Research Article

Probiotic Potential and Functional Properties of Lactobacillus Reuteri, Lactobacillus Rhamnosus and Lactobacillus Helveticus: A Comparative Study

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Abstract. This study was conducted to evaluate and comparethe probiotic propertiesof*Lactobacillus helveticus*NK1, *Lactobacillus rhamnosus*F and *Lactobacillus reuteri*LR1lactobacilli strains.Changes in pH, cell growth, proteolytic activity, antioxidantactivity, and angiotensin-converting enzyme(ACE)inhibitoryactivity were monitored during fermentation ofreconstituted skim milk (RSM) by pure cultures of lactobacilli.Among the tested strains, *L. helveticus*NK1 showed the highest proteolytic, ACE inhibitoryand antioxidantactivitiesduring milk fermentation,followed by *L. rhamnosus* F and *L. reuteri*LR1.The promising capability of all of the lactobacilli strains to release bioactivepeptides from the milk proteins was demonstrated.

Keywords: Lactobacillus, probiotic, milk fermentation, bioactive peptides

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1. Introduction

Probiotics are an important category of functional foods as theyprovide health benefits beyond the traditional nutrition value.Lactic acid bacteria (LAB) including *Lactobacillus* spp. are consideredto be probiotic microorganisms. Many Lactobacillusstrains display high proteolytic activity toward milk protein, whichin turn provides them with an ability to potentially release the bioactivepeptides.

Bioactive peptides with antioxidant activity and ACE-inhibitory(ACE-I) activity in food products and, particularly,their release during milk fermentationby lactobacilli were intensively studied[1].Various antioxidant and ACE-inhibitory peptides have been separatedfrom fermented milk types produced with different *Lactobacillus*spp.

The ability of individual lactobacillito produce bioactive peptides is most likely related to the completeness and efficiency of their proteolytic system[2, 3].As the proteolytic capacity of *Lactobacillus* is strain-specific, it is necessary to screen for suitable strains

based on their ability to produce bioactive peptides during fermentation and to test*in vitro* and *in vivo*antioxidant and ACE-I activity of the obtained fermented products for future development of novel functional products includingthat for management of hypertension.

In our previous work, we demonstrated that *Lactobacillus helveticus* NK1, *Lactobacillus rhamnosus* F and *Lactobacillus reuteri* LR1 strains exhibited safety and technological performance compatible with probiotic properties[4, 5].

This study assessed the proteolytic activity of these lactobacilli strains and their ability to release bioactive peptides capable of exerting antioxidative and ACE-inhibitory *in vitro* properties during incubation at 37°C in reconstituted skim milk (RSM).

2. Materials and Methods

2.1. Fermented milk sample preparation

*Lactobacillus helveticus*NK1 (GenBank: MT448799), *Lactobacillusrhamnosus* F(GenBank: MN994629), and *Lactobacillusreuteri*LR1(GenBank: MN994628)strainswere obtained from the Microorganism Collectionof the All-Russia Research Institute of the DairyIndustry (VNIMI, Moscow,Russia).

One liter of reconstitutedskim milk (RSM) was prepared by addingappropriate amount of skim milk powder towater, followed by stirring for full dispersionand heat treatment at 85°C for 30 min. The heat-treatedmilk was then cooled to approximately 40°C. Thesterile RSM was aseptically inoculated with 1% (vol/vol) of each activated strain (approximately 10^7 cells/mL) and incubated at 37°C for 96 h for growth.Samples were taken for analysis at 0, 6, 24,48,72and96h during incubation and the change in pH was measured at each time point.

Cell population of each lactobacilli strain was determined by counting of colony-forming units (CFU) on MRS agar after incubation at 37±1 °C for 48–72 h under anaerobic conditions using anaerobic kits.

2.2. Characterization of fermented milk samples

2.2.1. Preparation of water soluble extracts (WSE)

After fermentation, the pH of each sample was adjustedto 4.6 (if the pH of the fermented milk was above 4.6) by adding 0.75% TCA, and the sample was centrifuged at 10 000×g

for 20 min (4°C).The supernatant was filtered through a 0.45 μm syringe filterand stored at −80 °C until further analysis.

The protein content of WSE was determined usingthe PierceBSAProtein AssayKit (ThermoFisher, USA).

2.2.2. Proteolytic activity assay

The proteolytic activity was quantified by the measurement of the amount of released amino groups in WSE samples using 2,4,6-trinitrobenzenesulfonic acid solution (TNBS, Sigma-Aldrich, St. Louis, MO, USA) methodand D340 was measured using Synergy 2 microplate photometer–fluorometer (BioTek, Winooski, VT, USA). A calibration curve was prepared using L-leucine (L-Leu) as standard (0.1–2.0 mmol/L). The results were expressed as mmol/L of L-Leu equivalents.

