological properties of the developed beverage: Nutritional value includes the content of basic chemicals, the degree of their assimilation, taste, harmlessness. Determination of physical and chemical parameters was carried out on the milk analyzer Expert pro (Russia). The results of laboratory tests were conducted in compliance with the required guidelines for safety indications (table 2) [14], [34]. Test conditions: humidity - 23.0% and temperature - 22.73 °C.

Table 2. Physical and chemical properties.

Indicators	Results	Key Indicators According to Regulatory Documentation [34]
Mass fraction of fat, %	1.39 ± 0.1 _	0.1-9.9
Mass fraction of DSMR, %	9.57±0.01	Not less than 7.8
Acidity, % LA	0.63 ±0.02	
Density, kg/m^3	1.03508±0.01	2.6-4.0
Mass fraction of protein, %	3.65±0.2	8.0
Mass fraction of lactose, %	5.13±3.62	
Mass fraction of salts, %	0.80±0.01	
Freezing point, °C	0.59±0.01	
Mass fraction of water, %	0.0±0.01	
Conductivity, (Ms/cm)	9.09±0.1	
Sample temperature, °C	22.73 ± 0.0 1	

Mean ± SD, $p \leq 0.05$. DSMR - dry skimmed milk residue

According to Table 2, the main physical and chemical parameters of the developed beverage correspond to the regulatory document [14]. The main physical and chemical indicators of the beverage: fat 1.39 ± 0.1%, protein 3.65 ± 0.2%, acidity 0.63 ± 0.02% LA, DSMR - 9.57 ± 0.01%. These results are consistent with the results of other studies, where the physico-chemical characteristics of whey-based fermented beverage were demonstrated [8], [35-37].

The finished beverage contains in its composition $1x10^7$CFU/ml of probiotic cultures, which is an indicator that meets the standards [34]. Further, the number of cells of probiotic microorganisms and the change in titratable acidity in beverage were determined for 30 days when stored at 4 ± 1 °C.

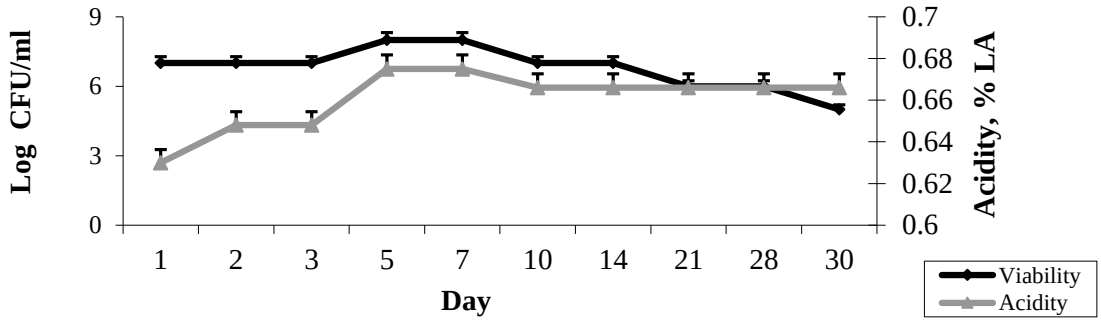


Figure 1. Determination of the number of viable cells of probiotics in the beverage and the acidity level during 30 days of storage at 4 ± 1 °C.

As displayed in Figure 1, the determination of the quantity of cells of probiotic microorganisms WB during the month depends on the duration of storage. Thus, in WB the greatest increase in the number of probiotic bacteria up to 10^8 CFU/ml on the 5th day is observed, but gradually decreases after 7 days of storage. The result obtained is consistent with the research of Silva e Alves [8] which reported a decrease in the titer of *Lactobacillus acidophilus* in whey-based beverage after 21 days of storage, at 4±1 °C. Another study showed stable survival of *Lactobacillus acidophilus* in a whey beverage at a shelf life of 56 days, 4 ± 1 ° C [38]. However, our prepared beverage had the required amount of viable probiotics and could be considered a functional dose for human consumption up to 7 days at 4° C.

The measurement of titratable acidity WB was carried out for a month. As can be seen from the data in Figure 2, after one month of storage, WB had a higher acidity (0.675 ± 0.01% LA) after 10 days of storage in the WB, the acidity level practically does not change during the month of storage. These data are consistent with the studies carried out in the study of Drgalic [36] and Islam [38] where acidity levels in probiotic drinks are demonstrated.

Thus, analyzing storage survival rate data of probiotics and the change in titratable acidity in the developed beverage, the recommended shelf life is 7-10 days at a storage temperature of 4 ± 1 °C.

