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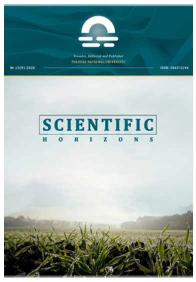

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Use of hop extract in the biotechnology of kefir beverage

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Abstract. The shelf life of fermented dairy products is only 3-5 days and is extended by the addition of preservatives to the production process, but the biological value of the kefir beverage is reduced. The purpose of this study was to determine the feasibility of using hop extract in the biotechnology of fermented milk beverage. Three samples of kefir beverage were produced: control (K) – using traditional technology based on dry starter "Kefir VIVO"; experimental (D1) – with the above type of starter in the amount of 0.05% by weight of milk and hop extract in the amount of 5% by weight of milk.

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The experimental sample (D2) was fermented using hop extract (10% by weight of milk). It was found that when using only hop extract for fermentation of the prototype kefir beverage, the duration of the technological process of its production is the same as when using dry starter and does not exceed 7-8 hours. The number of beneficial viable lactic acid microorganisms (*Lactococcus lactis ssp.*) in the fermented milk beverage made based on hop extract was $1.0 \cdot 10^7$ CFU/cm^3. These microorganisms have probiotic properties, which makes it possible to classify beverages enriched with hop extract as functional foods. In the experimental samples made using hop extract, yeast colonies were found in the amount of $3 \cdot 10^7$ and $8 \cdot 10^7$ CFU/cm^3, respectively. During the experimental storage period, the pH of all product samples was (4.71-4.46). Therewith, the acidity of the sample (D2) increased more slowly. Its organoleptic characteristics were improving. The shelf life has been extended to 10-15 days. The results of the study showed that milk can be fermented with hop extract, resulting in a new functional type of kefir beverage with high organoleptic characteristics, viability of beneficial microflora (lactic acid bacteria and yeast) and extended shelf life

Keywords: fermented milk beverage; shelf life; starter; lactic acid bacteria; yeast; fermentation; biotechnology

INTRODUCTION

Fermented milk products are made by fermenting milk. Therewith, its composition changes under the influence of microorganisms and the pH level decreases. The fermentation of lactose also leads to an increase in the bioavailability of nutrients and improves the quality of dairy products. At the same time, lactic acid microflora and its metabolic products inhibit the development of microorganisms that cause food spoilage and pathogens. The main products formed during the fermentation process in the production of fermented milk beverages are lactic acid, ethanol, and CO_2, which give them viscosity, sour taste, and lead to the accumulation of a low alcohol content. A prerequisite for fermented beverages is that the starter microorganisms are viable, active, and present in sufficient quantities in the product until the minimum shelf life. A considerable number of fermented dairy beverages are produced worldwide using mono- or complexes of starter cultures. Nevertheless, the issue of ensuring that such products have a longer shelf life is still relevant.

The principal function of fermented dairy starter cultures is to extend shelf life and increase safety due to the biopreservative function of metabolites produced during fermentation, as well as to improve the organoleptic, rheological, and nutritional properties of the finished product. Safety issues in fermented milk beverages arise from the presence of bacteriophages and antimicrobial agents that can inhibit the activity of bacterial starter cultures. This leads to a decrease in viscosity and a deterioration in the taste of the beverage. J. Tamang *et al.* (2020) found that the microbiota of fermentation starter products consists of selected and well-defined strains of lactic acid bacteria that are produced in concentrated and stable forms. Their wide availability, ease of use, and stable properties have made them widespread in many countries.

T. Bintsis and P. Papademas (2022) showed that the starter cultures used for milk fermentation include a variety of bacteria, dominated by *Lactobacillus species such as L. delbrueckii subsp. bulgaricus*, L. *helveticus*, L. *kefiranofaciens subsp. kefiranofaciens*, *L. kefiranofaciens subsp. kefirgranum* and *L. Acidophilus*, *Lactococcus spp.* such as *L. lactis subsp. lactis* and *L. lactis subsp. cremoris* and *Streptococcus thermophilus*. The microorganisms that make up the starter not only increase the acidity of the environment, but also affect the formation of texture, taste, and biochemical changes in the product.

