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In Vitro Assessment of Probiotic Potential and Functional Properties of *Lactobacillus reuteri* LR1

A. V. Begunova[a], O. S. Savinova[b], I. V. Rozhkova[a], Yu. I. Krysanova[a], and T. V. Fedorova[b, *]

[a]*All-Russia Scientific Research Institute of the Dairy Industry, Moscow, 115093 Russia*

[b]*Bach Institute of Biochemistry, Fundamentals of Biotechnology Federal Research Center, Russian Academy of Sciences, Moscow, 119071 Russia*

**e-mail: fedorova_tv@mail.ru*

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Abstract—Research on the effects of various antimicrobial agents on the strain *Lactobacillus reuteri* LR1 indicated its susceptibility to lincomycin, amoxicillin, and chloramphenicol, as well as resistance to other studied antibiotics. Upon milk fermentation by *L. reuteri* LR1 for 24 h, there was a significant increase in antioxidant and ACE-inhibitory activities, along with a decrease in L-leucine equivalents, as compared to the original milk. Further cultivation resulted in an increase in the inhibitory activity of the proteolytic, antioxidant, and angiotensin-converting enzyme (ACE), reaching its highest value after 96 h. HPLC-MS/MS analysis of the peptide profile of milk fermented by the lactobacillus showed the presence of peptides possessing ACE-inhibitory, antimicrobial, antioxidant, and immunomodulatory activities. Analysis of the substrate specificity of specificity of *L. reuteri* LR1 CEP proteinase toward the (f1-23) α_{s1}-casein fragment revealed its unique specificity, which may indicate the PI/PIII type.

Keywords: Lactobacillus reuteri LR1, resistance to antibacterial drugs, proteolytic activity, antioxidant activity, ACE-inhibitory activity, peptide profile, biologically active peptides

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INTRODUCTION

Modern science defines a microbial biocenosis as a single system of the human organism and its microbiome. At the same time, the microbiome is composed of the diverse microbial consortia and their metabolic products that inhabit a particular biotope [1].

There is growing evidence that various diets and environmental factors have a significant impact on the metabolism, immunoresponse, and human susceptibility to diseases, which results from changes in the species composition of microbial communities in the gastrointestinal tract (GIT) [2–4].

Since 2001, the term “probiotics” has referred to live microorganisms, the consumption of which in adequate amounts has a beneficial effect on the host organism [5]. Therefore, it is very important to understand the mechanism of action of a probiotic microorganism on a macroorganism, which has been continuously studied in experiments in vitro.

The beneficial characteristics of lactic-acid bacteria (LAB) for human health are intensively studied. There are currently studies and an accumulation of data on the broader spectrum of probiotic activity of microorganisms, which can normalize the functions of the GIT microbiota, boost immunity, reduce manifestations of food allergies, alleviate symptoms of lactose intolerance, and have hypocholesterolemic, anticarcinogenic, and antimutagenic effects [6, 7]. In the lower intestine, probiotic microorganisms are able to produce antioxidants, hormone-like substances, and enzymes, which are involved in the metabolic processes of the macroorganism [8].

The use of probiotic microorganisms, including LAB, in the production of fermented milk products of directed action makes it possible to add some functional properties to the product [9, 10]. The addition to the organism of probiotic strains as components of fermented milk products has been proven to be more effective than the consumption of probiotic strains as dosage forms [11]. At present, members of the genus *Lactobacillus* are the most widely studied probiotic microorganisms within the LAB group [12]. The genus *Lactobacillus* is known to include species with different spectra of physiological and biochemical features. *Lactobacillus reuteri*, a unique microorganism of the family *Lactobacillaceae* with various mechanisms of action, is of growing interest. Since the probiotic properties of lactobacilli are strain-specific, research on the features of a specific strain for its further use as a starter or probiotic starter culture is relevant.

The strain studied in this work, *Lactobacillus reuteri* LR1, was isolated in 2014 at the Central Labora-

tory of Microbiology of the All-Russia Scientific Research Institute of the Dairy Industry (Russia) from the feces of a healthy person and were identified with modern biochemical and molecular genetics methods [13]. *L. reuteri* LR1 has been shown to possess antimicrobial activity against gram-positive and gram-negative bacteria and to synthesize a bacteriocin-like compound, reuterin, in the presence of glycerol [13, 14].

The goal of this research was to study the probiotic potential and functional properties of *Lactobacillus reuteri* LR1.

EXPERIMENTAL

Cultures. The LAB strain *L. reuteri* LR1 from the Collection of Microorganisms of the All-Russia Scientific Research Institute of the Dairy Industry (Moscow, Russia) was used in this study. Sterile skim milk was used to cultivate lactobacilli.

