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ORIGINAL PAPER

Antimicrobial activity of goat's milk fermented by single strain of kefir grain microflora

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Abstract

The aim of the study was to assess the antibacterial properties of bioactive compounds released during the fermentation of goat's milk by selected bacterial strains that are part of the kefir grain microflora. The material used in the experiments was kefir grain microflora (*Lactobacillus kefiranofaciens* subsp. *kefirgranum* DSM 10550*, Lactobacillus kefiri* PCM 2501*, Lactobacillus parakefiri* DSM 10551*, Lactobacillus brevis* PCM 488, *Lactobacillus delbrueckii* subsp*. lactis* PCM 2611), goat's and cow's milk and whey from these kinds of milk. The antimicrobial activity was tested against *E. coli, Salmonella, Micrococcus luteus* and *Proteus mirabilis*. Based on the experiments, it was found that during the fermentation of whey and goat's milk, bioactive substances were released, which inhibit the growth of indicator microorganisms by up to 8 logarithmic cycles.

Keywords Goat's milk · Kefir grain microflora · Antimicrobial activity · Whey protein · Casein

Introduction

Goat's milk is an attractive food because of its high nutritional values, easy digestibility and hypoallergenicity [9]. It is similar in composition to cow's milk regarding its protein, fat and lactose concentration, but there are differences between them that affect their digestibility and nutritional value. The differences between amino acid composition and proteins' secondary structure of goat's milk helps to reduce its allergenic potential compared with cow's milk [6].

The basic goat's milk proteins are casein proteins: β-casein, κ-casein, αs2-casein and whey protein: α-lactalbumin, β-lactoglobulin. Casein is a phosphoprotein that is found in milk in a soluble and micellar form. The structure of casein in goat's milk is more delicate and less succinct than in cow's milk. Thus, this protein is more susceptible to the action of digestive enzymes, which facilitate amino acid bioavailability [5]. The dominant protein in goat's milk is β-casein (approx. 54.8% of total casein), while the dominant casein fraction of cow's milk is αs1-casein (approx. 38% of total casein). The absence or very low content of αs1-casein and the content of αs2-casein higher than in cow's milk are characteristic of the casein fractions of goat's milk. The αs1-casein fraction is the main allergenic factor in cow's milk. Its lack or low content in goat's milk makes it a good alternative for people with a protein intolerance to consume this milk without the risk of allergic disorders. Approx. 40% of children allergic to cow's milk do not exhibit allergic reactions after consuming goat's milk [18].

The main whey protein of goat's and cow's milk is β-lactoglobulin, which accounts for 54.2 and 59.3% of all whey proteins, respectively. It is a rich source of threonine and serine. Goat's milk, compared to cow's milk, has a slightly higher content of α-lactalbumin rich in cystine and cysteine. The individual fractions of goat's milk and cow's milk proteins differ in the structure and composition of amino acids. The research conducted by Kycia and Szymczak, 2013 showed that the allergenicity of goat's milk is approx. 72–73% lower than that of cow's milk in relation to α-lactalbumin and by approx. 96% in relation to β-lactoglobulin. Goat's milk, like sheep's milk and human milk, is an excellent source of all exogenous amino acids,

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while in cow's milk, the limiting amino acids are methionine and cysteine [18].

Goat's and cow's milk fat is an emulsion of individual fat globules suspended in water [5]. Fat globules of goat's milk, however, have a smaller diameter than fat globules of cow's milk (on average 2.76 and 3.51 µm, respectively), are more dispersed and take up a larger area in 1 cm^3 of milk. Thanks to this, lipolytic enzymes can easier access to fat globules; therefore, goat's milk fat is characterized by higher digestibility. In addition, fat globules of goat's milk do not tend to agglomerate due to the lack of specific agglutinin protein, as is the case of cow's milk. This makes difficulties with centrifugation and it is responsible for poor standing ability of goat's milk. The cholesterol content of goat's milk is on average 11 mg/100 cm^3. In cow's milk, this amount is slightly larger—about 14 mg/100 cm^3 [18]. Moreover, goat's milk possesses health-promoting compounds, such as bioactive peptides and conjugated linoleic acid [24]. Goat's milk can be used to produce a wide variety of products, such as beverages, buttermilk, yoghurt, kefir and cheese.

In recent years, researchers have focused on bioactive peptides released from milk during the fermentation process. Experiments done in vivo and in vitro in this area indicated an impact on cancer prevention [8], blood pressure management [20] and in treating type 2 diabetes [15]. These health-promoting properties are related to the bioactive properties of proteins such as antioxidants, opioid, antihypertensive, antimicrobial and antitumor [23].