Acute toxicity assessment of whey beverage on mice: To evaluate efficacy, correct dosage, and long-term effects, formulated formulations should be tested *invivo* [39]. The purpose of this stage was to determine the tolerable, toxic and lethal amounts of the developed WB and the causes of death of animals.

Each study group contained 6 animals, with 12 female and 12 male mice in total. Since WB are of low toxicity and it was not possible to determine LD_{50}. Due to

the lack of dead animals, mice were applied the greatest achievable volume, which was 50 mL/kg of body weight of mice in the amount of $1x10^7$CFU/mL probiotic cultures.

Rodent groups for the experiment were distributed as follows: group 1 (6 male mice) - injected with WB, once; group 2 (6 male mice) - drinking water was injected in a volume of 2.0 ml (manipulation control); group 3 (6 female mice) - injected with WB, once; group 4 (6 female mice) - drinking water was injected in a volume of 2.0 ml (manipulation control).

With a single intragastric injection of the WB at the highest achievable dose of 50 ml/kg body weight of mice, animal death was not observed. The results of the impact of WB on the dynamics of body weight of mice table 3.

Table 3. The effect WB on mice's body weight.

Experimental group, n=6	Body weight (g) of mice		
	At the start of the experiment	After 1 week	After 2 weeks
Group1,♂	31.7±0.9 p =0.7821	31.0±0.9 p =0.6467	32.1±0.9 p =0.5699
Group2, control, ♂	32.1±1.2	31.8±1.5	31.1±1.4
Group 3, ♀	28.3±1.0 p=0.9542	27.4±0.8 p=0.9614	27.9±0.9 p=0.3375
Group 4, control, ♀	28.4±0.4	27.4±1.5	29.7±1.5

Mean ±SD; p - significance level, p <0.05 - statistically significant differences compared to the corresponding numbers in the control group of animals; ♂ - male; ♀ - female; n - number of animals in the group.

The outcomes seen in Table 3, display no effect of the WB on the dynamics of the body weight of mice with them receiving the maximum achievable dose of 50 mL/kg of mice body weight.

The overall condition and responses of the animals, possible death, and the expression of symptoms of intoxication, were observed over the course of the experiment (Table 4).

Table 4. The effect of a whey beverage on the state of mice observed for two weeks.

Parameters	Experimental group, n=6			
	Group 1, ♂	**Group 2, control, ♂**	**Group 3,♀**	**Group 4, control, ♀**
Level and nature of motor activity	The mice are moving, and their coordination is not impacted.			
The existence and nature of seizures	Missing			
State of hair and skin	No changes seen (wool is white, clean, smooth)			
Condition and color of mucous membrane	No changes found			
Response to sound, pain stimuli	React			
Animal death	0			
Urination (color of urine)	No changes found			
Defecation (consistency, color)	No changes found			

♂ - male; ♀ - female; n - quantity of rodents in the group

On the first day after the introduction of WB and over the course of the whole study, no changes were observed in the actions, appearance, and motor activity of mice. The study did not lead to the death of rodents or any other significant behavioral alterations.

After 2 weeks of WB, rodents were removed from the experiment, and their internal organs were examined and removed to be weighed (Table 5).

The masses of the organs of the mice that were impacted by the WB did not differ from the mass of the internal organs of the control mice. The internal organs of the mice that were administered the drink failed to be different from the internal organs of the control mice.

Table 5. Influence of WB on the mass of internal organs of rodents.

Experimental group, n=6	Mass of internal organs, g						
	Brain	**Heart**	**Lung**	**Liver**	**Spleen**	**Kidneys**	**Gonads**
Group 1, ♂	0.380±0.020 p=0.4441	0.201±0.012 p=0.2948	0.275±0.022 p=0.3861	2.539±0.170 p=0.3918	0.228±0.009 p=0.2098	0.332±0.011 p=0.2647	0.097±0.003 p=0.2900
Group 2, control, ♂	0.398±0.008	0.186±0.006	0.296±0.009	2.364±0.098	0.214±0.006	0.349±0.010	0.101±0.003
Group 3, ♀	0.408±0.008 p=0.2816	0.178±0.007 p=0.2905	0.248±0.008 p=0.4139	2.004±0.002 p=0.8196	0.193±0.006 p=0.4294	0.264±0.048 p=0.5401	0.017±0.001 p=0.4452
Group 4, control, ♀	0.420±0.007	0.169±0.004	0.299±0.060	1.980±0.103	0.183±0.011	0.232±0.016	0.015±0.002

Mean ±SD; ♂ - male; ♀ - female; n - number of animals in the group; p - significance level, p <0.05 - statistically significant differences compared to the corresponding values in the control group of animals.