Cell counting. The number of *L. reuteri* LR1 cells was enumerated via plating on MRS agar culture medium (Biokompas-S, Russia). The cultivation was carried out under anaerobic conditions in an OXOID anaerojar and GasPak gas pouches (BD Biosciences, United States) at 37 ± 1°C. All colonies grown on the medium for 72 h were counted.

Antibiotic resistance. The resistance of *L. reuteri* LR1 to antibiotics was determined in vitro with the disk-diffusion method, which is based on the ability of antimicrobial agents to diffuse into the nutrient medium from paper disks soaked in agents, which inhibits microbial growth on the surface of the agar medium. The results of the assessment of lactobacillus susceptibility were interpreted according to the recommended practices 2.3.2.2789-10, “Guidelines for Sanitary-Epidemiological Assessment of the Safety and Functional Potential of Probiotic Microorganisms Used for Food Production” (https://ohranatruda.ru/upload/iblock/846/4293757373.pdf).

Obtainment of protein-peptide fractions. An aliquot (15 mL) of the fermented milk sample was centrifuged (10000 *g*) for 30 min at 4°C with a 5702R centrifuge (Eppendorf, Germany). The supernatant, which contained a fat layer, was filtered through folded filter paper (MN 640W, Macherey-Nagel, Germany). The pH of the filtrate was adjusted to 4.6 with 0.1 M sodium hydroxide. The obtained mixture was subsequently centrifuged (10000 *g*) for 30 min at 4°C with a 5702R centrifuge (Eppendorf, Germany), and the supernatant was filtered through syringe filters with a hydrophilic membrane with a pore diameter of 0.2 μm (Sartorius, Germany). The protein-peptide fractions were frozen and stored at –73°C prior to analysis.

Before the analysis, samples of the protein-peptide fractions of fermented milk were thawed and additionally filtered through syringe filters with a hydrophilic PVDF membrane with a pore diameter of 0.45 μm (Carl Roth, Germany).

The proteolytic, antioxidant, and ACE-inhibitory (hypotensive) activities, as well as the organic-acid content, in these samples of protein-peptide fractions of fermented milk were determined.

Proteolytic activity. The proteolytic activity was quantified via measurement of the amount of released amino groups in supernatants with the previously described method [15] with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Sigma-Aldrich, United States). The optical density of solutions was determined on a Synergy2 microplate photometer/fluorometer (BioTek, United States) at a wavelength of 340 nm. L-leucine (Sigma-Aldrich, United States) and used as the standard for the determination of proteolytic activity. The results of the measurements were expressed as mmol/L leucine equivalents.

Antioxidant activity. Antioxidant activity was determined in vitro with the Oxygen Radical Absorbance Capacity (ORAC) fluorescence method via the generation of peroxyl radical in the reaction medium with a BioTek Synergy 2 microplate photometer/fluorometer as described earlier [16, 17]. The peroxyl radical was generated directly in the reaction medium as a result of the thermal decomposition of the 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) azo compound (Sigma, United States) during incubation for 10 min at 37°C. The fluorescence-decay kinetics was recorded for 1 h at 60-s intervals on a Synergy 2 photometer/fluorometer in the fluorescence-intensity detection mode (485-nm excitation wavelength; 528-nm emission wavelength) at 37°C. The antioxidant activity of fermented milk samples, relative to the peroxyl radical, was expressed as mM of Trolox equivalents (TE).

ACE-inhibitory activity. The ACE-inhibitory activity was analyzed in 96-well, black, nonbinding polypropylene microplates (Greiner Bio One, Germany). The kinetics of increasing fluorescence intensity was studied for 15 min at 20-s intervals on a Synergy 2 microplate photometer/fluorometer (excitation wavelength of 320 nm; fluorescence registration wavelength of 420 nm) at 37 ± 1°C.

The graph of the dependence of ACE inhibition (%) on the common logarithm of the dilution factor of the original fermented milk sample was used to determine via linear interpolation the dilution factor of the sample at which 50% of the ACE activity was inhibited (IC_{50}). For fermented milk samples, the IC_{50} value, expressed as mg protein/mL, was calculated from the division of the protein concentration in the sample (mg/mL) by the dilution factor value at which 50% of the ACE activity was inhibited.

Organic-acid content. The content of organic (lactic and acetic) acids during milk fermentation by *L. reuteri* LR1 was determined via high-performance liquid chromatography (HPLC) on an Agilent 1290 Infinity instrument (Agilent Technologies, United States) with diode array detector at 210 nm and a Zorbax SB-C8 column (4.6 × 50 mm, 1.8 μm).

