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Production of probiotic kvass beverages enriched with pine nut shell extract and propionic acid bacteria

Andrey Germanovich KHANTURGAEV[1,2], Irina Sergeevna KHAMAGAEVA[1], Valentina Germanovna SHIRETOROVA[1,3*]

Abstract

The aim of this study was to develop a new functional product: a probiotic kvass beverage (PKB) based on curd whey with the addition of polyphenols from *Pinus sibirica* shell extract and propionic acid bacteria (PAB). The studies conducted have shown that replacing water with curd whey in the traditional technology for preparing kvass wort contributes to the active growth of yeast and the accumulation of alcohol. Therefore, it has been established that the separate cultivation of yeast and PAB is optimal. The optimal parameters for producing a product with the best consumer properties were determined: the dose of yeast starter, the amount of PAB concentrates, the quantity of pine shell extract, and the duration of the cultivation stages. Fermentation of whey-based fermented kvass wort with a concentrate of PAB and pine shell extract enriches it with propionic, acetic, and free amino acids (FAA) and B vitamins. Also, it improves the consumer properties of beverages and the quality and functional properties of the finished product. Tannins give the product a brown color and a pleasant tart taste. The high antioxidant activity of tannins contributes to preserving the quality of beverages during long-term storage. Pronounced antagonistic and antimutagenic activities of the resulting drink were established due to the combined action of yeast, PAB, and pine shell extract. Based on the research, a technology for producing a PKB was developed.

Keywords: probiotic beverage; kvass; curd whey; yeast; propionic acid bacteria; pine nutshell.

Practical application: This research expands the range of functional beverages with probiotic and antioxidant properties.

1 INTRODUCTION

Fermented functional food products are among the most significant segments of the food processing industry. Fermentation improves organoleptic properties, extends shelf life, produces new health-promoting compounds, and enhances the bioavailability of vitamins and minerals (Sharma et al., 2022). Traditionally, fermentation has been used as an efficient way to preserve various types of foods, such as dairy, vegetables, meat, grains, and fruits (Tamang et al., 2020). The growing interest in research and development of new fermented foods among nutritionists, biologists, and consumers is because fermented foods are rich in unique bioactive ingredients that can provide health benefits (Şanlier et al., 2019). Researchers have shown the effectiveness of fermented foods against diseases such as cancer (Bermejo et al., 2019; Zhang et al., 2019), cardiovascular diseases (Companys et al., 2021; Zhang et al., 2020), bowel dysbacteriosis (Stiemsma et al., 2020), obesity (Mohammadi et al., 2021), diabetes (Almeida Souza, 2020), and also immunosuppression (Sharma et al., 2019), as well as an increase in life expectancy (Das et al., 2020).

Functional products are produced by adding exogenous natural compounds, probiotics, or other microorganisms that produce biogenic compounds (Lamsal & Faubion, 2009). Polyphenols are known for their antioxidant and antimicrobial properties in raw food preservation (Martinengo et al., 2021). Studies have shown that plant polyphenols inhibit the growth of pathogenic bacteria while stimulating the growth of probiotic microorganisms (Pacheco-Ordaz et al., 2018; Ray & Mukherjee, 2021; Sharma et al., 2022). Furthermore, it has been demonstrated that association with polyphenols can increase the degree of survival of probiotic bacteria both during food storage and in the intestine (Vodnar & Socaciu, 2012), and vice versa. As a result of biotransformation during incubation with probiotics, qualitative and quantitative changes in the polyphenol profile are possible, improving the biological properties (for example, antioxidant activity) of polyphenolic products (López de Lacey et al., 2014). Thus, developing new functional products where polyphenols are combined with probiotic bacteria, which contribute to the suppression of pathogenic species and the survival of probiotic bacteria, as well as having their biological activity, is a promising direction.