Numerous studies by V. Braccini *et al.* (2021) have shown that kefir is produced using bacteria and yeast, which exist in symbiotic association in kefir fungi. It is a traditional example of the coexistence of bacteria and yeast, which produce compounds that are beneficial to health. As a rule, kefir fungi contain a relatively stable and specific microbiota enclosed in a matrix of polysaccharides and proteins. Microbial interactions in kefir are complex due to the composition of kefir fungi. Furthermore, certain populations of individual kefir grains shape the organoleptic characteristics of fermented beverages. Kefir is characterised by a distinctive taste typical of yeast and a noticeable fizzy effect.

Yeast and acetic acid bacteria practically do not ferment lactose, but they contribute to flavour formation by producing ethanol and CO_2. The properties of kefir yeast are less studied than those of bacteria. T. Srimahaeak *et al.* (2022) showed that *Kluyveromyces marxianus/Candida kefir*, *Kluyveromyces lactis var. lactis*, *Debaryomyces hansenii*, *Dekkera anomala* can ferment lactose. *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Pichia fermentans*, *Kazachstania unispora*, *Saccharomyces turicenysis*, *Issac* do not *ferment* lactose. Some authors have proposed alternative ways to produce kefir beverages using non-dairy substrates such as fruit and molasses (Farag *et al.*, 2020), agro-industrial waste (Aiello *et al.*, 2020), and plant extracts (Setiyoningrum *et al.*, 2019). However, only the organoleptic and some physicochemical properties of the product changed.

A potential source of antimicrobial activity is hops (*Humulus lupulus*), whose antibacterial effect is partly explained by the presence of humulone (alpha-acid), lupulone (beta-acid), and the polyphenol xanthohumol.

Hops are commonly used in baking and brewing. L. Nionelli *et al.* (2018) showed that hop starter inhibited the growth of fungi in bread for 14 days, characterised by a concentration of free amino acids, antioxidant and phytase activity higher than that of bread made with baker's yeast alone). Beer containing hops has also been found to be less perishable, indicating the antimicrobial potential of this plant (Blaxland *et al.*, 2022).

Furthermore, a considerable amount of "wild" yeast is concentrated on the surface of hop cones. D. Watanabe (2023) showed that during growth, yeast enriches the environment with a number of essential metabolic products, making it more suitable for the development of lactic acid bacteria. Thus, lactic acid bacteria, when co-cultured with yeast, use growth factors that are absent in milk but present in yeast autolysates. Furthermore, the alcohol produced (due to alcoholic fermentation caused by yeast) inhibits the rate of cell division of lactic acid microorganisms, thereby slowing down the aging of the population, as a result of which lactic acid bacteria stay active longer.

Considering the high antimicrobial effect of hops, their availability and distribution throughout Ukraine, they can be considered a promising raw material for use in the food industry, including in the production of fermented milk beverages. Therefore, the purpose of this study was to determine the feasibility of using hop extract in the biotechnology of fermented milk beverage.

MATERIALS AND METHODS

The study was conducted at the educational and scientific laboratory of craft technologies and gastronomic innovations of Sumy National Agrarian University in January 2023. Three samples of fermented milk beverages such as kefir were produced using the thermostatic method. The main raw material used was ultra-pasteurised cow's milk with a mass fraction (g/100 g) of 2.8 proteins, 2.5 fats, and 4.5 carbohydrates.

As a control (C), the kefir beverage (sample 1) was produced using direct application starter cultures for kefir "Kefir VIVO", which include kefir fungi microflora standardised with additional mesophilic and thermophilic microflora (0.1% by weight of milk, which is the amount recommended by the manufacturer). The prototype of product 2 (D1) was fermented with the direct application starter "Kefir VIVO" (0.05% by weight of milk) and hop extract (5% by weight of milk). Sample 3 (D2) was fermented with hop extract only (10% by weight of milk). This amount of extract had no negative effect on the organoleptic properties of the finished product (Fig. 1).