2.2.3. Antioxidant activity assay

The *in vitro* antioxidant activity in WSEsamples was determined by the Oxygen Radical Absorbance Capacity fluorescence method (ORAC)using a Synergy 2 microplate photometer–fluorometeras described in[6].The peroxyl radical was generated directly in the reaction medium during the thermal decomposition of the azo compound 2,20-azobis (2-methylpropionamidine) dihydrochloride (AAPH, Sigma-Aldrich, St. Louis, MO, USA), initiated by incubation at 37 °C for 10 min. The antioxidant activity was expressed as the amount of Trolox (Sigma-Aldrich, St. Louis, MO, USA) molar equivalents (TE, μM).

2.2.4. ACE-I assay

Angiotensin converting enzyme inhibitory activity (ACE-I) in WSE samples was determined by their ability to inhibit angiotensin I-converting enzyme (Sigma-Aldrich, St. Louis, MO, USA). o-Aminobenzoyl-Phe-Arg-Lys(dinitrophenyl)-Pro (Sigma-Aldrich, St. Louis, MO, USA) was used as a substrate with internal fluorescence quenching as described in[6]. The 96-well, black, nonbinding polypropylene microplates (Greiner Bio One, Germany) were used. The measurements were carried out on a Synergy 2 microplate photometer–fluorometer. The concentration IC_{50} was determined at which ACE activity decreased by 50%. IC_{50} was expressed as mg of protein permL.

TABLE 1: Fermentation characteristics of different lactic acid bacteria (LAB): *Lactobacillus reuteri*(LrLR1), *Lactobacillusrhamnosus*(LrhF), and *Lactobacillus helveticus* (LhelNK1).

Fermentation time, (h)	pH			Cellnumbers (CFU×mL^{-1})		
	Lr LR1	Lrh F	Lhel NK1	Lr LR1	Lrh F	Lhel NK1
0	6.46	6.55	6.47	1.2×10^{7}	1.5×10^{8}	2.5×10^{8}
6	6.06	6.45	5.67	1.3×10^{7}	1.6×10^{8}	3.7×10^{8}
24	4.87	5.02	3.42	1.9×10^{7}	4.62×10^{8}	7.0×10^{8}
48	4.33	3.89	3.26	3×10^{9}	1.36×10^{9}	2.5×10^{9}
72	4.01	3.69	3.25	9.4×10^{8}	2.6×10^{9}	2.5×10^{9}
96	4.00	3.67	3.19	9.3×10^{8}	1.27×10^{9}	2.5×10^{9}

2.3. Peptide profile analysis

2.3.1. Identification of peptides

The peptide profile of the WSE samples was analyzedby liquid chromatography-tandem mass spectrometry (LC-MS/MS) in a system consisted of Agilent 1100 chromatograph (Agilent Technologies, United States) and LTQ-FT Ultra mass spectrometer (Thermo, Germany) as described in[6].

2.3.2. Heat map ofpeptidomics data construction from MS analysis

Heat maps were constructed forβ-, α_{S1}-, κ- and α_{S2}-casein peptides of bovine milk using Peptigram: a web-based application for peptidomics data visualization[7]. The color intensity is proportional to the summed ion intensities of peptides, with dark green indicating high peptide intensity and light green indicating low peptide intensity.

3. Results and Discussion

3.1. Lactobacillus fermentation activity

The fermentation capacity of *L.helveticus* NK1,*L. rhamnosus*Fand *L.reuteri* LR1strains was investigated after reconstituted skim milk (RSM) inoculation (Table 1).

Overall, *L. helveticus*NK1strain showed a much faster growth than *L. rhamnosus*Fand *L.reuteri* LR1.As expected, this high bacterial growth resulted in astrong acidification of the medium, which was higher than that for samples inoculatedwith *L. rhamnosus*Fand *L.reuteri* LR1 strains.Lactobacillus strains displayed distinct acidification capabilities. *L. helveticus*NK1strain showed a pH decrease of ∼3 pH units, reaching a mean of 3.42

TABLE 2: Antioxidant, ACE-land proteolytic activities ofmilk samplesfermented by *Lactobacillus reuteri*(LrLR1), *Lactobacillusrhamnosus*(LrhF), and *Lactobacillus helveticus* (LhelNK1).

Ferme time, (h)	ORAC, μM TE/mL			ACE-I (IC_{50})*			L-Leu equivalents,mmol/L		
	Lr LR1	Lrh F	Lhel NK1	Lr LR1	Lrh F	LhelNK1	Lr LR1	Lrh F	Lhel NK1
Milk	206.0–218.0			26.2			7.9–8.1		
24	422.7	750.8	721. 4	12.6	0.95	0.6	3.7	6.0	12.2
48	414.6	828.7	898.3	11.2	0.71	0.29	3.9	8.4	14.1
72	400.6	854.7	1045.5	7.1	0.21	0.18	4.1	10.9	14.6
96	797.3	991.3	1447.0	1.6	0.18	0.15	8.5	11.1	14.5

*IC_{50} is defined as the protein concentration (mg/mL) required inhibiting 50% ofACE activity.

