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QUANTITATIVE ANALYSIS OF ENTEROCOCCI ISOLATED FROM DAIRY PRODUCTS

КІЛЬКІСНИЙ АНАЛІЗ ЕНТЕРОКОКІВ, ВИДІЛЕНИХ З МОЛОЧНИХ ПРОДУКТІВ

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ABSTRACT

Purpose of the work. To evaluate the number of enterococci in the dairy products of industrial manufacturing.

Methodology. Molecular-genetic methods were used to analyze enterococci in milk, kefir and sour cream samples. Metagenomic DNA was isolated from each product and used for amplification. PCR with specific primers to *Enterococcus* genus was carried out to amplify specific genome region and to detect enterococci in dairy products. Quantitative PCR (qPCR) with SYBR Green dye solution was performed to enumerate *Enterococcus* bacteria presented in three milk products. The melting curve analysis was used to define the specificity of amplification. The genome-equivalent values of enterococci in milk, kefir and sour cream were calculated using the standard curve analysis.

Scientific novelty. In the present research the quantitative analysis of enterococci in the three milk products of industrial manufacturing was conducted using metagenomic DNA and qPCR-analysis. There were no significant differences of enterococci number between dairy products.

Conclusions. The development of molecular-genetic methods and approaches gives a possibility to estimate the number of enterococci in dairy products by culture-independent way. The results suggested that similar amount of enterococci in all three dairy products can be due to their industrial production and using the same preservation agents. Although the genotyping and species identification of *Enterococcus* should be performed in the next researches.

The fermented milk products have an important and in general beneficial effect on human health. Despite qualitative and quantitative variability of microorganisms comprising the composition in milk products, lactic acid bacteria represent the most numerous group. The ambiguous features characterize bacteria belonging to *Enterococcus* genus: they contribute to the specific flavor and taste of dairy products and can be used as a probiotic and adjunct starters but, at the same time, they can be the source of virulence factors and be an indicator of poor hygienic conditions during production. Thus, it is important to perform quantitative analysis and control of enterococci in dairy products.

Key words: dairy products, enterococci, quantitative PCR

АНОТАЦІЯ

Мета роботи. Оцінити кількість ентерококів у молочних продуктах промислового виробництва.

Методологія. Молекулярно-генетичні методи були використані для аналізу ентерококів у зразках молока, кефіру та сметани. Метагеномна ДНК виділяли з кожного продукту та використовували для ампліфікації. ПЛР з праймерами, специфічними до роду *Enterococcus* проводили з метою ампліфікації специфічної ділянки геному та для визначення ентерококів у молочних продуктах. Кількісну ПЛР (кПЛР) з використанням розчину барвника SYBR Green виконували з метою підрахунку бактерій *Enterococcus*, наявних у трьох молочних продуктах. Аналіз кривої плавління використовували для визначення специфічності ампліфікації.

Показники геном-еквівалентів ентерококів у молоці, кефіру та сметані розраховували із застосуванням аналізу стандартної кривої.

Наукова новизна. У представленому дослідженні кількісний аналіз ентерококів у трьох молочних продуктах промислового виробництва був виконаний із використанням метагеномної ДНК та кПЛР-аналізу. Між молочними продуктами не виявлено відмінностей у кількості ентерококів.

Висновки. Розвиток молекулярно-генетичних методів і підходів надає можливість оцінювати кількість ентерококів у молочних продуктах без їх культивування. Результати показали, що однакова кількість ентерококів у всіх трьох молочних продуктах може бути спричинена їх промисловим виробництвом та використанням однакових консервантів. Хоча генотипування та видова ідентифікація *Enterococcus* має бути проведена у наступних дослідженнях.