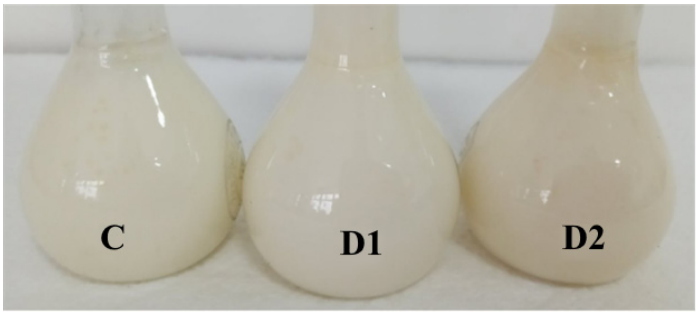


***Figure 1**. Samples of fermented milk beverages*
***Notes**: C – based on dry starter; D1 – based on a mixture of dry starter and hop extract; D2 – based on hop extract*
***Source**: compiled by the authors of this study*

The amount of added extract was determined by the analysis of literature sources and a range of preliminary laboratory studies. When the amount of extract was increased to 15% and 20%, the product had a too liquid consistency and a noticeable bitter taste. When less than 5% hop extract was used for the fermentation, the fermentation time was around 16 hours. The hop extract was prepared by infusion. For this, the cones of wild-growing *Humulus* hop were mixed with hot distilled water (85°C) in a ratio of 10:1 and infused for 24 hours. The resulting extract was filtered and then added to milk heated to the fermentation temperature (25°C), and the starter was added. The mixture was mixed thoroughly and placed in the thermostat. The duration of fermentation of all kefir beverage samples was 7-8 hours.

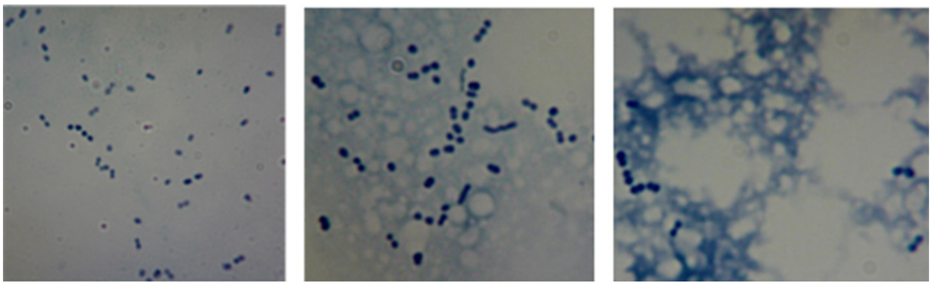
Study of microbiological parameters of kefir beverages. The number of lactic acid bacteria and yeasts was determined by plating serial dilutions on selective medium. Bacteria were counted according to DSTU 7999:2015 (2017), yeast – according to DSTU ISO 7954:2006 (2007). The active acidity was determined following DSTU 8550:2015 (2017), which is based on the determination of hydrogen ion activity using a potentiometric analyser, "pH-500" pH meter (Italy).

To determine the microbiological composition of the samples by morphological features, the samples were microscoped according to the generally accepted methodology "Instruction on microbiological control at dairy enterprises". The staining was carried out using a simple method. In this case, a few drops of methylene blue solution were poured onto a preserved smear for 3-5 minutes, then the paint was washed with water, and the smears were dried with filter paper. The samples were microscoped using a Konus Academy ×1000 immersion microscope system (Italy), and the samples were captured in the microscope field of view using a Delta Optical 2-megapixel camera (Poland). To determine the live lactic acid bacteria, on Day 3 of storage, using the cup method, plating was carried out on a special selective medium MPA (meat-peptone agar), followed by cultivation at 30±1°C for 72 hours. Plating for the detection of yeast and mould colonies was carried out on Sabouraud selective medium, followed by cultivation at 27±2°C for 72 hours.

Statistical analysis. Statistical data were calculated using the Fisher-Student method, considering statistical errors and the reliability of comparable analogous indicators. The values were considered significant at the level of more than 95% ($p<0.05$).