The release of single- or multi-functional activity proteins via microbial proteolysis during fermentation of cow's milk is well documented [1, 10, 17, 22]. There are few data describing the functional properties of bioactive compounds released during the fermentation of goat's milk with kefir grain microflora. Kefir grain microflora consisting of bacteria of the genus *Lactobacillus, Lactococcus* and yeast of the genus *Kluvermyces* and *Saccharomyces* connected extruded exopolysaccharide matrix—kefiran produced by *Lactobacillus kefiranofaciens* [14]. The aim of the study was to evaluate the antibacterial properties of biologically active compounds released during the fermentation of goat's milk by selected bacterial strains that are part of the kefir grain microflora.

Materials and methods

The experiment material was: goat's milk, whey from goat's milk from the "Kózka" Organic Farm, Łubowo, Poland and cow's milk, whey from cow's milk from the Dairy Cooperative Piątnica, Poland. The kefir grain microflora used in the experiments ware: *Lactobacillus kefiranofaciens* subsp. *kefirgranum* DSM 10550*, Lactobacillus kefiri* PCM 2501*, Lactobacillus parakefiri* DSM 10551*, Lactobacillus brevis* PCM 488, *Lactobacillus delbrueckii* subsp. *lactis* PCM 2611. Indicator microorganisms used in the experimental part were: *Escherichia coli* PCM 2793, *Salmonella* PCM 2548, *Micrococcus luteu PCM 525s, Proteus mirabilis PCM 1361.*

Microorganisms were obtained from the Polish Collection of Microorganisms at the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Leibniz Institut, Germany.

Evaluation of bacterial metabolic activity

The evaluation of bacterial metabolic activity was performed by a direct method by registering impedance changes directly in the growth medium using the BacTrac 4100 Automatic Microorganism Growth Analyzer [19].

Testing of antimicrobial properties

Culture preparation method

By culturing in a liquid medium, it was investigated whether peptides with antimicrobial properties against selected bacteria were formed during fermentation. The research material was goat's milk and whey from goat's milk with the addition of kefir grain microflora. The control sample contained 2.5 ml water, 2 ml broth and 0.5 ml bacteria. The actual test sample contained 2.5 ml of the research material, 2 ml of broth and 0.5 ml of bacteria. Samples were incubated in an incubator for 24 h in 37 °C. Then, the smear was plated on an agar plate using a loop. The next stage of the experiment was incubation of the samples in an incubator for 24 h at 37 °C. Innoculum of *Salmonella*, *Micrococcus luteus, Proteus mirabilis* was prepared at a dilution of 10^6 cfu/ml, and an inoculum of *E. coli* at a dilution of 10^5 cfu/ml.

Determination of reduction in number of indicator microorganisms

Quantitative determination of the reduction in the number of indicator microorganisms was carried out by means of plate cultures on selective media appropriate for the indicator microorganism (VRBG medium, nutrient agar) being analysed. Two ml of broth and 0.5 ml of indicator bacteria in the amount of 10^8 cfu/ml was added to 2.5 ml of milk or whey fermented with the selected microorganisms from kefir microflora. The samples thus obtained were inoculated for 24 h at 37 °C. Then, after decimal dilutions, the samples were put on selective media and inoculated at 37 °C for 48 h.

Optical density measurement using a bioscreen C

Optical density measurement of whey and goat's milk fermented with single microorganisms from kefir grain microflora inoculated with indicator bacteria: *Salmonella*, *E. coli*, *Micrococcus luteus* and *Proteus mirabilis* was done using Bioscreen C. The measurement principle is based on measuring the optical density of the sample. The experiment analysed samples were prepared using 30 µl of inoculum of the test indicator bacterium, 150 µl of whey or goat's milk fermented with selected bacterial strains from kefir grain microflora and 120 µl of nutrient broth. The samples were then incubated at 37 °C for 72 h. Changes in the optical density of the sample were recorded automatically every 30 min during the experiment.

Results and discussion

The scope of inhibition of the growth of microorganisms by bacterial strains of the genus *Lactobacillus* includes both bacteria of the same and other species, as well as fungi, pathogenic and toxin-forming [3, 12]. The spectrum of bacterial antagonist activity is associated with the presence of such metabolites as lactic acid, acetic acid, hydrogen peroxide, diacetyl and bacteriocins. Measurements of the changes in electrical impedance in milk and whey were used to assess the metabolic activity of selected strains of kefir grain microflora. The detection time of impedance changes at 5% (value chosen based on literature and previous experiments) in the medium resulting from the metabolic activity of selected microorganisms was analysed [19]. It was found that selected microorganisms needed the most time to create impedance changes of 5% in cow's milk. Comparing the detection time of impedance changes at 5% in whey from cow's and goat's milk, it was found that a significantly lower detection time occurred in whey from goat's milk (Fig. 1). Based on these experiments, a significantly lower detection time of changes in whey was found in comparison with milk both goat's and cow's. The shortest detection time was found for *Lactobacillus kefiri* inoculated whey from goat's milk. The longest time was found for *Lactobacillus kefiranofaciens* subsp. *kefirgranum* inoculated cow's milk (Fig. 1).