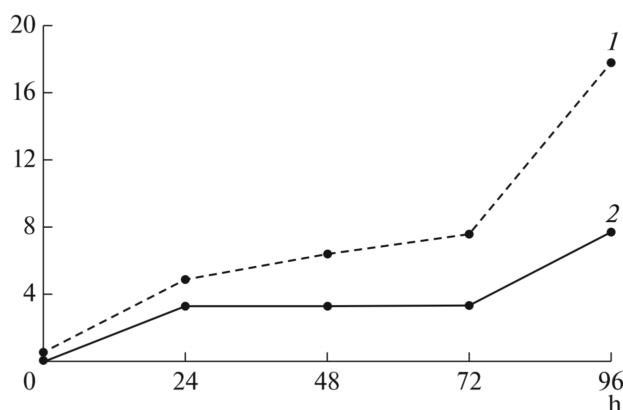

Fig. 1. Dynamics of changes in the organic-acid content (mg/mL) during the cultivation of *L. reuteri* LR1 in milk: lactic acid (*1*) and acetic acid (*2*).

Determination of the peptide profile. The peptide profile was analyzed with a system consisting of an Agilent 1100 chromatograph (Agilent Technologies, United States) and an LTQ-FT Ultra tandem mass spectrometer (Thermo, Germany). The peptides were separated via reversed-phase gradient chromatography [18]. The QualBrowser software was used to monitor the results of HLPC–mass spectrometry (HPLC-MS). Lists of the exact masses of peptides and peptide fragments were obtained from the mass chromatograms with the Raw2msm software; they were used for the database search and protein identification with the Peaks Studio software package (Bioinformatics Solutions Inc., United States, version 8.5). Peptides were identified by the amino-acid sequences of proteins according to the UniProtKB database. The search parameters for the identification were as follows: enzyme, none (native peptides); mass accuracy for the parent ion, 15 ppm; mass accuracy for MS/MS fragments, 0.50 Da. De novo sequencing was performed with the Peaks Studio software package.

The protein concentration in the samples was determined with a BCA assay kit (ThermoFisher, United States) according to the manufacturer's recommendations.

All experimental studies were carried out in three to five replicates. The Microsoft Office and Statistica 10 software packages were used for diagram plotting, table construction, and statistical processing of the experimental data.

RESULTS AND DISCUSSION

Numerous studies have proven that biological effects and, therefore, the probiotic potential of different strains of the same *Lactobacillus* species can vary significantly and may be a strain-specific feature [19–21]. To increase the efficiency of the use of probiotic microorganisms, an understanding of the mechanisms underlying the biological effect of a specific strain is also required.

Previous studies have shown pronounced antimicrobial properties for *L. reuteri* LR1 in a coculture with opportunistic microorganisms that cause nosocomial and foodborne toxicoinfections, which are among the main indicators of the probiotic activity of this strain [13, 22].

Antibiotic resistance. With the use of probiotics, antibiotic resistance plays a crucial role. The susceptibility of *L. reuteri* LR1 to 15 antimicrobial agents belonging to different groups (penicillins, tetracyclines, aminoglycosides, fluoroquinolones, macrolides, and others; Table 1) was therefore studied. These drugs are used in clinical practice for the treatment of various intestinal, respiratory, and urinary tract, and other infections. As a result, *L. reuteri* LR1 was shown to be sensitive to lincomycin, amoxicillin, and chloramphenicol, to possess intermediate resistance to ampicillin and neomycin, and to be resistant to other tested antimicrobial drugs. These results indicate that *L. reuteri* LR1 is relatively resistant to antibiotics and may be recommended as a bacterial preparation or as a component of a fermented-milk product to normalize the gastrointestinal microbiota during antibiotic therapy for various infections.

In vitro biological and proteolytic activities. Active acidity decreased during milk fermentation by *L. reuteri* LR1. This resulted from the formation of organic acids (primarily lactic acid) during the fermentation of milk carbohydrates, as well as the enzymatic hydrolysis of milk proteins (mainly various casein fractions) by proteases synthesized by *L. reuteri* LR1, with the formation of peptides. Figure 1 shows our data on the change in the organic-acid content during the cultivation of *L. reuteri* LR1 in sterile skim milk. At the same time, a significant increase in antioxidant and ACE-inhibitory activity was observed within 24 h of milk fermentation, along with a decrease in L-leucine equivalents, as compared to the original milk (Table 2). This was due to the rather low proteolytic activity of *L. reuteri* LR1 against milk casein proteins; therefore, a slow growth of cell biomass was observed, and the number of *L. reuteri* LR1 cells increased insignificantly from 1.2×10^7 to 1.9×10^7 CFU/mL. In the next 24 h of cultivation, an increase in *L. reuteri* LR1 cell abundance occurred at a higher rate and reached the highest value of 3×10^9 CFU/mL. At the same time, there was a slight decrease in the antioxidant and ACE-inhibitory activities, along with a constant number of L-leucine equivalents. Further cultivation resulted in an increase in the proteolytic, antioxidant, and ACE-inhibitory activities; the highest values occurred after 96 h of cultivation (Table 2). Moreover, the number of viable *L. reuteri* LR1 cells remained almost unchanged after 72 h (Table 2), which may be explained by the stationary growth phase reached by the culture and the pos-