RESULTS AND DISCUSSION

Results of the microbiological study. Microscopy of the experimental samples was conducted after the fermentation process. Mesophilic lactic acid bacteria represented the microflora of the experimental samples (Fig. 2).



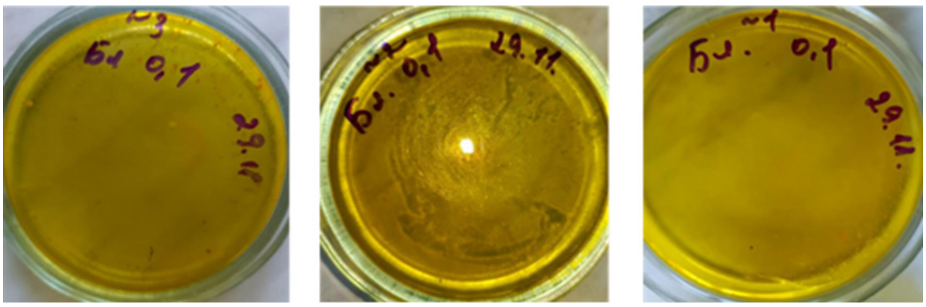
a b c

***Figure 2**. Sample microscopy results*

***Notes**: a – sample 1 (C); b – sample 2 (D1); c – sample 3 (D2)*

***Source**: compiled by the authors of this study*

The dominant strains of microorganisms detected in the control sample (C) were *Lactococcus lactis ssp*. lactobacilli. The cells of *Lactococcus lactis ssp*. are spherical in shape, usually grouped into diplococci and short chains of 0.5-1.5 μm in length. The species composition of the microflora of samples D1 and D2 was comparable, but the number of lactobacilli in the field of view in sample D2 was lower. In the control sample (C) and sample 2 (D1), 50-80 cells were observed in the field of view. This may indicate a high initial concentration of lactic acid microorganisms in the product, which is also confirmed by the cup test. In sample 3 (D2), single cells and clusters were observed in the field of view, which can be explained by the use of only hop cone extract as a starter, i.e., a low initial concentration of *Lactococcus lactis ssp*. cells, but not their absence. Since ultra-pasteurised milk was used to prepare the test samples, no microbiological tests were carried out on it before fermentation. The results of determining the content of living microorganisms are presented in Figure 3.



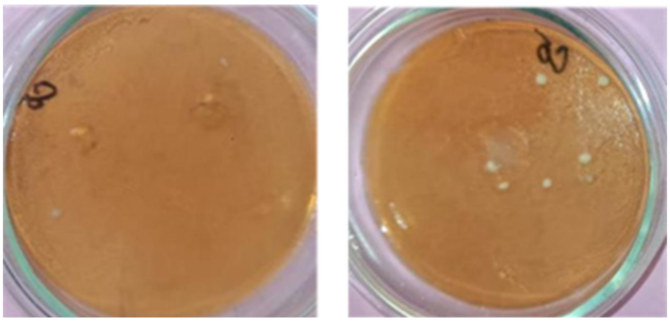
a b c

***Figure 3**. Results of surface plating*

***Notes**: a – sample 1 (C); b – sample 2 (D1); c – sample 3 (D2)*

***Source**: compiled by the authors of this study*

According to the results of surface plating (Fig. 3), samples 1 (C) and 2 (D1) contain live lactic acid bacteria in the amount of $1.0 \cdot 10^8$ CFU/cm^3. Sample 3 (D2), made only from hop extract, contained $1.0 \cdot 10^7$ CFU/cm^3 of live lactic acid bacteria. In other words, all samples were within the limits set by the requirements (DSTU 4417:2005, 2006). The morphology of the colonies when cultivated on media and the morphology of the cells under microscopy was typical of kefir beverages. Plating for the detection of yeast and mould colonies was carried out on Sabouraud selective medium, followed by cultivation at 27±2°C for 72 hours. The results are presented in Figure 4.



b c

Figure 4. *Number of yeast colonies*

Notes: *b – sample 2 (D1); c – sample 3 (D2)*
Source: *compiled by the authors of this study*

No yeast growth was observed in the control sample (C). On Sabouraud selective medium, the strain *Kluyveromyces lactis var. lactis* grew with the formation of white matte convex colonies with smooth edges and a smooth shiny surface. This is a desirable phenotype for large-scale biosynthesis of biomass-related products, with a unique set of beneficial properties such as the fastest growth, heat tolerance, and a wide range of substrates (Bilal *et al.*, 2022).