There is a clear difference in the course of impedance changes in the medium containing milk or whey inoculated with *Lactobacillus kefiri, Lactobacillus parakefiri* and *Lactobacillus kefiranofaciens* subsp. *kefirgranum* Figs. (2, 3, 4, 5, 6). Based on the course of the impedance change curves, two groups can be distinguished. The first group is goat's and cow's milk and the second is whey from goat's and cow's milk. The course of the impedance change curves of these microorganisms in cow's and goat's milk is similar in the case of whey from cow's and goat's milk. The curve of impedance changes caused by the increase in *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus brevis* is clearly different for cow's milk (Fig. 3, 5). Impedance changes in other substrates are similar. Peptides from casein and whey proteins are responsible for many biological activities, including antimicrobial and antioxidant activity. Bioactive peptides are fragments rich in specific amino acids like arginine and lysine [26].

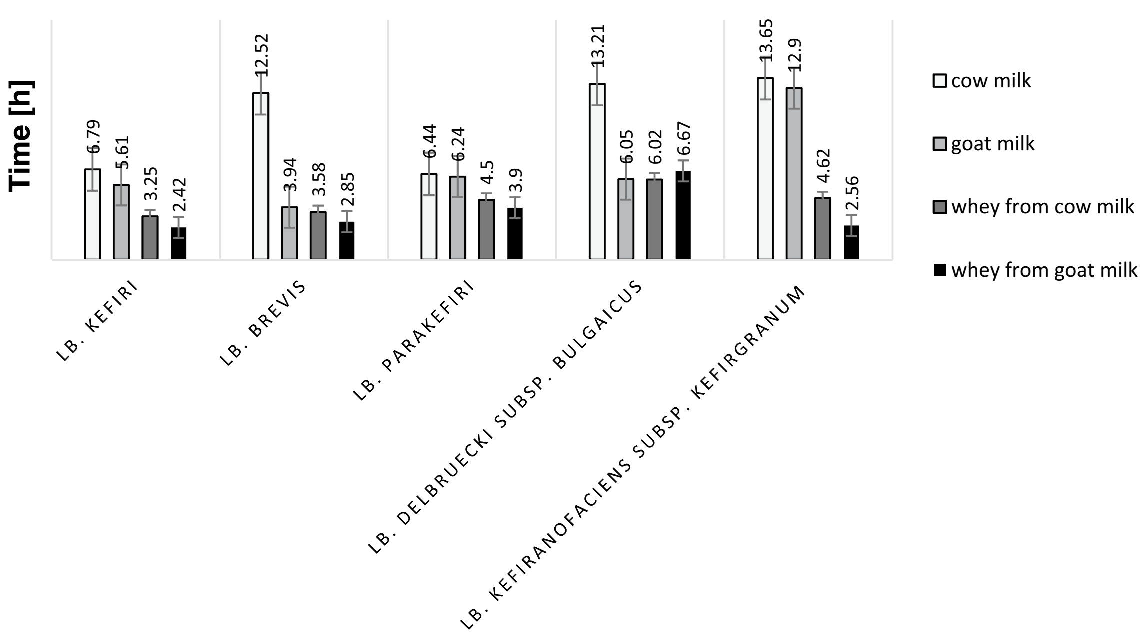

Fig. 1 The detection time of impedance changes at 5% in the medium

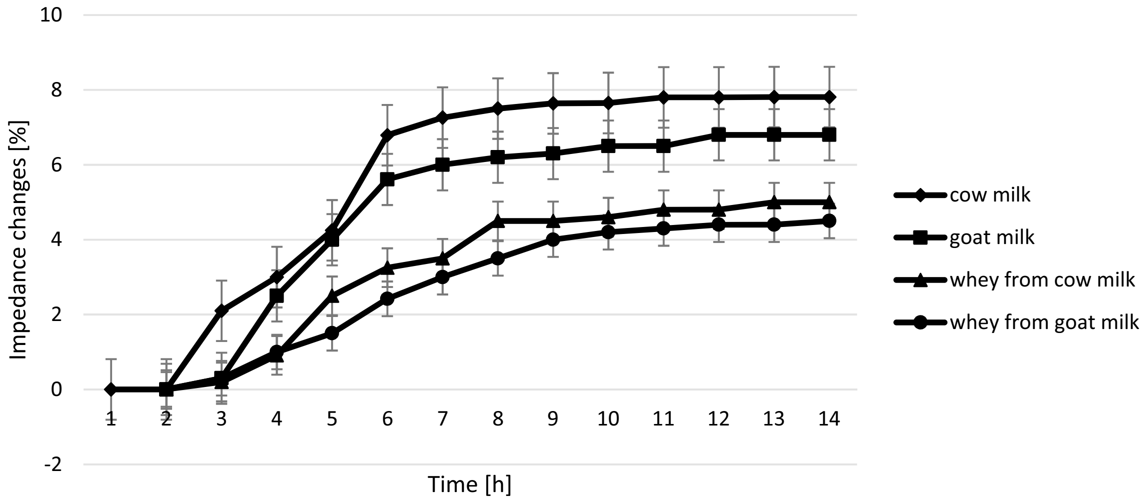

Fig. 2 Impedance changes in different medium caused by *Lactobacillus kefiri* growth