Table 1. Resistance of *Lactobacillus reuteri* LR1 to antimicrobial agents

Serial no.	Sample	Substance amount in disc, μg	Diameter of zone of growth inhibition, mm	Assessment of susceptibility of lactobacilli
		Aminoglycoside group		
1	Gentamicin	120 μg	12	Resistant
2	Kanamycin	30 μg	8–10	Resistant
3	Neomycin	30 μg	12–14	Intermediate resistant
		Penicillin group		
4	Amoxicillin	20 μg	25–26	Sensitive
5	Ampicillin	10 μg	15–16	Intermediate resistant
6	Benzylpenicillin	10 U	10	Resistant
7	Oxacillin	1 μg	9–10	Resistant
		Tetracycline group		
8	Doxycycline	30 μg	8	Resistant
9	Tetracycline	30 μg	–	Resistant
		Fluoroquinolone group		
10	Levofloxacin	5 μg	8–10	Resistant
11	Pefloxacin	5 μg	–	Resistant
		Azalide/macrolide group		
12	Azithromycin	15 μg	9	Resistant
		Others		
13	Lincomycin	15 μg	27	Sensitive
14	Chloramphenicol	30 μg	30–32	Sensitive
15	Fosfomycin	200 μg	10	Resistant

Table 2. Dynamics of changes in the proteolytic, antioxidant, and ACE-inhibitory activities during *Lactobacillus reuteri* LR1 cultivation in milk

Duration of cultivation, h	*L. reuteri* LR1 cell number, CFU/mL	Antioxidant activity (ORAC), μM TE/mg protein	ACE-inhibitory activity, (IC_{50}) mg protein/mL	Proteolytic activity, (L-leucine equivalents), mM
0	1.2×10^7	205.6	26.2	8.1
24	1.9×10^7	374.9	12.6	6.2
48	3×10^9	295.1	11.2	6.0
72	9.4×10^8	361.5	7.1	8.5
96	9.3×10^8	453.6	1.6	10.4

sible inhibition of growth by the lactic and acetic acids that accumulated in the medium (Fig. 1).

Peptide profile and substrate specificity of proteases. HPLC-MS/MS analysis of peptide fractions of non-fermented milk samples (control) and milk fermented by *L. reuteri* LR1 resulted in the identification of 277 peptides (Table 3). The peptides mainly belonged to casein proteins; of the noncasein proteins, α-lactalbumin and β-lactoglobulin, lactoferrin, osteopontin, and others were identified in the largest amounts.

After 24 h of *L. reuteri* LR1 cultivation, ~150 oligopeptides containing 5–52 amino-acid residues were identified in fermented milk (Table 3, Figs. 2 and 3). Analysis of the amino-acid sequences of oligopeptides showed that most of them were also present in the original milk before the addition of the starter culture, and the other part was composed of fragments of these peptides (Figs. 2 and 3). Only 7 unique oligopeptides identified in *L. reuteri* LR1 fermented milk were formed as a result of the hydrolysis of β-casein itself, and not of the oligopeptides present in the original milk (Fig. 2). The presence of peptides in the original unfermented milk indicated the activity of endogenous milk proteases, such as plasmin and elastase, as