As a result, it was found that all samples of kefir beverages did not contain mould. The yeast *Kluyveromyces lactis var. Lactis* was detected in the experimental samples of the kefir beverage. In the experimental product sample 2 (D1), their content was $3.0 \cdot 10^1$ CFU/cm^3, while in sample 3 (D2) – $8.0 \cdot 10^1$ CFU/cm^3. That is, the number of yeasts increased with the amount of hop extract added to the milk prepared for the fermentation process. This indicates that the hop extract contains yeast that can ferment lactose, as the fermentation time for all samples was the same, and in all cases a dense lactic acid curd, typical of fermented milk beverages, was formed. Hop extract can therefore be an alternative to bacterial leaven. The active acidity is an important indicator of the quality of fermented dairy products during storage. A decrease in pH indicates the formation of lactic acid. The results of the active acidity study are presented in Figure 5.

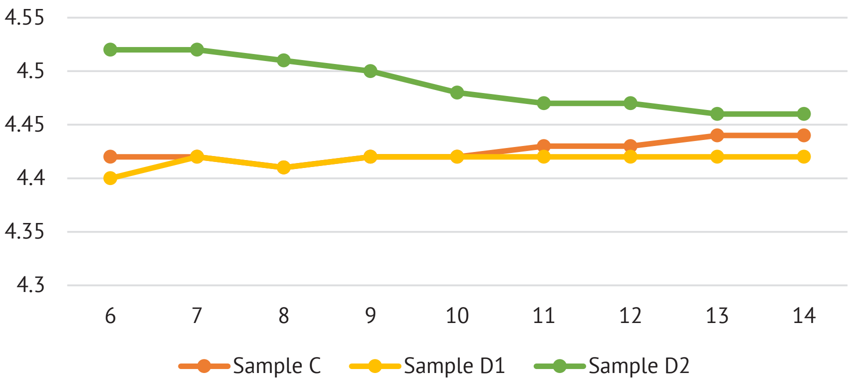


Figure 5. *Changes in pH during storage*

Source: *compiled by the authors of this study*

The study showed that after 8 hours of fermentation, all samples had sufficient acidity, which corresponds to the acidity of kefir beverages. However, already on Day 2 of storage, a rapid decrease in pH was observed in samples 1 (C) and 2 (D1), indicating a significant increase in the concentration of lactic acid. It is known that with an increase in the concentration of lactic acid, the development of lactic acid bacteria is inhibited, and their number begins to decline. This creates conditions for the development of pseudo-yeast and mould fungi.

In contrast to the control (C) and the experimental product sample (D1), the acidity of the sample 3 (D2) produced by fermenting the milk mixture with hop extract decreased more slowly. Even though the concentration of lactic acid in sample D2 was lower, there were no signs of spoilage. This is explained by the antimicrobial properties of hop extract

(Kumar Verma *et al.*, 2020; Kolenc *et al.*, 2022). Due to the biostabilisation of the fermented milk beverage with yeast, the product was suitable for longer storage for 14 days at 6±2°C.

The organoleptic characteristics of the experimental sample of fermented milk beverage – 3 (D2), enriched with hop extract, were the best. The consistency was homogeneous and viscous. The taste was sour-milk, tangy, with a pleasant, barely noticeable hop aroma. Moderate gas formation was observed. In samples 1 (C) and 2 (D1), a slight separation of serum was observed on Days 2-3 of storage. There was no gas formation in sample 1 (C). This led to the development of an industrial technology for a kefir beverage using hop extract. The scheme of production of kefir beverages based on hop extract is presented in Figure 6.