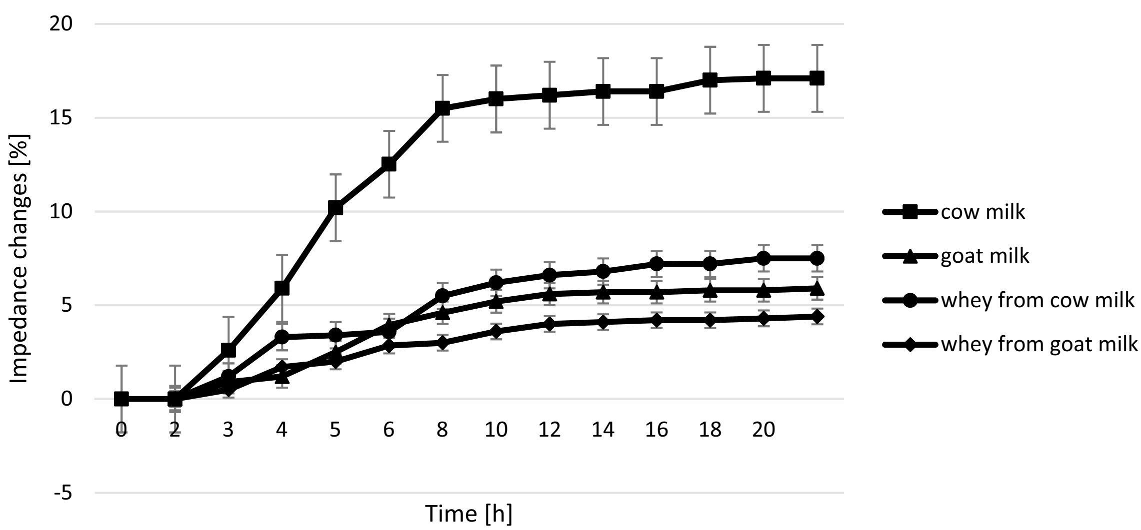

Fig. 3 Impedance changes in different medium caused by *Lactobacillus brevis* growth

Bacteriocins have the strongest antagonistic activity against related species, an example of which is plantaricin ZJ008, produced by *Lactbacillus plantarum* ZJ008 characterized by high antibacterial activity, also against pathogenic bacteria. The mechanism of antagonist bacteriocin activity may be associated with inhibition of DNA, RNA, ATP synthesis, reduction in protein synthesis, disruption in membrane potential or disruption in ionic potential [25]. Bacteriocins are used as natural food preservatives. An example is nisin, a polycyclic peptide of 34 amino acids produced by *Lactococcus lactis* subsp. *lactis* and is used in the production of ripening cheese, whipped cream and Mascarpone cheese [16].

Based on these analyses, higher metabolic activity of the single strains analysed from kefir grain microflora in milk and whey from goat's milk was found; therefore only this raw material was used for further research.

The evaluation of the antibacterial activity of milk and whey fermented with selected bacterial strains of kefir grains against *Proteus mirabilis, Micrococcus luteus, Salmonella* and *E. coli* was performed using Bioscreen C. Analysis of curves showing the changes in optical density indicated that milk and whey fermented with *Lactobacillus brevis* strain had growth-inhibiting properties for *Micrococcus, Proteus, Salmonella* and *E. coli* (Fig. 7, 8*)*. Fermentation of milk and whey from goat's milk by *Lactobacillus brevis* allowed