The shell of pine nuts is a large-scale waste during their processing to obtain kernels used in food, and pine nut oil is a valuable source of polyunsaturated fatty acids. More than one million tons of pine nuts (*Pinus sibirica* Du Tour seeds) are harvested annually in Siberia for industrial processing, where a large amount of waste accumulates in the form of shells, which

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[1]*East-Siberian State University of Technology and Management, Ulan-Ude City, Russian Federation.*
[2]*ITMO University, Faculty of Biotechnology, St. Petersburg, Russian Federation.*
[3]*Baikal Institute of Nature Management of Siberian Branch of the Russian Academy of Sciences, Ulan-Ude City, Russian Federation.*
*Corresponding author: vgshiretorova@mail.ru

make up 51–59% of the mass of nuts. Siberian pine's seed shell contains various polyphenolic compounds, such as flavonoids, phenolic acids, tannins, and lignans (Khamidullina et al., 2005; Pozharitskaya et al., 2006; Pozharitskaya et al., 2007; Rogachev & Salakhutdinov, 2015).

In folk medicine, pine shells in tinctures and decoctions were used to treat gastrointestinal tract, articular, skin diseases, deafness, haemorrhoids, leukaemia, etc. (Telyatiev, 1976). Studies have established the anti-inflammatory and choleretic activity of a dry aqueous-alcoholic extract obtained through microwave aqueous-alcoholic extraction of the Siberian pine seed shell (Shiretorova, 2014; Shiretorova et al., 2005).

Kvass is a soft beverage traditional to Eastern European countries, typically produced by fermenting kvass mash with yeast (traditionally *Saccharomyces cerevisiae*) and lactic acid bacteria. Kvass is a traditional Russian beverage made by fermenting kvass wort with a combined starter culture of yeast and lactic acid bacteria. It should be noted that the preparation of yeast and lactic acid bacteria breeding is a laborious and lengthy process associated with microorganism transfers in the laboratory and then with the separation of pure cultures. Previous studies have shown that propionic acid bacteria (PAB) multiply well in curd whey and enrich it with biologically active substances with a broad spectrum of activity (Orlova, 2021; Ranaei et al., 2020). The high nutritional value of curd whey makes it a promising raw material for non-alcoholic refreshing beverages (Russian National Standard, 2013).

In connection with the above, the study aimed to develop a new probiotic kvass beverage (PKB) based on curd whey by adding polyphenols from pine shell extract and PAB.

2 MATERIALS AND METHODS

Experimental studies were conducted in the laboratories of the Small Innovative Enterprise "Bifivit" and the Small Innovative Enterprise "Baikal-EcoProduct" of the East Siberian State University of Technology and Management (ESSUTM).

Materials and methods were preferred for this study to meet the Russian National Standard Methods (GOST) requirements.

2.1 Materials

Kvass wort concentrate (Russian National Standard 28538-2017) is a product obtained by mashing with water rye and barley malt, rye or corn flour, or freshly germinated stewed (fermented) rye malt with the addition of rye flour and enzyme preparations, followed by clarification, thickening the resulting wort in a vacuum apparatus, and heat treatment of the product. In appearance, it is a thick viscous liquid of dark brown color, with sweet and sour taste, slightly pronounced bitterness, and the aroma of rye bread. It is highly soluble in water and has a mass fraction of solids of 70±2% and a titratable acidity of 16±4.0 cm^3 of NaOH solution with a concentration of 1.0 mol/L per 100 g of concentrate.

Curd whey (Russian National Standard 34352-2017) is a raw material obtained as a by-product in the manufacture of cheese, cottage cheese, and casein and intended for further processing and use for feed purposes.

Whey was fermented using baker's yeast (Russian National Standard 171-2015), which is a technically pure culture of Saccharomycetes yeast fungi, and bacterial concentrate "Propionix" TS (Technic Specifications) 9229-007-02069473-2005 (manufacturer SIE "Bifivit", ESSUTM, Russia). This probiotic starter is a concentrated microbial mass of *Propionibacterium freudenreichii* subsp. *shermani* - KM 186, whose bacteria are in a live-action form. PAB strains were obtained from the All-Russian Collection of Microorganisms Fund of the Institute of Biochemistry and Physiology of Microorganisms (Moscow, Russia), activated by the biotechnological method developed at ESSUTM.

As a source of polyphenols, a dry extract of the Siberian pine seed shell *Pinus sibirica* shell extract (PSSE), obtained by micro-wave-assisted extraction with 60% ethyl alcohol, was used. The method of obtaining it is described in Patent RU 2351641 (Zalutsky et al., 2007). The content of tannins in this extract was 12%.