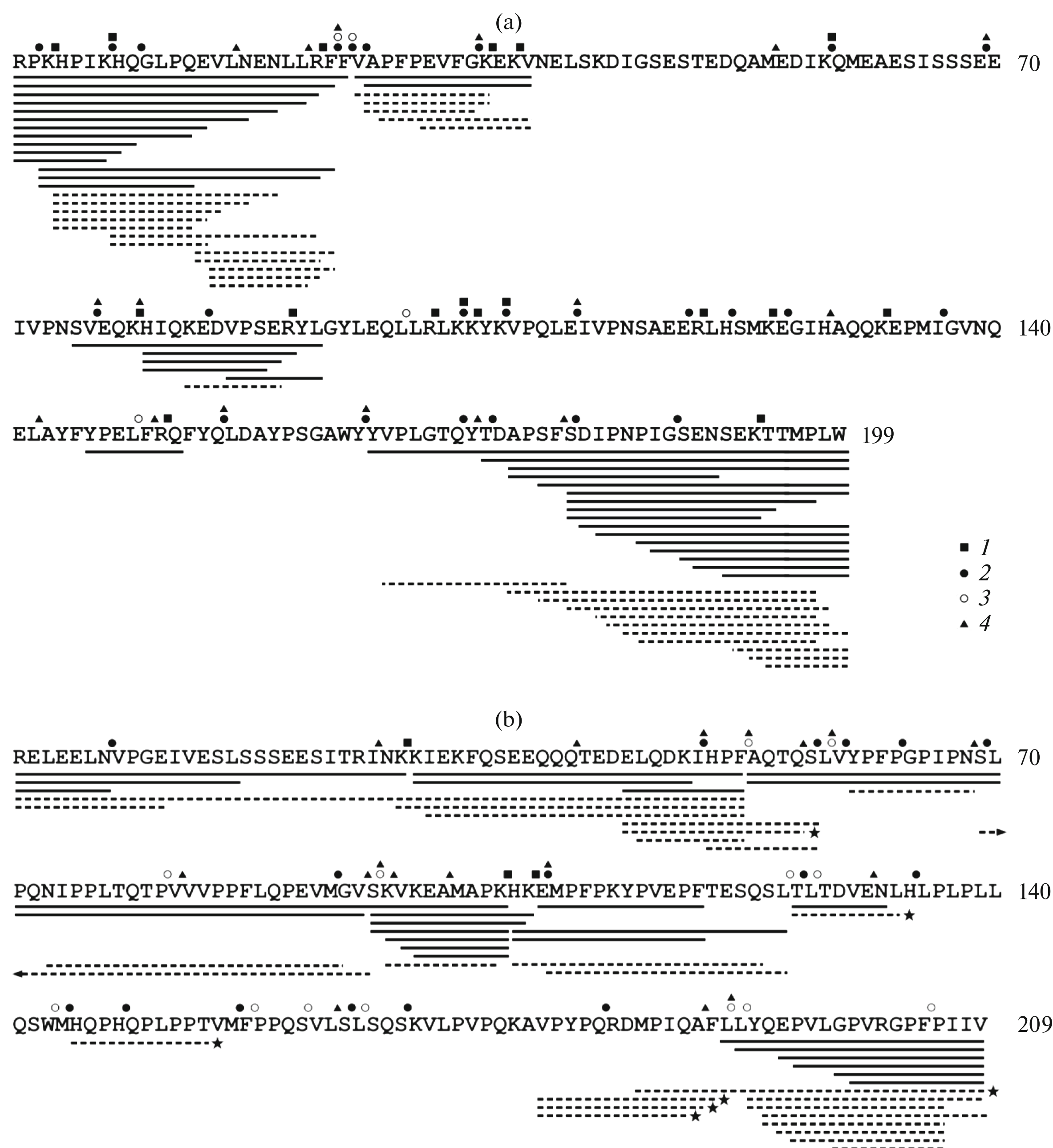

Fig. 2. Peptides of α_{s1}-casein (a) and β-casein (b) identified in the nonfermented (control) milk samples and milk fermented by *L. reuteri* LR1. Solid lines indicate peptides that were identified in both the control milk sample and the sample of milk fermented by *L. reuteri* LR1; dashed lines indicate unique peptides that were identified only in the sample of fermented milk; the peptides that formed as a result of the hydrolysis of β-casein, not its oligopeptides present in the original milk, are indicated by asterisk. Cleavage sites of endogenous milk proteases: plasmin (*1*); cathepsin B (*2*); cathepsin D (*3*); cathepsin G (*4*) [23].

Table 3. Numbers of identified unique peptides of casein and noncasein proteins in original milk samples and milk fermented by *Lactobacillus reuteri* LR1

Samples	Identified (total)	Casein β	Casein α_{s1}	Casein κ	Casein α_{s2}	Noncasein proteins
Control (milk)	126	39	61	6	12	8
Fermented milk	151	54	75	10	3	9