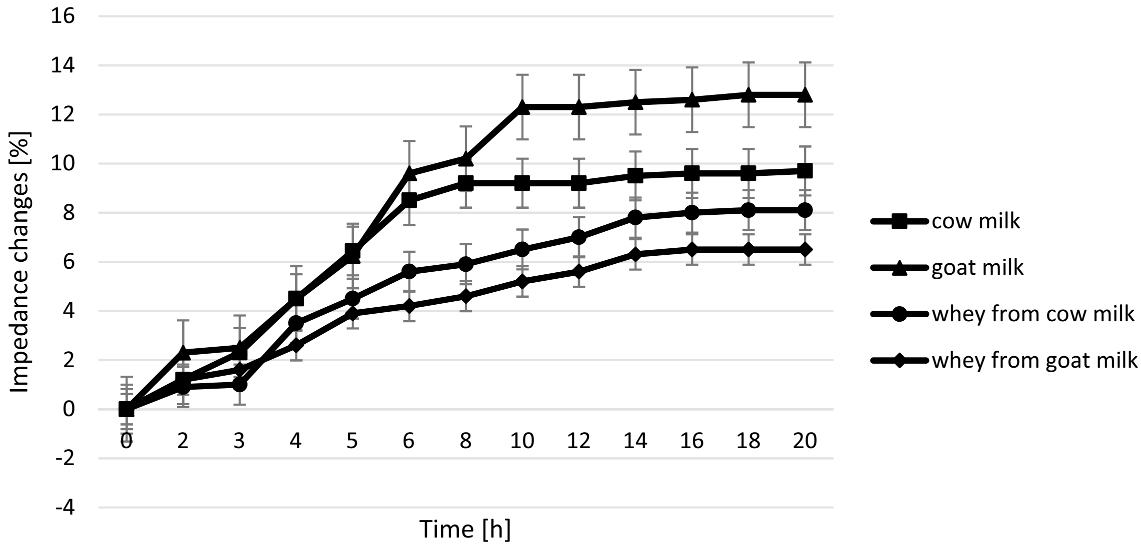

Fig. 4 Impedance changes in different medium caused by *Lactobacillus parakefiri* growth

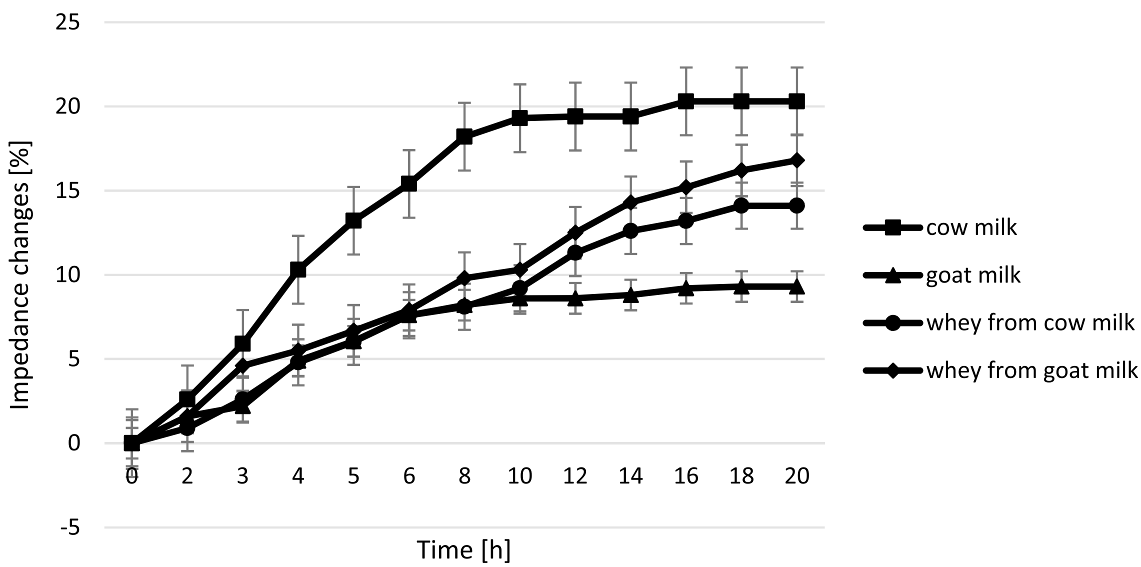

Fig. 5 Impedance changes in different medium caused by *Lactobacillus delbrueckii* subsp. *lactis* growth

release of bioactive compounds that limit the growth of *Micrococcus* during the process (Fig. 7, 8). Antibacterial properties were found based on the assessment of the growth rate of indicator microorganisms in fermented goat's milk and whey with kefir grain microflora (Table 1). Growth inhibition of the indicator microorganism was found in most samples. To confirm the antibacterial properties of fermented goat's milk and whey, culture with a defined number of microorganism was made on selective media suitable for bacteria against which growth inhibitory properties were tested. The initial concentration of bacteria for all the microorganisms being analysed (*Micrococcus, Proteus, Salmonella, E. coli*) was 10^8 cfu/ml. It was found that during the fermentation of goat's milk and whey from goat's milk, bioactive compounds were released which inhibit the growth of test indicator microorganisms. The growth of tests indicator microorganisms was inhibited, which was probably due to release of bioactive compounds. The largest reductions in indicator microorganisms were observed in whey from goat's milk products. For indicator microorganisms, a reduction of at least 2 logarithmic cycles was found for *Micrococcus* and for other microorganisms (*Proteus, Salmonella* and *E. coli*) by 8 logarithmic cycles (Table 2). Fermented goat's milk with individual strains of kefir grain microflora showed