Titratable acidity was determined using the titrimetric method outlined in the Russian National Standard 3624-92, with the phenolphthalein indicator. The alcohol content was determined using a pycnometer of the relative mass of the distillate solution obtained from the product, according to Russian National Standard 3629-97. The carbon dioxide content was determined by measuring the pressure in a sealed bottle and calculating the mass fraction of carbon dioxide depending on the estimated pressure and temperature of the beverage, according to Russian National Standard 51153-98.

The antioxidant activity of the dry extract of pine shell was determined by the accumulation of malondialdehyde (MDA), which is one of the products of free radical lipid oxidation. This measurement employed the TBARS method (Thiobarbituric Acid Reactive Substances), where MDA reacts with thiobarbituric acid. At high temperatures in an acidic medium, MDA reacts with two thiobarbituric acids to form a colored trimethine complex with an absorption maximum of 532 nm (Stalnaya & Garishvili, 1977). Butter with a mass fraction of fat of 82.5% was used as a model system.

The analysis was compared with standard amino acid hydrolysates according to ISO 13903 (ISO, 2005). The research was conducted on the amino acid analyser AAA 339 T N (Czech Republic).

Water-soluble B vitamins in their free forms and their concentrations in the probiotic beverage were analyzed with a capillary electrophoresis system "Kapel-105M" (Lumex LLC Ltd., St. Petersburg, Russia).

The microbiological parameters of the PKB were determined according to Russian National Standard 32901-2014.

The antimutagenicity of the plant extracts against 4-nitroquinoline 1-oxide (4-NQO) was determined using the Ames test (Maron & Ames, 1983).

The agar diffusion method determined the antagonistic activity against *Shigella sonnei* and *Escherichia coli* test cultures.

The quantitative account of PAB (Propionic acid bacteria) was determined by limiting dilutions on a nutritional hydrolyzate-milk medium according to Technic Specifications (TS) 10-10-02-789-192-95.

The obtained data were statistically processed using the Statistica 6 software package. The non-parametric Mann-Whitney test was used to compare the independent samples. Differences were considered significant if the error probability was $p \leq 0.05$.

3 RESEARCH RESULTS

In the first stage of the work, the authors studied the biochemical activity of yeast in the preparation of kvass wort on curd whey. First, kvass wort was prepared by diluting pasteurized kvass wort concentrates in curd whey with added sugar syrup to a solid concentration of 6.5%. Then, a 2, 4, and 6% starter containing a prepared culture of yeast Saccharomices minor of race M was added. Cultivation was carried out for 16 h at a temperature of 30°C using the standard technology for producing kvass. For comparison, we used the control, in which the kvass wort was prepared in water and the dose of yeast starter was 4%. During cultivation, the accumulation of alcohol and carbon dioxide in kvass wort was also determined. The results of the studies presented in Figure 1 showed that yeast cells develop more actively in a medium prepared using curd whey. Furthermore, a proportional relationship exists between the amount of starter and the accumulation of alcohol and carbon dioxide.

At the next stage of the study, dry *Pinus sibirica* shell extract (PSSE) was added to improve the quality of the probiotic beverage. As a result of the study (Figure 2), it was found that PSSE has antioxidant properties. Furthermore, when PSSE was added in amounts of 0.05, 0.1, and 0.15% to the model system (butter), a significant slowdown in the rate of MDA accumulation was observed compared to the control (without PSSE), which indicates a decrease in the lipid oxidation rate.

The authors introduced PSSE in the amount of 0.1–0.3 g/L and conducted fermentation to establish the optimal doses and their effect on the formation of the properties of the kvass beverage. The introduction of 0.1 g/L PSSE increases the organoleptic properties of the glass; at 0.2 g/L, an improvement in organoleptic properties is observed; and at 0.3 g/L, excessive astringency appears in the taste of the kvass beverage, which reduces its organoleptic properties. Thus, the samples with the addition of PSSE in the amount of 0.2 g/L are characterized by the highest organoleptic properties.