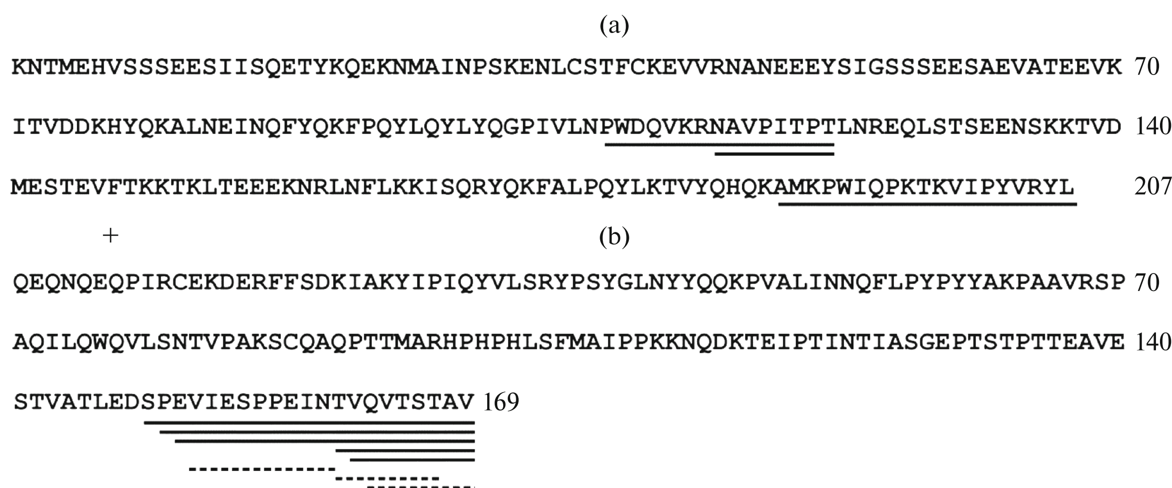

Fig. 3. Peptides of α_{s2}-casein (a) and κ-casein (b) identified in the nonfermented (control) milk samples and milk fermented by *L. reuteri* LR1. Solid lines correspond to the peptides found in both the control milk sample and the sample of milk fermented by the lactobacillus; dashed lines indicate the peptides found only in the fermented milk sample.

well as cathepsins D, B, and G (Fig. 2) [23]. At the same time, plasmin is slightly active or completely inactive against κ-caseins and serum proteins, which were found in significantly smaller amounts, as compared to peptides from β- and α_{s1}-caseins (Table 2, Fig. 3). The absence of some peptides after milk fermentation might be due to the *L. reuteri* LR1 hydrolysis by extracellular proteases or their consumption by cells.

The data on the peptide profile agreed with the results of the determination of proteolytic activity. A decrease in the number of L-leucine equivalents from 8.1 mM (original milk) to 6.0–6.2 mM was observed in 48 h of *L. reuteri* LR1 cultivation (Table 2). This indicated a decrease in the total amount of peptides in milk. Thus, *L. reuteri* LR1 cells used peptides that were originally present in milk as a source of nitrogen nutrition. Moreover, no conglomerates formed during milk fermentation by *L. reuteri* LR1. Similar results were reported earlier [24]. The authors have shown that the growth and development of *L. reuteri* JCM 1112 and *Lactobacillus gasseri* JCM 1131 improved significantly in prehydrolyzed milk as compared to whole skim milk. These strains required oligopeptides, rather than proteins or free amino acids, as a source of nitrogen for their growth in milk.

LAB contain various cell envelope proteinases (CEPs), which are involved in the hydrolysis of mainly casein proteins and the release of various bioactive peptides. To date, five different CEP types of LAB have been characterized: PrtP from *Lactococcus lactis* and *Lactobacillus paracasei*, PrtH from *Lactobacillus helveticus*, PrtR from *Lactobacillus rhamnosus*, PrtS from *Streptococcus thermophilus,* and PrtB from *Lactobacillus bulgaricus* [25]. CEPs are usually classified according to their pattern of hydrolysis of the α_{s1}-casein fragment, which contain 1–23 residues [26]. Typically, two types of CEP are found: PI and PIII. The preferred substrates for PI-type proteinases are β-casein and κ-casein (to a lesser extent), while PIII-type proteinases possess equal ability to cleave α_{s1}-, β-, and κ-caseins. For lactobacilli, PI and PIII proteinase types, as well as an intermediate PI/PIII type with a new type of substrate specificity, were shown [27].