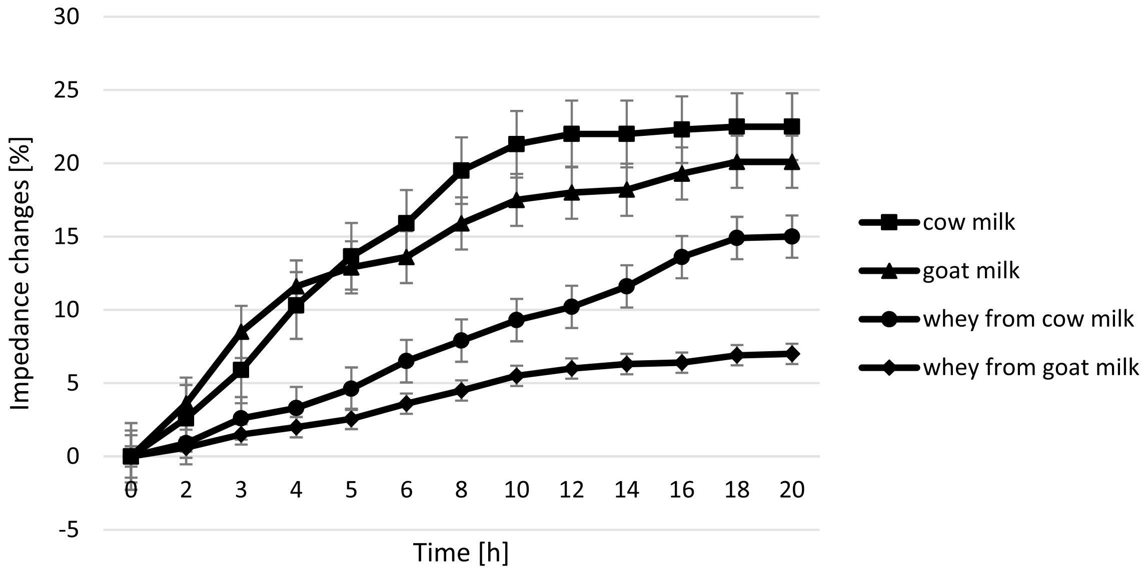

Fig. 6 Impedance changes in different medium caused by *Lactobacillus kefiranofaciens* subsp. *kefirgranum* growth

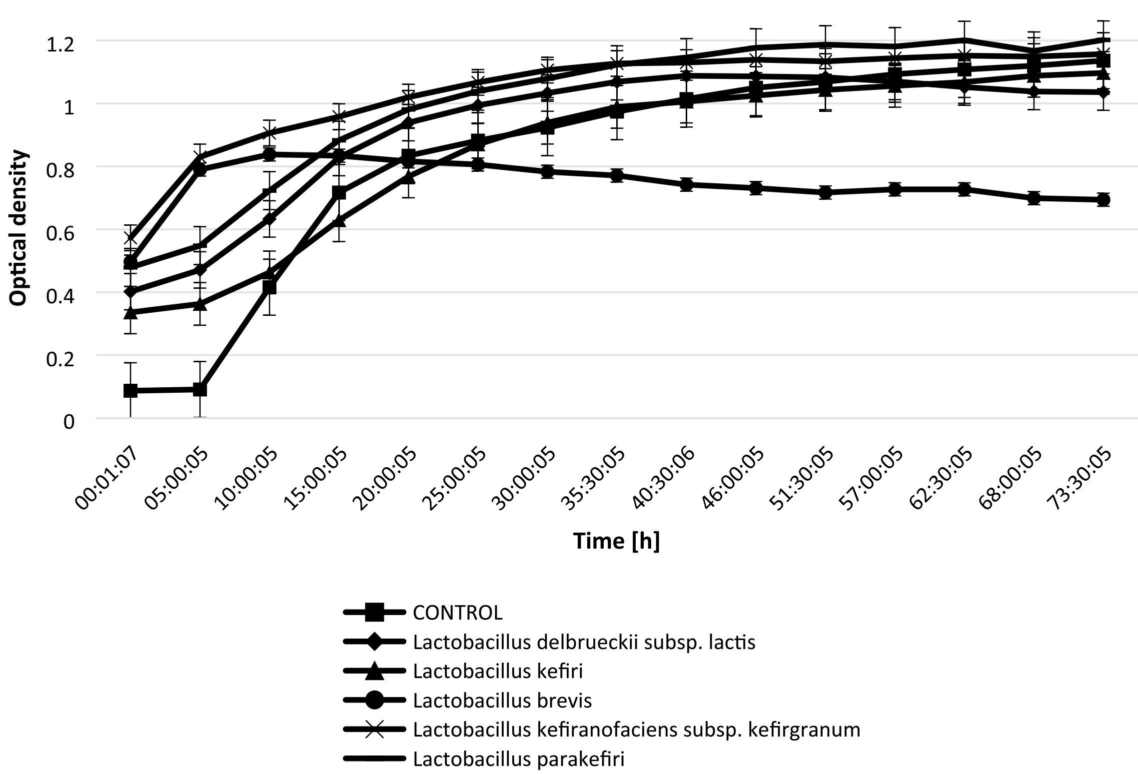

Fig. 7 Influence of presence of product of fermentation of whey from goat's milk by kefir grain microflora on *Micrococcus luteus* growth