Next, the authors studied two ways to apply the PAB concentrate. The first method involved the fermentation of fermented kvass wort with PAB concentrate, which was added 4 h before the end of kvass wort fermentation. It has been established that the number of viable PAB cells reaches $3*10^{10}$ CFU/cm^3. The titratable acidity of fermented and fermented kvass wort is 7.5 mL of 1 N NaOH solution per 100 mL of beverage. The active growth of PAB is explained by the fact that the waste products of yeast stimulate their growth. At the same time, it should be noted that the active development of PAB leads to a decrease in the evolution of yeast microflora and the accumulation of alcohol. The number of viable yeast cells was 10^7 CFU/cm^3.

The second method consisted of various doses of the bacterial concentrate added to the fermented kvass wort and cooled to 8°C. The study results of PAB cell growth dynamics and titratable acidity with the introduction of various doses of the concentrate, illustrated in Figure 3, show that increasing the concentrate amount leads to the activation of the fermentation process.

The authors studied the change in the physicochemical, microbiological, and organoleptic parameters of PKB during storage for 30 days at a temperature of 4±2°C to determine the shelf life. The difference in the number of viable PAB cells and titratable acidity during storage is shown in Figure 4. Significant

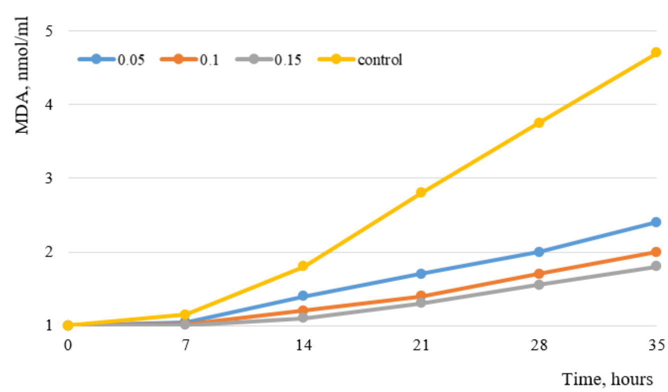

Figure 2. Antioxidant activity of PSSE.

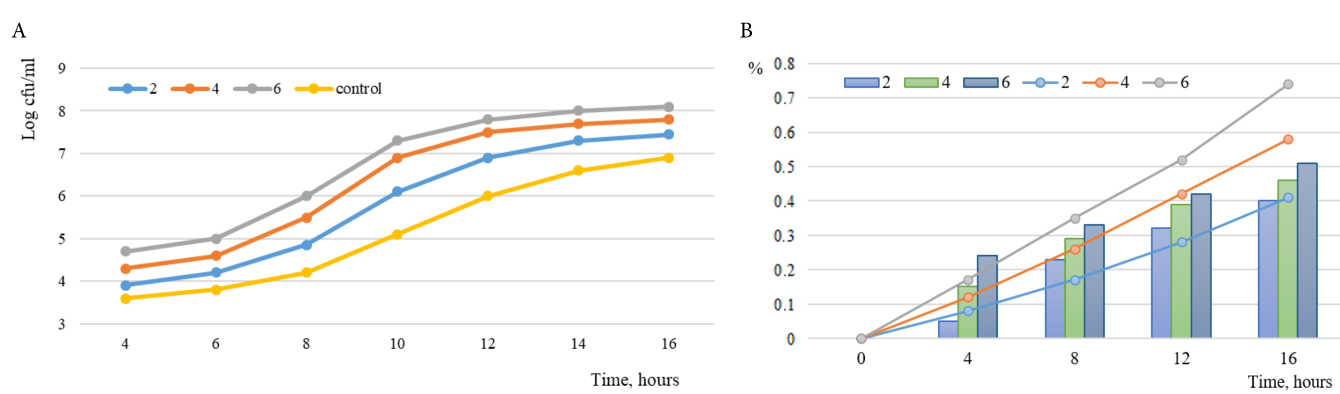

Figure 1. Influence of the yeast starter dose on the number of yeast cells (A) and the accumulation of alcohol (lines) and carbon dioxide (bars) (B) during cultivation with curd whey.

changes in these indicators are noted after 16 days of storage. Changes in organoleptic indicators, such as sharpness, taste, aroma, appearance, and color, were also determined.

Next, the content of free amino acids (FAA) and B vitamins in the obtained PKB was determined in comparison with clarified curd whey (control) and curd whey fermented by PAB (probiotic beverage (PB)). The results of the analysis are given in Table 1.