L. reuteri LR1 hydrolyzes the (f1-23) α_{s1}-casein fragment at the following positions: K3-H4, K7-H8, Q9-G10, Q13-E14, E14-V15, V15-L16, and N17-E18 (Fig. 4). Most of these cleavage sites are typical for PI/PIII-type CEPs isolated from LAB, such as *S. thermophilus* CNRZ385, *Lactobacillus delbrueckii* subsp. *lactis* CRL581, *L. helveticus* L89, *L. casei* PRA205, and *L. rhamnosus* PRA331 [25, 28, 29]. The restriction sites at positions H8-Q9, L16-N17, and E18-N19, which are described for proteinases of other LAB, are not characteristic of the *L. reuteri* LR1 CEP (Fig. 4). However, three additional sites were found at positions K3-H4, K7-H8, and V15-L16. The (f1–9) sequence of the amino-terminal region of α_{s1}-casein is known to be cleaved only by the intracellular PepO2 and PepO3 endopeptidases from *Lactobacillus helveticus* CNRZ32 through the P5-I6 bond [30]. Furthermore, the same authors have shown that K3-H4 and K7-H8 bonds are sensitive to the action of the PepE endopeptidase of *L. helveticus* CNRZ32. Nevertheless, the bacterial biomass growth and the absence of cell lysis (Table 2, Fig. 1) in the first 24 h of fermentation excluded the possibility of the effect of intracellular proteases on casein hydrolysis by *L. reuteri* LR1. Moreover, the substrate specificity of the *L. helveticus* Zuc2 CEP to the K3-H4 site was shown previously [31]. The K7-H8 and V15-L16 restriction sites were shown for lactobacillus CEPs for the first time, which emphasizes the unique specificity of this proteinase of *L. reu-*

Lb. reuteri LR1

R-P-K-H-P-I-K-H-Q-G-L-P-Q-E-V-L-N-E-N-L-L-R-F

Lb. hel. CRNZ32(PrtH)

Lb. hel. CRNZ32(PrtH2)

Lb. del. CRL581

Lb. casei PRA205

Lb. rhamn. PRA331

Lc. lactis Wg2(PrtPI)

Lc. lactis SK11(PrtPIII)

Fig. 4. Specificity of CEPs of various LAB, including *L. reuteri* LR1, toward the (f1-23) α_{s1}-casein fragment. Cleavage sites are indicated by arrows; cleavage sites that are not characteristic of LAB CEPs are indicated by asterisk. *Lb. hel.*, *L. helveticus* CRNZ32 [30]; *Lb. del.*, *L. delbrueckii* subsp. *lactis* CRL581 [28]; *Lb. casei*, *L. casei* PRA205 [29]; *Lb. rhamn.*, *L. rhamnosus* PRA331 [29]; *Lc. lactis* Wg2, *Lactococcus lactis* Wg2 [28]; *Lc. lactis* SK11, *Lactococcus lactis* subsp. *cremoris* SK11 [32].

teri LR1. It can be classified as a PI/PIII-type proteinase that shows higher activity against β- and α_{s1}-caseins than κ- and α_{s2}-caseins of cow's milk.

Biologically active peptides. Peptides the functional properties previously described in the scientific literature [33] were discovered in a comparison of the peptides identified in the Milk Bioactive Peptide Database: the β-casein YQEPVLGPVRGPFPIIV (f193–209), YQEPVLGPVRGPFP (f193–206), and QEPVLGPVRGPFPIIV (f194–209) peptides, which possess ACE-inhibitory, antimicrobial, and immunomodulatory activities; the κ-casein peptide VQVTSTAV (f162–169), which exerts antimicrobial activity; and the β-casein peptide YPFPGPIPN (f60–68), which exhibits ACE-inhibitory activity. The α_{s1}-casein EVLNENLLRF (f14–23) peptide detected in the study [34] was shown to have ACE-inhibitory activity; the peptide fraction containing this peptide had an IC_{50} of <10 μg/mL.

Among the identified peptides, peptides containing residues of redox-active amino acids (tyrosine, tryptophan, methionine, cysteine, and histidine), which determine the antioxidant properties of these peptides, were found. In fermented milk, the β-casein VKEAMAPK (f98–104) octapeptide was identified and shown to possess antioxidant activity.

Analysis of the identified peptides indicated that milk fermented by *L. reuteri* LR1 was also composed of peptides containing fragments with antihypertensive properties associated with their ACE-inhibitory effect, according to the literature data (Table 4). In contrast to peptides exhibiting ACE-inhibitory activity, the immunomodulatory peptides released from β-casein are rarely described. However, those peptides that have been determined to be multifunctional peptides, such as β-casein peptide (f59–68), which exerts antioxidant, opioid, and ACE-inhibitory activities, and peptide (f193–209), which possesses antimicrobial, immunomodulatory, antithrombotic, ACE-inhibitory, and antioxidant activities, originate from the C-terminal region of β-casein [33]. The β-casein peptide DMPIQAFLLYQEPVLGPVR (f184–202), which has an anti-inflammatory effect, was recently characterized [33]. Peptides from the C-terminal region of β-casein, such as MPIQAFLLYQEPVLGPVRGPFPIIV (f185–209), VPYPQRDMPIQ (f178–188), VPYPQRDMPIQA (f178–189), and VPYPQRDMPIQAF (f178–190), were also found in milk fermented by *L. reuteri* LR1. All of these peptides formed as a result of hydrolysis of the protein sequence of β-casein (and not its oligopeptides present in the original milk); they are marked in Fig. 2 with an asterisk. We propose that the peptides released from this part of β-casein may be responsible for the possible anti-inflammatory and/or immunomodulatory effects of milk fermented by *L. reuteri* LR1.