antibacterial properties against the indicator microorganisms analysed, although the reduction was significantly smaller than in the case of fermented whey from goat's milk with the same kefir grain microflora. The highest quantitative reduction of indicator microorganisms in the case of fermented whey was found in the fermented whey by *Lactobacillus parakefiri*. The study conducted by Izquierdo-González et al. [14] indicated that 24 h fermentation of goat's milk by kefir microflora maximizes the variety of peptides with potential biological activity.

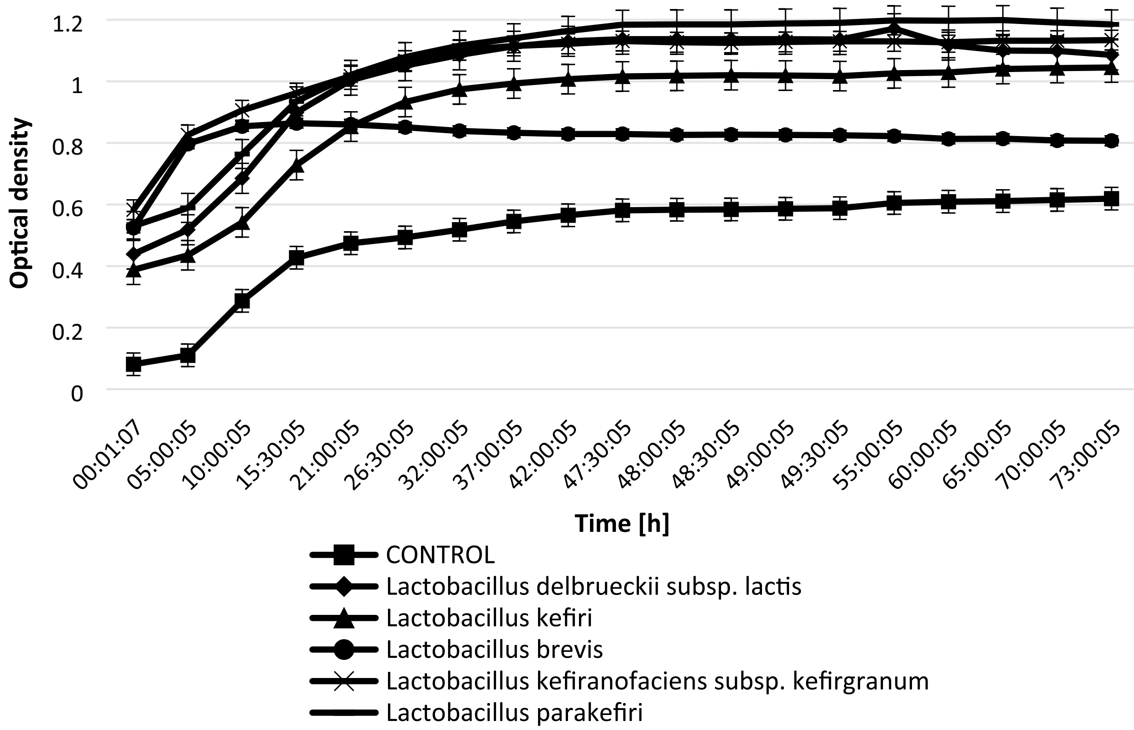

Fig. 8 Influence of presence of product of fermentation of goat's milk by kefir grain microflora on *Micrococcus luteus* growth

Table 1 The antimicrobial activity of whey and goat's milk fermented by single strains of kefir grain microflora against indicator microorganisms expressed as late growth

	Proteus	*E. coli*	*Micrococcus luteus*	*Salmonella*
Goat's milk fermented by *Lactobacillus delbrueckii subsp. lactis*	I	II	0	I
Goat's milk fermented by *Lactobacillus kefiri*	0	I	0	I
Goat's milk fermented by *Lactobacillus brevis*	I	II	I	I
Goat's milk fermented by *Lactobacillus kefiranofaciens subsp. kefirgranum*	III	II	III	I
Goat's milk fermented by *Lactobacillus parakefiri*	II	II	II	I
Whey from goat's milk fermented by *Lactobacillus delbrueckii subsp. lactis*	III	III	III	III
Whey from goat's milk fermented by *Lactobacillus kefiri*	III	III	III	III
Whey from goat's milk fermented by *Lactobacillus brevis*	I	II	II	I
Whey from goat's milk fermented by *Lactobacillus kefiranofaciens subsp. kefirgranum*	II	I	II	0
Whey from goat's milk fermented by *Lactobacillus parakefiri*	I	I	III	III