Analysis of the amino acid composition of PB showed that the content of isoleucine increased, compared with the control sample, by 29.5%, leucine by 46%, tyrosine by 86%, phenylalanine by 58.7%, lysine by 16.7%, histidine by 41.8%, and tryptophan by 27.6%. The FAA content in PKB is slightly lower than in PB but higher than in curd whey.

The study results of antagonistic and antimutagenic activities (Figure 5) indicate an increasing trend in these activities in the order of curd whey < PB < PKB.

4 DISCUSSION

The proposed method for producing a probiotic beverage similar to kvass involves using curd whey instead of water for preparing kvass wort, replacing lactic acid with PAB, and adding a shell extract with polyphenols. This approach will further improve the benefits of this widely used beverage.

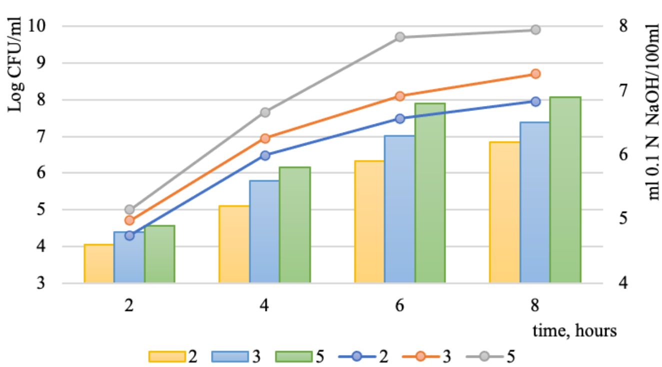

Figure 3. Growth dynamics of PAB (lines) and titratable acidity (bars) during cultivation in fermented kvass wort at 8°C depending on the dose of bacterial concentrate (2, 3, 5 EA).

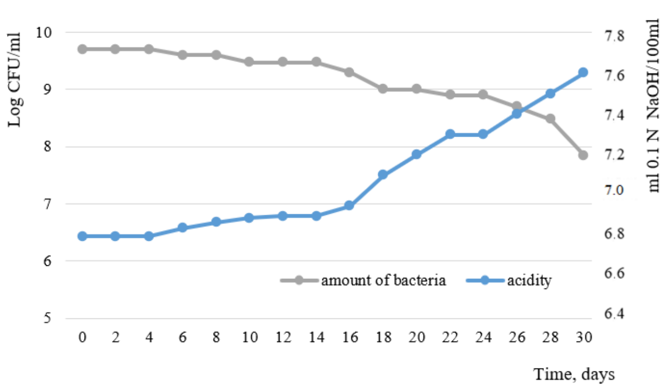

Figure 4. Dynamics of PAB amount and titratable acidity during storage of PKB.

The study results (Figure 1A) showed that replacing water with curd whey in the traditional technology for preparing kvass wort has a positive effect on the development of yeast cells. Compared to the control (water), curd whey contains additional nutrients, accelerating the accumulation of yeast biomass and reducing the fermentation process. At the end of cultivation, the viable cells were 1×10^8–4×10^9 CFU/cm^3, one or two orders of magnitude higher than in the medium prepared by the traditional method (control). In addition, the change in the fermentation process of such important consumer properties of kvass, such as alcohol and carbon dioxide contents that characterize the beverage's sharpness, was also studied. The authors observed a proportional relationship between the amount of yeast starter and the accumulation of alcohol and carbon dioxide (Figure 1B). The optimal values of the parameters were chosen for the cultivation duration of 10 h and the dose of yeast sourdough of 4%, since as a result of replacing water with curd whey, the accumulation of yeast biomass is accelerated, which leads to a reduction in the duration of the fermentation process to achieve the required values of parameters characteristic of traditional kvass (content alcohol: 0.3–0.6% and carbon dioxide: at least 0.3%).