Ферментовані молочні продукти мають важливий та, загалом, сприятливий вплив на здоров'я людини. Незважаючи на якісну та кількісну варіабельність мікроорганізмів, що входять до складу молочних продуктів, молочнокислі бактерії є найчисленнішою групою. Бактерії роду *Enterococcus* характеризуються неоднозначними властивостями: вони сприяють специфічному аромату та смаку молочних продуктів і можуть використовуватися як пробіотики та додаткові закваски, але водночас вони можуть бути джерелом факторів вірулентності та бути індикатором поганих гігієнічних умов під час виробництва. Таким чином, важливо проводити кількісний аналіз і контроль ентерококів у молочних продуктах.

Ключові слова: молочні продукти, ентерококи, кількісна ПЛР

Statement of the problem

The fermented foods are defined as the products of controlled microbial growth and enzymatic transformation of food components [16]. Dairy products are the group of the fermented food products that affect human health and, in general, have beneficial consequences. Consumption of fermented milk products have been going on for thousand years and the biochemical activity of microorganisms from a raw milk was used to produce the dairy products for thousand years too. Nowadays many researches have been devoted to the study of the effect of fermented dairy products on human health. The results of these studies demonstrated the positive effect of kefir consumption on the density and strength of bone tissue [19], suggested the decreasing of the risk of type 2 diabetes that was associated with yogurt consumption [7; 9], and also revealed the influence of fermented dairy products containing various microorganisms on the cognitive functions, in particular preventive effects against dementia, Alzheimer's disease [2]. These and other properties of fermented dairy products, having a positive effect on the physiological and mental state of a person, contribute to the growing popularity of milk food [20], such as yogurts, kefir, acidophilic milk, koumiss, cheeses and other fermented milk products. The dairy products manufacturing occurs as homemade as industrial. The composition of microorganisms that are used for production varies in the species variability and in their quantity.

Lactic acid bacteria (LAB) play an important role in the food, agricultural and medical fields. Bacteria of the group are characterized as gram-positive, not forming spores, cocci or bacilli, and they produce lactic acid [12]. The group of LAB mainly includes representatives of four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Besides, LAB include representatives of the genus *Enterococcus* that can possess opposite features: produce unique organoleptic peculiarities and protect against spoilage or as a probiotic, and at the same time enterococci can carry virulence factors and be an indicator of poor hygienic condition of manufacturing. The conflicting characteristics of *Enterococcus* species make the detection and control of these bacteria an important and essential stage of dairy products manufacturing.

Detection and identification of microorganisms inhabiting various environments are performed both culture-dependent and culture-independent methods. The culture-independent methods are based on the molecular-genetic approaches and allow fast and accurate estimation of bacteria in the complex substrates and microorganisms' conglomerates.

The purpose of the presented study was to evaluate the number of enterococci in the dairy products of industrial manufacturing.

Dairy products. To perform the present research samples of three products manufactured by the same commercial company were chosen: milk, kefir and sour cream. 1 mL / mg of each dairy product was taken for the analyses.

DNA isolation. 0,1 mL or 0,1 mg of the dairy product was used for DNA isolation. Total bulk DNA was isolated using Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer's protocol with some modifications. Briefly, the samples were incubated with 0,4 mL of lysis buffer and 20 µL of Proteinase K at 55 °C for 30 min. Then 0,5 mL of chloroform was added and the solution was mix by tube inverting. After centrifugation at 10000 rpm for 10 min the upper phase was transferred to the new tube and 2,5 V of 96 % ethanol was added. DNA was precipitated at -20 ^{0}C overnight. Then DNA was spinned down at 10000 rpm for 5 min, ethanol was removed and the pellet was washed with 70 % ethanol. After centrifugation at the same conditions, the pellet was dissolved in 40 µL of deionized water. DNA concentration was measured using DS-11 FX+ DeNovix spectrophotometer / fluorometer.

PCR-analysis. To detect bacteria belonging to enterococci in dairy products PCR with genus-specific primers was performed. The sequences of forward and reverse primers were reported in [22]: 5'-TACTGACAAACCATTCATGATG-3' and 5'-ACTTCGTCACCAACGCGAAC-3'. The components of the reaction mix were 10 µL of 2x DreamTaq PCR Master Mix (Thermo Scientific), 40 pmol of each primer and 50 ng of DNA. The total volume of 20 µL was adjusted with deionized water. The PCR was run for 1 cycle at 95 °C, 2 min; 30 cycles at 95 °C, 20 sec; 55 °C, 30 sec; 72 °C, 45 sec; and the final elongation step at 72 °C, 7 min. The Mastercycler Personal 5332 (Eppendorf) was used for amplification.