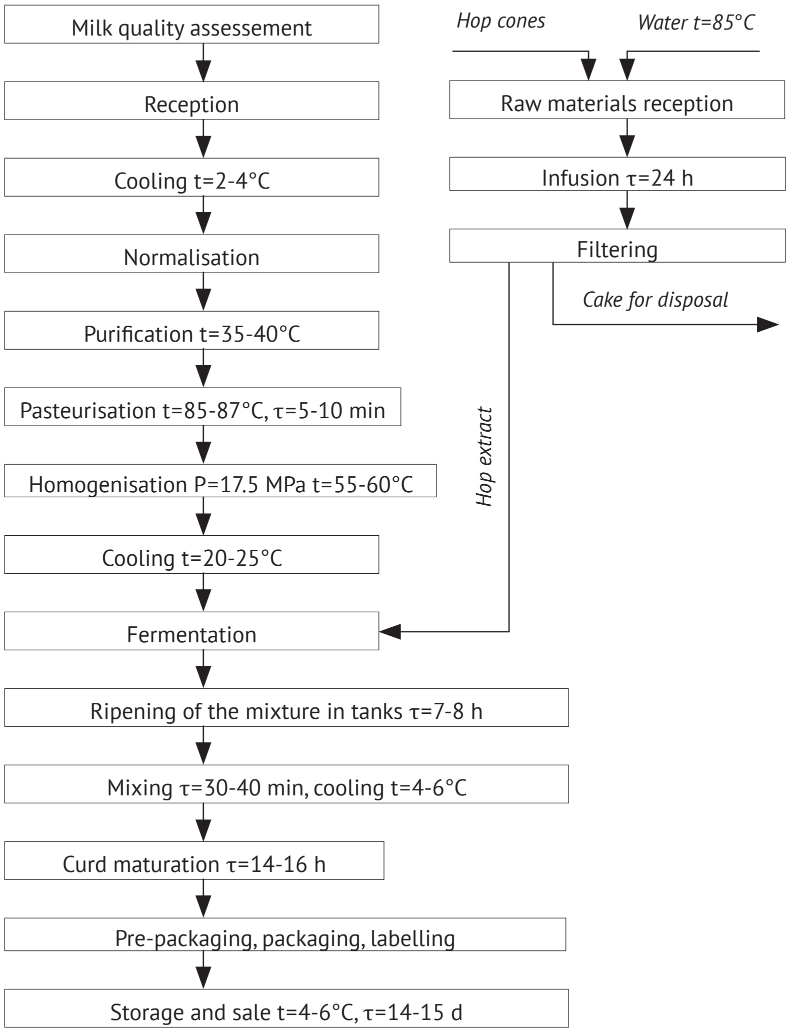


Figure 6. *Flow chart of production of fermented milk beverage with hop extract*
Source*: compiled by the authors of this study*

According to the proposed scheme, fermentation in industrial conditions is recommended to be carried out using the tank method, as this method is the most common in the production of kefir. The purified milk is cooled and normalised to a fat mass fraction of 2.5%. The normalised milk mixture is heated and homogenised to improve the structure of the finished product. The homogenised mixture is cooled to the fermentation temperature. It is recommended to add 10% hop extract at 25°C to the fermentation tank. After the hop extract has been added, the mixture is stirred thoroughly. The fermentation time is 7-8 hours. The resulting product is then thoroughly mixed to achieve a homogeneous consistency and cooled to the maturation temperature. After 14-16 hours of curd maturation, the kefir beverage is prepackaged and packaged. The recommended shelf life of the fermented milk beverage with hop extract is 14-15 days.

The results of the experiment showed that milk fermentation can be carried out using hop extracts. Since the ultra-pasteurised milk used for the study is free of lactic acid bacteria, the fermentation occurs due to the yeast present in the extracts made from wild hop cones. According to J. Varela *et al.* (2019), humans have been using yeast to make cheese and kefir for thousands of years, but the ability to ferment the milk sugar lactose has only been found in a few species of yeast, the first of which is *Kluyveromyces lactis*. Two genes, *LAC12* (lactose permease) and *LAC4* (lactase), are sufficient to take up lactose and hydrolyse it to glucose and galactose. The ability to grow on lactose as the sole carbon source is a defining feature of the nutritional yeasts *Kluyveromyces lactis* and *Kluyveromyces marxianus*. These are the strains that were detected on the experimental samples D1 and D2 (Fig. 4).