The authors introduced a dry extract of pine shells, a source of polyphenols known for their antioxidant properties, to improve the quality of the PKB. The study results showed high antioxidant activity of the pine shell extract (Figure 2), and the addition of PSSE significantly reduced the rate of lipid oxidation. It should be noted that antioxidants not only inhibit the oxidation of food products during storage but also simultaneously have a therapeutic and prophylactic effect, making their use in the formulations of a wide range of products relevant. Also, the addition of PSSE makes it possible to abandon the dye traditionally used in the production of kvass, i.e., burnt sugar since it gives the necessary dark brown color to the beverage. It should be noted that the pH of the medium significantly affects the color. In an acidic environment, the beverage's color stabilizes and becomes brown. The optimal dose was experimentally determined; the highest organoleptic properties are characterized by samples with the introduction of PSSE in the amount of 0.2 g/L.

Table 1. Contains free amino acids and B vitamins in curd and fermented whey beverages.

Component	Content of amino acids, nmol/mL		
	Control	PB	PKB
Isoleucine	62.250	80.640	74.539
Leucine	47.716	69.841	78.519
Tyrosine	16.737	31.15	30.751
Phenylalanine	13.900	22.063	23.789
Lysine	73.445	85.714	80.168
Histidine	25.279	35.847	34.780
Tryptophan	33.254	42.438	39.950
	Content of vitamins, µg/kg		
B_1	300	450	540
B_2	1,100	2,000	2,700
B_{12}	2.9	4.1	4.5

Further research was directed towards selecting the PAB concentrate application method for PKB production. The co-cultivation of yeast and PAB is complicated because aerobic conditions are required for yeast development while anaerobic conditions are necessary for PAB. Fermentation in kvass production is carried out with periodic stirring with a centrifugal pump (every 2 h) for 30 min. However, constant air saturation in kvass wort creates unfavorable conditions for PAB growth. In addition, PAB's high fungicidal properties can lead to yeast microflora inhibition. In this regard, two methods of introducing PAB concentrate were studied: co-cultivation and separate cultivation.

In co-cultivation, the introduction of PAB was carried out 4 h before the end of the process. The resulting PKB was characterized by high acidity (7.5 mL of 1 N NaOH solution per 100 mL of beverage), which is higher than the established norm for kvass (no more than 7.0) and is explained by the fact that yeast waste products stimulate the growth of PAB, and at the same time, the development of yeast microflora is suppressed.

The second method consisted of various doses of the bacterial concentrate added to the fermented kvass wort and cooled to 8°C. This method was considered because the technology for the production of traditional kvass provides for holding the latter after fermentation at a temperature of 6±2°C for sedimentation and draining of yeast. Analysis of the data in Figure 3 showed that PABs develop well at low temperatures. With an increase in the dose of the concentrate, activation of the fermentation process was observed. The optimal amount of the concentrate was five activity units, and the process duration was 6 h. Fermentation of fermented kvass wort at a temperature of 8°C for 6 h provides PKB with lower acidity (6.8 mL of 1 N NaOH solution per 100 mL beverage) than co-cultivation. Therefore, it contributes to the dissolution of CO_2 and the saturation of the product with it.

Thus, the studies have shown that the most appropriate method for obtaining PKB with the best consumer properties is the separate cultivation of yeast and PAB.

Analysing organoleptic indicators, it can be noted that PSSE, when added to PKB, enhances its color and participates in the formation of taste, giving it astringency, fullness, and softness. In addition, PSSE polyphenols interact with proteins to form a residue, which is removed during processing and ensures the transparency and stability of PKB during long-term storage. Moreover, PSSE, due to its antioxidant properties, contributes to the extended storage of PKB. During PKB ageing, phenolic substances are oxidized to form quinone forms of catechins and other polyphenols with a high redox potential, creating favorable conditions for developing PAB *(Sharma et al., 2022)*. The taste richness is also influenced by the fermentation products of PAB. Short-chain fatty acids (SCFAs): propionic and acetic are end products of lactate fermentation by PAB. The SCFAs have many health benefits, such as anti-inflammatory, immunoregulatory, anti-obesity, anti-diabetes, anti-cancer, cardiovascular protective, hepatoprotective, and neuroprotective activities (Xiong et al., 2022). Thus, the metabiotics of PAB and PSSE increase the therapeutic and prophylactic properties of the fermented beverage.