CONCLUSIONS

Thus, a detailed characterization of the peptide profile of the protein-peptide fraction of milk fermented by the strain *L. reuteri* LR1 resulted in the identification of the peptides that are responsible for antioxidant, hypotensive, and antimicrobial properties. This was confirmed by the corresponding activities of fermented milk in tests in vitro (Table 2). The values of proteolytic and antioxidant activities obtained for *L. reuteri* LR1 agree with the literature data for other probiotic strains of lactobacilli. Milk fermented by *L. plantarum* after 48 h of cultivation exhibited comparable antioxidant activity to that obtained for *L. reuteri* LR1 in this study: 250–350 μM Trolox equivalents (ORAC) [45]. The proteolytic activity of *L. reuteri* LR1

Table 4. Predicted hypotensive peptides possessing ACE-inhibitory activity identified in the composition of milk fermented by *L. reuteri* LR1

Identified peptide*	Peptide with hypotensive activity	IC_{50}, μM	Reference
SLPQNIPPLTQTPVVVPPFLQPEVMGV	LVYPFPGPIPNSLPQNIPP	5.3	[35]
CASB (f69–95)	NIPPLTQTPV	173	[36]
	TPVVVPPFLQP	749	[37]
YPFPGPIPN CASB (f60-68)	VYPFPGPI	500	[36]
	SKVYPFPGPI	1.7	[38]
	YPFPGPIPN	15	[39]
NIPPLTQ**TPVVVPPFLQP**EVM	TPVVVPPFLQP	749	[37]
CASB (f73–93)	IPPLTQTPVVVPP	9.0	[40]
ELQDKIHP**FAQTQ** CASB (f44–56)	FAQTQSLVYP	25	[41]
ELQDKIHP**FAQTQS** CASB (f44–57)	DKIHPF	257	[36]
LQ**DKIHPF** CASB (f45–55)	KIHPFAQTQSLVYP	39	[41]
	ELQDKIHPF	–	[42]
VAPFPEVFGK CASA1 (f25–34)	VAPFPEVF	363	[43]
VAPFPEVFGKEKV CASA1 (f25–37)	FVAPFPEV	476	
APFPEVFGK CASA1 (f26–34)	FFVAPFPEVFGK	18	[44]
APFPEVFGKEKV CASA1 (f26–37)			
APFPEVFG CASA1 (f26–33)			
PFPEVFGKEKV CASA1 (f27–37)			
EVFGKEKV CASA1 (f30–37)			
VL**NENLLR** CASA1 (f30–37)	NENLLRFFVAPFPEVFG	55	[42]
V**LNENLLRF** CASA1 (f30–38)	ENLLRFFVAPFPEVFG		
LNENLLR CASA1 (f31–37)	LNENLLRFFVAPFPEVFG		

*CASB, β-casein; CASA1, α_{s1}-casein.

after 72 h of milk fermentation was 8.5 mM L-leucine equivalents, which was comparable to that of *L. rhamnosus* PRA331 and *L. casei* PRA205 after 72 h of milk fermentation: 6.4 and 10.9 mM L-leucine equivalents, respectively [29]. The ACE-inhibitory activity of milk fermented by *L. reuteri* LR1 (IC_{50} = 7–11 mg/mL) did not differ in the range of IC_{50} values from those described for fermented-milk beverages in the literature [46]. However, the ACE-inhibitory activity of milk fermented by *L. reuteri* LR1 was one order of magnitude lower than that shown for *L. acidophilus* ATCC 4356: IC_{50} = 0.42 mg/mL after 24 h of milk fermentation [47]. It may be explained by the lower proteolytic activity of *L. reuteri* LR1 as compared to *L. acidophilus* ATCC 4356. In the latter, after 24 h of growth, the proteolytic activity was 10 mM L-leucine equivalents (pH 5.6), and the number of viable cells was 6.4×10^8 CFU, while *L. reuteri* LR1 was characterized by 6.2 mM L-leucine equivalents and 1.9×10^7 CFU.

These in vitro studies suggest that *L. reuteri* LR1 exhibits pronounced antioxidant, hypotensive, and antimicrobial properties and may therefore be used as a starter culture that possesses probiotic properties.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflict of interest. This article does not contain any studies involving animals or human participants performed by the authors.

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