The results of the PCR were visualized with the agarose gel electrophoresis and ethidium bromide dye solution.

Quantitative PCR. To evaluate total amount of bacteria and enterococci in dairy products qPCR with SYBR Green dye solution was carried out. The amplification mix contained 12,5 µL of 2x Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific), 20 pmol of each primer, 5 µL of DNA template and the mix was adjusted up to 25 µL with deionized water. Primers to 16S rRNA gene [23] were used to evaluate the total numbers of bacteria and genus-specific primers were used for *Enterococcus* quantification. Amplification was carried out using QuantStudio ™ 3 Real-Time PCR System (Applied Biosystems). PCR cycling conditions were as follows: 1 cycle – 50 °C, 2 min; 95 °C, 10 min; 40 cycles – 95 °C, 15 sec; 58 °C, 15 sec; 72 °C, 1 min. The amplification was followed with the melting curve analysis: 95 °C, 15 sec; 60 °C, 1 min; 95 °C, 15 sec. Fluorescence of the DNA/SYBR Green complex was detected and measured at the elongation step (72 °C) of each reaction cycle. Amplification for each sample was carried out in duplicate and each amplification run included no template control (NTC).

The melting curve analysis was used to analyze the specificity of amplification. The standard curve analysis was used to calculate the reaction efficiency and the coefficient of determination (R^2). The data were analyzed only if R^2 was greater than 0,93 and PCR was repeated in case R^2 was lower.

Data analysis. To calculate the relative (in comparison to the total numbers of bacteria) and absolute (genome / equivalent) quantity of enterococci the calibration curve method was used. Relative amount was estimated based on threshold cycle (Ct value) of 16S rDNA amplification and the weight / volume of dairy products taken for analysis.

The calibration curve analysis that represents the correlation between the threshold cycle (Ct value) and the logarithm of the standard sample concentration was used for the quantitative evaluation as described in [22]. 10-fold dilutions of DNA isolated from the type strain, *Enterococcus faecalis* CCM 7000^T, was used as standard samples. The number of genome-equivalents of enterococci presented in the sample was calculated as it was reported in [22].

Statistical significance of the results was estimated with t-test.

Research results

Detection of enterococci in dairy products. Bacteria of *Enterococcus* genus like other lactic acid bacteria are the common component of the dairy products. In the presented study, the three dairy products were analyzed by PCR-analysis with primers specific to enterococci. The amplicon of 112 bp characteristic to *Enterococcus* was revealed in all dairy products as shown in Fig. 1.

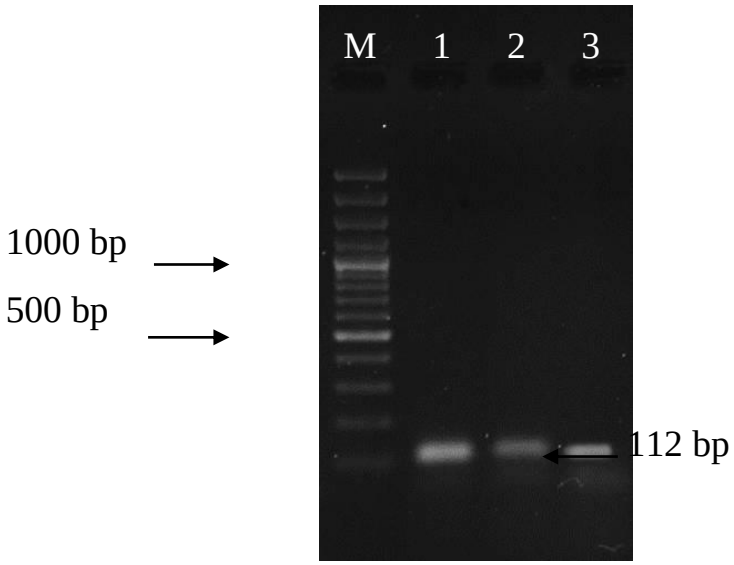

Fig. 1. Electrophoregram of the amplification with primers to *Enterococcus* genus.