The authors studied the change in the physicochemical, microbiological, and organoleptic parameters of PKB during storage for 30 days at a temperature of 4±2°C to establish reasonable storage periods. Also, the following characteristics of organoleptic indicators for freshly produced PKB were determined. These include sharpness, characterized by abundant

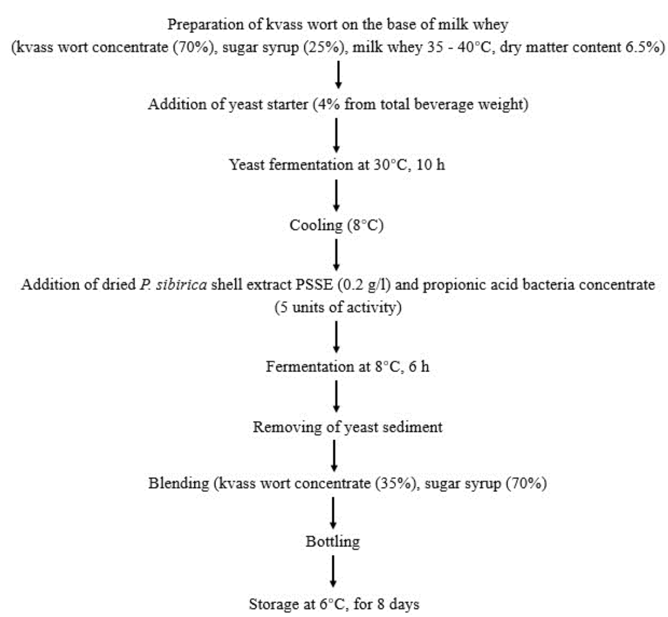

Figure 6. Protocol for the manufacture of PKB.

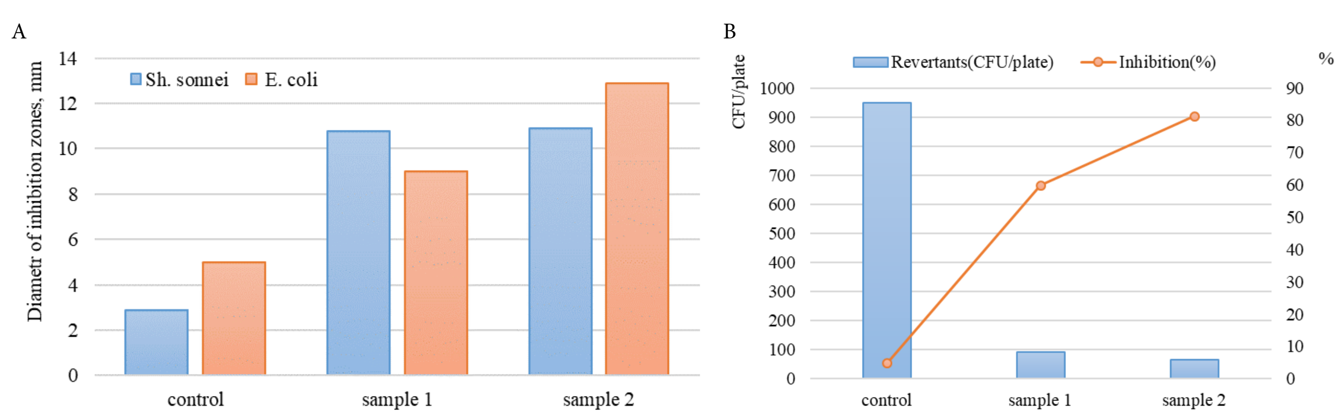

Figure 5. (A) Antagonistic and (B) antimutagenic activity of milk whey beverages.

release of bubbles, slight tingling on the tongue, and prolonged CO_2 release; taste and aroma, with a sweet and sour flavor, moderate tartness, and a distinct rye bread fragrance; color, dark brown; and appearance, the beverage is transparent and no sedimentation is observed during storage. Analysis of the data in Figure 4 shows that during 16 days of storage of PKB, there is no significant change in the content of viable PAB cells, titratable acidity, or organoleptic parameters. After 1 month of storage, the content of PAB cells remains at the same level, but a decrease in the sharpness of the product is observed. Upon further storage, the product loses its edge, has a weak aroma, and has an incomplete taste. The cell content decreases by two orders of magnitude but remains relatively high. The shelf life of PKB significantly exceeds the shelf life of kvass produced by traditional technology. This is probably due to the formation of propionic acid during the fermentation process, which is a natural preservative with a high fungicidal effect on extraneous microflora and the high antioxidant activity of PSSE.