A biochemical study of urine, which was performed one day after the administration of WB, no advances from normal values of physiological functioning were observed. The studied parameters in the experimental groups that were administered WB did not differ from outcomes of the control group of mice (Table 6).

Table 6. The effect of WB on the biochemical parameters of urine**.**

Experimental group, n=6	Parameters				
	Erythrocytes, units µL	**Ketones, mmol/L**	**Protein, g/L**	**Glucose, mmol/L**	**pH**
Group1, ♂	6/6 - negative	0.2±0.1 p=0.1503	0.7±0.2 p=0.4316	6/6 - negative	6.5±0.1 p=0.9055
Group 2, control, ♂	6/6 - negative	0.1±0.0	0.5±0.1	6/6 - negative	6.5±0.1
Group 3, ♀	6/6 - negative	0.2±0.1 p=0.9091	0.7±0.2 p=0.3126	6/6 - negative	6.3±0.1 p=0.3134
Group 4 , control, ♀	6/6 - negative	0.2±0.1	0.5±0.2	6/6 - negative	6.2±0.1

Mean ±SD, n – quantity of animals in the group**.**

Based on the results of acute toxicity studies on outbred laboratory rodents of the CD -1 line, according to the generally accepted hygienic classification, WB is associated with the 4th hazard class - low-hazard substances [40].

These results match that of other studies where the safety assessment of probiotic in vivo was demonstrated, so Chaudhari K [41] showed the safety of probiotic *B. coagulans* on acute toxicity studies in mice, Lee [42] studied the toxicity of probiotic doses in male and female mice for 2 weeks, which did not reveal abnormal clinical signs of body mass, hematological parameters.

The results of the assessment of the allergenic properties of the beverage: The results of the study of the allergenic properties of the beverage in the conjunctival test are shown in Table 7.

Table 7. The outcomes of the study of the allergenic properties of WB in the conjunctival test in rabbits.

Experimental group, n=3	Animals with positive reaction in the conjunctival test		
	After 15 minutes	After 24 hours	After 48 hours
Group 1, ♂	0	0	0
Group 2, control, ♂	0	0	0

♂ - male; n - quantity of rodents in the group

Utilizing the data found in Table 7, it can be seen that the WB in the conjunctival test does not have an allergenic property. To assess the allergenic effect of WB, the method of skin applications on albino guinea pigs was also used. The total number of applications was 10. The skin reaction was taken into account daily according to the skin test rating scale. This experiment allows ~~you~~ to identify the risk of developing non-allergic contact dermatitis.

The study outcomes of the allergenic effect of WB by the method of skin applications are presented in Table 8.

Table 8. The outcomes of the experiment of the allergenic effect of WB by the method of skin applications in guinea pigs

Experimental group, n=5	Number of applications	Animals with positive reaction (presence of erythema/edema)
Group1, ♂	10	0/0

♂ - male; n - the number of animals in the group

From the data found in Table 8, it is implied that the whey beverage during the course of skin application failed to cause hyperemia and skin edema, which could indicate the development of a skin allergic reaction. Thus, it has been shown that the WB in the studied tests (conjunctival test and the method of skin applications) does not show allergenic properties.

Among people prone to allergies, long-term use of probiotics or fermented foods can lead to side effects and the risk of rhinitis, severe asthma attacks and atopic dermatitis, allergies and sensitization [43]. Animal studies of the allergenic properties of whey-based beverages *in vivo* may be useful in predicting the effectiveness of hypoallergenic beverages in humans in preventing allergy to milk products [44-47].

CONCLUSION

Whey has highly beneficial nutritional characteristics due to its high content of essential amino acids with high bioavailability. In this regard, the formulation of a drink based on milk whey with the addition of probiotic bacteria *Lactobacillus casei* 1A*, Lactobacillus paracasei* 2A*, Lactobacillus brevis* 4 LB, prebiotic inulin, vitamins (A, C) and mineral (potassium iodide) has been developed.

The study confirmed the potential of whey as a favorable environment for preserving the growth of probiotic microorganisms. The technology of preparation of a potential probiotic drink based on whey has been developed. The drink has good organoleptic characteristics, contains stable therapeutically significant levels of beneficial microorganisms and is safe in terms of toxicity and allergenic properties, which opens up prospects for further research in the development of functional fermented beverages based on whey.

Abbreviations: WB – whey beverage; CFU - colony forming units, SD – standard deviation

Authors Contribution: All authors contributed to this study and wrote this paper.

Competing Interests: The authors declare no conflict of interest.

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