Microbiological testing showed that no pathogens were found in all samples, including those made with kefir starter. The predominant microflora of kefir beverages was *Lactococcus lactis ssp*. (Fig. 2). It is likely that the acidic pH prevents the growth of these microorganisms in this particular microenvironment. Other researchers have obtained analogous results. D. Kim *et al*. (2017), G. Aprea *et al*. (2023), investigating the tolerance of probiotic microorganisms to acidic pH, found that a decrease in acidity leads to the inhibition of pathogenic microflora.

It is known that the acidity of fermented foods is usually maintained or reduced during storage, which is related to microbial activity, which is a continuous fermentation process where lactic acid bacteria and yeast assimilate carbohydrates (Łopusiewicz *et al*., 2019; dos Santos *et al*., 2019). The study found that during the storage of fermented milk beverages made with hop extract, the pH decreased much more slowly than in samples C and D1 (Fig. 5). This means that the formation of lactic acid is slower, and the action of lactic acid bacteria is inhibited. The beverage did not spoil, and no moulds developed in it. It is likely that this effect was also caused by "wild" yeasts that developed on the surface of hop cones, which can inhibit the development of moulds. E. Sohlberg *et al*. (2022) showed that "wild" yeast can proliferate during the initial phases of fermentation, outnumbering other species of microorganisms, including lactic acid bacteria, which are producers of lactic acid. These results confirm the assumption made.

All kefir samples had a pH value of less than 4.7. This value is the maximum for microbial stability. Observations during the study indicate that during the storage of the product, the active acidity (pH) of the sample based on hop extract decreased gradually and did not exceed the permissible limits. Notably, the high organoleptic characteristics of the kefir beverage were maintained, and the consistency stayed homogeneous. No syneresis was observed. In contrast, the pH of the control sample 1 (C), made based on dry fungal starter, decreased sharply on the second day of storage. On Day 6 of the study, control sample 1 (C) showed the first signs of spoilage (curd structure was disturbed, sour taste and smell appeared). Such changes occurred in experimental sample 2 (D1) and 3 (D2) on Day 11 and Day 14 of storage, respectively. Analogous results of the antimicrobial activity of fermented milk beverages were obtained by other researchers (Ortiz-Merino *et al*., 2018; Al-Mohammadi *et al*., 2021) and were explained only by a decrease in the active acidity in the milk medium and the effect of the resulting lactic acid. However, the experiments conducted in this study indicate that the inhibitory activity of the experimental samples of fermented milk beverages against mould was also conditioned by substances present in hop extract, including an increased level of yeast in its composition, which cannot ferment lactose to lactic acid. During the storage of two kefir experimental samples 2 (D1) and 3 (D2), no accumulation of high concentrations of fermentation products – lactic acid – was observed.

No yeast was found in the control. This indicates their absence in the composition of dry kefir starter. Control sample 1 (C) was already unfit for consumption on Day 6 of storage. Yeasts have a higher resistance to lactic acid than other microorganisms, which allows them to grow dominantly (Sohlberg *et al*., 2022). As a result, the shelf life of fermented dairy products is extended. The number of yeast colonies in the freshly brewed test samples with different amounts of hop extract differed substantially. A positive effect of yeast on improving the quality of experimental samples 2 (D1) and 3 (D2) was observed. Thus, an increase in the amount of yeast under the influence of hop extract in the kefir beverage improved its organoleptic characteristics. The improvement of the organoleptic characteristics of kefir beverage made based on hop extract is probably conditioned by the presence of essential oils and hop resins in the composition of hop extracts. These biochemical compounds give the product a pleasant taste and aroma. According to the findings of A. Díaz *et al*. (2022), a considerable number of aromatically active compounds are related not only to the composition of hops, but also to specific fermentation conditions and to the concrete yeast strain used, which can produce interesting aromatic components such as higher alcohols or compound esters. Thus, by using certain types of hops in the production of fermented milk beverages, it is possible to give them unique organoleptic properties.