Based on the research results, a technological scheme for the production of PKB was developed, as shown in Figure 6. First, due to the active growth of yeast and the sufficient accumulation of alcohol in the whey wort, fermentation was reduced to 10 h. Then the fermented wort is cooled to 8 °C, and five units of PAB concentrate activity are added per 100 deciliters of the beverage. Finally, fermentation was carried out for 6 h, which allows for keeping within the technological cycle to produce kvass beverages (16 h).

PKB quality indicators have also been developed:

- Organoleptic characteristics: color, dark brown; taste, refreshing, sweet, and sour; aroma, a distinct rye bread fragrance; appearance, transparent and forms a slight precipitate upon settling; and sharpness, abundant release of bubbles, slight tingling on the tongue, and prolonged CO_2 release;
- Physical and chemical parameters: solids content>11.5%; titratable acidity, 6.8–7 mL 0.1 N NaOH/100 mL; mass fraction of alcohol, 0.3–0.6%; mass fraction of carbon dioxide, 0.3–0.6%.
- Microbiological parameters: number of viable PAB cells>10^7 CFU/mL; total coliform count, not detected in 1 mL; pathogenic microorganisms, including *Salmonella* spp., not seen in 25 mL.

The spectrum of FAA in a probiotic beverage after the development of microorganisms in it depends on the amino acid composition of the serum, the proteolytic activity of microorganisms, and their ability to consume and accumulate certain amino acids in the process of life. The results of the analysis (Table 1) showed that curd whey contains a relatively large amount of FAA, and fermentation with PAB increases their content (sample PB) by 16.7–86% because PAB supplies themselves with the necessary amino acids not only due to the breakdown of protein and nitrogenous substances in the environment but also due to their synthesis (Vorobjeva et al., 2008). On the contrary, the FAA content in PKB is slightly lower than in PB but also higher than in curd whey. This may be due to yeast utilizing relatively large amounts of amino acids and their weak acid-synthesizing activity.

It is known from the literature that PAB are capable of synthesizing large amounts of B vitamins (Piwowarek et al., 2018). Particularly in this regard, the synthesis of vitamin B_{12} attracts attention since this vitamin is not synthesized by yeast. The research results presented in Table 1 show that PAB cultivated in the fermented kvass wort of a probiotic beverage synthesize many B vitamins: the content of vitamin B_1 increased by 80%, B_2 by 145%, and B_{12} by 55% in comparison with curd whey.

It is known that classical PABs produce several protein bacteriocins that inhibit the growth of several gram-positive and gram-negative bacteria (Antone et al., 2023). Also, a notable property of PAB is their ability to biosynthesize compounds with antimutagenic and anticarcinogenic effects (Vorobjeva et al., 2008). In connection with the preceding, the authors studied the antagonistic and antimutagenic activities of the PKB developed. The results of experimental studies (Figure 6) show that PKB has a high hostile activity against *Shigella sonnei* and *Escherichia coli* test cultures and has an antimutagenic effect against mutagenesis induced by 4-nitroquinoline-N-oxide. These activities increased in the series curd whey <PB<PKB; among them, PKB exhibited the most powerful impact due to the combination of yeast, PAB, and PSSE.

5 CONCLUSION

As a result of the research, it was found that replacing water with curd whey in the traditional technology for preparing kvass wort contributes to the active growth of yeast and the accumulation of alcohol. Fermentation of whey-based fermented kvass wort with PAB concentrate and the addition of tannins enriches it with propionic, acetic, and free amino acids and B vitamins. Also, it improves the consumer properties of beverages and the quality and functional properties of the finished product. Tannins give the product a brown color and a pleasant tart taste. The high antioxidant activity of tannins contributes to preserving the quality of beverages during long-term storage. Pronounced antagonistic and antimutagenic activities of PKB have been established due to the combined action of yeast, PAB, and PSSE.

Thus, based on the research, a technology was developed for obtaining PKB enriched with tannins from pine nut shells. With the help of a complex of biochemical and physicochemical studies, it was established that PKB has high probiotic properties.

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