M – DNA-ladder, 1 – milk, 2 – kefir, 3 – sour cream

Evaluation of enterococci in dairy products. The microbiome of the dairy products consists of various microorganisms, the majority of which are presented by lactic acid bacteria. Different representatives of these bacteria, including enterococci, were detected in many dairy products: raw milk [15; 21], kefir [5; 13], yogurt [18], cheese [1], cottage cheese [24], sour cream [6] and others. Results of metagenomic analyses performed in these studies revealed that the percentage of enterococci among all microorganisms varied in a wide range, from 0 to 15%, and depended on the type of dairy products and the way of their production (home-made or industrial). In our research we have analyzed the amount of *Enterococcus* in milk, kefir and sour cream relatively to the total amount of bacteria in each product. In milk and kefir the amount of enterococci in relation to the total number of bacteria was the same but in sour cream enterococci amounted to 1,6 times larger number. Despite differences in the representation of enterococci between three milk products the absolute number of *Enterococcus* genus was not varied: in all samples analyzed the quantity of bacteria was almost the same (Table 1).

Table 1

The number of genome-equivalents / mL (mg) of bacterial DNA in the dairy products

Bacterial genus	Milk	Kefir	Sour cream
Enterococcus	$(5,27 \pm 0,04) \times 10^5$	$(4,19 \pm 0,04) \times 10^5$	$(6,65 \pm 0,05) \times 10^5$

Note: The data are mean value ± StD

The results obtained by qPCR-analysis revealed slight not significant variability of the numbers of enterococci between three dairy products manufactured by the same company. The microorganisms' compositions of milk and fermented milk products consist of various microbial species and differ by their qualitative and quantitative features. Enterococci have been often revealed in bacterial mixtures of many fermented products [3; 21]. As it was mentioned enterococci may be presented up to 10^8 colony-forming unit (CFU) / g in dairy products [8]. The level of these bacteria varied in different dairy products. It was shown that in raw bovine milk they were evaluated with an average count of 2,48 $\log_{10}$ CFU / mL [17] and in European raw milk enterococci varied from 10^3 cells / mL to 10^5 cells / mL [4]. The larger number of bacteria belonging to *Enterococcus* genus was observed in different type of cheeses: 5,77 log CFU / g in civil cheese [10]; 5,52 – 6,48 log CFU / g in Iran cheeses and 6,12 log CFU / g in Turkish cheese [11].

Enterococci are widely distributed in the environment and are the important part of food products. They are known for their beneficial role of being starter or adjuncts starter cultures, of having probiotic peculiarities and developing of the organoleptic characteristics of the dairy products [4]. At the same time, enterococci can possess resistance to multiple antibiotics, carry potential virulence factors [6]. Besides, some of them are opportunistic pathogens that cause diseases and thus, are harmful to human health [14].

The number of enterococci revealed in the research has been smaller than that detected in the previous study of homemade milk products [22]. The considerable difference between these results can be caused by the way of manufacturing: the homemade production might be accompanied with poor hygiene during milk handling and processing while industrial manufacturing might be associated with using some conservative agents against spoilage including induced by enterococci.

Conclusions

The development of modern molecular-genetic methods and approaches allows fast and accurate detection of microorganisms in complex substrates, environmental samples, food products without their cultivation. Quantitative PCR (qPCR) is one of the culture-independent methods that was used in the presented study for estimation of enterococci in the dairy products. The results revealed insufficient differences of *Enterococcus* level between milk, kefir and sour cream that can be due to the same way of industrial production. Although *Enterococcus* species identification and genotyping should be performed further to analyze their safety.

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