0—No growth of the microorganisms

I—very slight microorganism growth

II—growth of the microorganism, smaller than the standard

III—growth of the microorganism as a standard

Lactobacillus kefiranofaciens subsp. *kefiranofaciens* is a microorganism primarily responsible for the production of kefiran, a water-soluble polysaccharide in which the kefir grain microbial consortium is embedded and that has been reported as having antibacterial, antitumoral and antidiabetic properties, as well as several technological roles as a thickener, gelling agent and emulsifier [2, 13]

Similar studies on cow's milk were performed by Boughera et al. [4]. Researchers analysed antibacterial properties against *E. coli, Salmonella enteritidis, Micrococcus luteus*

Table 2 The quantitative analysis of antimicrobial activity of whey and goat's milk fermented by single strains of kefir grain microflora against indicator microorganisms

	Proteus	*E. coli*	*Micrococcus luteus*	*Salmonella*
goat's milk fermented by *Lactobacillus delbrueckii subsp. lactis*	1.1*107	$1.7*10^7$	9.5*102	2*107
goat's milk fermented by *Lactobacillus kefiri*	NG	NG	3.0*102	1.1*104
goat's milk fermented by *Lactobacillus brevis*	$2*10^3$	$3.2*10^6$	$1.1*10^4$	NG
goat's milk fermented by *Lactobacillus kefiranofaciens subsp. kefirgranum*	$2.5*10^7$	$7.5*10^5$	$3.0*10^4$	1.2*107
goat's milk fermented by *Lactobacillus parakefiri*	$1.6*10^7$	$5.0*10^3$	$3.0*10^4$	7.5*104
whey from goat's milk fermented by *Lactobacillus delbrueckii subsp. lactis*	NG	NG	$4.2*10^6$	NG
whey from goat's milk fermented by *Lactobacillus kefiri*	NG	NG	2.0*106	NG
whey from goat's milk fermented by *Lactobacillus brevis*	NG	NG	3.2*106	NG
whey from goat's milk fermented by *Lactobacillus kefiranofaciens subsp. kefirgranum*	NG	NG	1.6*106	NG
whey from goat's milk fermented by *Lactobacillus parakefiri*	NG	NG	1.2*104	NG

NG no growth

and *Listeria innocua* of casein of cow's milk fermented by *Lactococcus lactis* subsp. *lactis*. Clear zones of inhibited growth above 10 mm were found. The same subject was investigated by Aguilar-Toalá et al. [1], who analyzed the antibacterial activity of cow's milk fermented by *Lactobacillus plantarum* towards *E. coli, Listeria innocua, Salmonella*. The highest effectiveness was found for *Listeria*. Lin and Pan [21] proved that *Lactobacillus plantarum* NTU 102 had strong antimicrobial activity against *Klebisella pneumoniae* subsp. *pneumoniae* and *V. parahemolyticus*. The researchers found that antimicrobial activity was stable at pH 1.0–4.0. A complete loss of activity was observed at a pH value ranging from 5.0 to 11.0. Forestier et al. [11] studied the antibacterial activity of *Lactobacillus rhamnosus* using nine pathogenic bacteria, both Gram (−) and Gram (+), aerobes and anaerobes. The viability of all these microorganisms was verified during the 7-h incubation period with the *Lactobacillus rhamnosus* strain. After 3 h of incubation, little or no inhibitory effect was observed, but after 5 h the growth of all bacterial species was slowed down in the presence of *Lactobacillus rhamnosus*. The inhibitory effect of *Lactobacillus* strains varies even within the same species. *Lactobacillus acidophilus* and *Lactobacillus helveticus* have extracellular serine proteases which release functional peptides [7].

Conclusion

These experiments showed that during the fermentation of goat's milk with individual microorganisms of kefir grains, bioactive substances are formed, which have antibacterial properties against the most deadly strains in the field of food hygiene. Accordingly, further studies are in progress to characterize in greater detail what kind of substances are responsible for this antimicrobial activity.

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Compliance with ethical standards

Conflict of interest This is an original work of the authors and all of them mutually agree that it should be submitted to European Food Research and Technology journal. The manuscript has not been published, is not currently submitted for review to any other journal, and will not be submitted elsewhere before a decision is made by this journal. Authors are responsible for disclosing all financial and personal relationship between themselves and others that might bias their work.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects which require permission from ethics committees or other institutions.

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