According to the findings of this study, it can be argued that hop extract, introduced into the process of manufacturing prototypes of the fermented milk beverage, has a bacteriostatic effect that can delay the development of negative microflora, which leads to product spoilage and has a positive effect on improving organoleptic characteristics and increasing its shelf life to 10-15 days.

CONCLUSIONS

The findings of this study showed that the use of hop extract for fermentation of milk mixture is appropriate and timely, since the partial or complete exclusion of starter cultures from the production of fermented milk beverages will improve the economic performance of Ukrainian dairy processing enterprises. A scheme for the production of a fermented milk beverage was developed, which is carried out according to the technological parameters that are usually used in industrial conditions. Therewith, the duration of milk fermentation in the production of the test samples of the beverage is practically the same as in the control.

The experimental product sample 3 (D2), made using hop extract, contains a sufficient amount of viable lactic acid microflora and has prominent organoleptic properties. This experimental sample contains yeast, probably a strain of *Kluyveromyces lactis var. Lactis*, which can ferment other milk carbohydrates, except lactose, the fermentation of which is accompanied by the formation of a high concentration of lactic acid. This causes product defects and reduces its shelf life. Given that yeast is a source of B vitamins, the presence of yeast in a fermented milk beverage will have a positive impact on consumer health.

The use of hop extract in the production of experimental samples of the fermented milk beverage contributed to a slow decrease in their active acidity, which was within the normal range. This allowed extending the shelf life of the above samples to 10-15 days. A technology for the production of fermented milk beverages based on hop extract for industrial use was developed, which will expand the range of fermented natural beverages with biologically active ingredients. The prospect of further research is to investigate the antioxidant properties of fermented milk beverages made based on hop extracts.

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None.

CONFLICT OF INTEREST

The authors of this study declare no conflict of interest.

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Використання хмелевого екстракту в біотехнології кефірного напою

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Анотація. Термін придатності до зберігання кисломолочних продуктів становить лише 3-5 діб і збільшується веденням в процес їх виробництва консервантів, проте, при цьому біологічна цінність кефірного напою знижується. Мета роботи – визначення доцільності використання хмелевого екстракту в біотехнології кисломолочного напою. Було виготовлено 3 зразки кефірного напою: контрольний (К) – за традиційною технологією на основі сухої закваски «Кефір VIVO»; дослідний (Д1) – із вище вказаним видом закваски в кількості 0,05 % до маси молока та екстрактом хмелю в кількості 5 % до маси молока. Ферментація дослідного зразка (Д2) проводилась із використанням хмелевого екстракту (10 % до маси молока). Встановлено, що при використанні лише хмелевого екстракту для заквашування дослідного зразка кефірного напою, тривалість технологічного процесу його виробництва є такою ж, як і при використанні сухої закваски та не перевищує 7-8 годин. Кількість корисних життєздатних молочнокислих мікроорганізмів (*Lactococcus lactis ssp.*) у кисломолочному напої, виготовленому на основі хмелевого екстракту, становила $1{,}0 \cdot 10^7$ КУО в 1 $см^3$. Ці мікроорганізми мають пробіотичні властивості, що дозволяє віднести напої, збагачені екстрактом хмелю до продуктів функціонального призначення. У дослідних зразках, виготовлених із використанням хмелевого екстракту, виявлено колонії дріжджів у кількості $3 \cdot 10^7$ та $8 \cdot 10^7$ КУО в 1 $см^3$ відповідно. Протягом дослідного періоду зберігання усіх зразків продукту рівень pH становив (4,71-4,46). При цьому кислотність зразка (Д2) наростала повільніше. Покращувалися його органолептичні показники. Термін придатності до зберігання збільшився до 10-15 діб. Результати дослідження показали, що молоко можна ферментувати хмелевим екстрактом, в результаті чого отримується новий функціональний тип кефірного напою з високими органолептичними показниками, життєздатністю корисної мікрофлори (молочнокислих бактерій та дріжджів) та подовженим терміном придатності

Ключові слова: кисломолочний напій; термін придатності; закваска; молочнокислі бактерії; дріжджі; ферментація; біотехнологія