± 0.08after 24 h of culture, whereas the pH of milk inoculated with *L. rhamnosus*Fand *L.reuteri* LR1decreased by a near of 2 pH units, reaching a mean of 5.02 ± 0.07 and 4.87 ± 0.05 respectively (Table 1).The acid production stopped around pH 4.0 during *L. reuteri* LR1 fermentation, whereas the more acid-tolerant Lactobacillus species continued acid production to a pH below 4 with the *L. helveticus* giving the lowest final pH (3.2–3.3).As expected, all milk fermentations led to milk protein coagulation because of casein precipitation at pH lower than 4.6[8]. The *L. helveticus*NK1 strain gave the fastest acidification to pH 4.6 (10–13 h), followed by the *L. rhamnosus*Fand*L. reuteri* LR1. Among the tested strains, *L. helveticus* NK1 was the most efficient for both bacterial growth and acidification capacity.

3.2. Proteolytic,antioxidant, and ACEinhibitory activities of Lactobacillus during milk fermentation

Strain growth and acidification capacities correlate with proteolytic activity(Table 1 and 2). In the same time a correlation between proteolytic activity and ACE inhibitory (ACE-I) activity was found with all strains: milk samples of 96 hours fermentation exhibited the highest proteolytic and ACE-I activities (Table1 and 2).A similartrend in correlation between ACE-I activity and proteolysis degreewas reported by[9] forfermented milk produced by Lactobacillus strains.

L.helveticus NK1 showed the highest proteolytic and ACE-I activities compared with*L. rhamnosus*F and *L.reuteri*LR1strains (Table 2). At the end of fermentation, strain *L.helveticus* NK1 showed value of leucine equivalents of 14.5-14.6 mmol/L, whereas *L. rhamnosus* F and *L. reuteri*LR1– about 11.0 and 8.5mmol/L respectively (Table 2).

In *in vitro* experiments, fermentation of RSM with *L.helveticus* NK1 and *L. rhamnosus*Fstrains produced intense ACE-Iactivity whereas *L.reuteri* LR1 strain was less

efficient(Table 2).*L.reuteri*LR1 strain slowly produced ACE inhibitory compounds reachinghigheractivity after 96 h of cultivation. *L.helveticus* NK1 and *L. rhamnosus* Fshowed the ratherhigh ACE-I activity after 24 and 48 h fermentation respectively.

The development of antioxidant activity correlated to bacterial growth and proteolysis degree for *L. helveticus* NK1 and *L. rhamnosus*F strains whereas no correlation was found for*L. reuteri* LR1 strain during 72 h fermentation. At the end of fermentation (96 h) the milk samples exhibited the highestantioxidantactivity, whichwas slightly higher for strain*L. helveticus* NK1 thanfor*L. rhamnosus*F (1447 versus991 μM×mL^{-1} of Trolox, respectively).*L. reuteri* LR1 strain had the lowest antioxidantactivity of 797 μM/mL of Trolox.

*L.helveticus*NK1 was the most proteolytic among the Lactobacillus studied in this work, and it also showed the highestantioxidantand ACE-I activities*in vitro*. *L. helveticus* is considered as the species displaying the highest proteolytic activity among all Lactobacillus[2].Milk fermentation with different*L. helveticus* strains showed IC_{50} values of 0.16–1.1 mg/mL [10]whichis in line with the results obtained in this study (0.15–0.6 mg/mL).The other LAB showing a significant proteolytic, antioxidantand ACE-I activities in our research was *L. rhamnosus*strain F.

3.3. Peptideprofile

Figure 1 shows the peptide profile of three lactobacilli strains after 24 h fermentation on RSM. In such type of graphical representation, all peptides identified are repositioned (multiple green bars) on the β-, α_{S1}-, α_{S2}- and κ-casein sequence, clearly illustrating the proteolysis patterns of casein fractions. The repartition of peptides on the backbone of casein fractions defines a specific proteolysis signature. For the *L. helveticus* NK1, *L. rhamnosus*F, and *L. reuteri* LR1 strains these signatures were clearly different: the preferential areas of cleavage (dark green regions on the heat maps) were not situated in the same regions of the protein. Differences in proteolysis signatureswere associated with the cell wall-associated proteinase (CEP) profiles of LAB. CEPhave differences regardingcleavage sites specificities in different Lactobacillus strains. Most frequently, LAB possess only one CEP, but the presenceof two and more CEPs has been described in*L. helveticus*[2].However, in general, they cleave more efficiently the β- and α_{S1}-caseins, and to a lesser extent, the α_{S2} and κ fractions [11].

Peptideproductioncorrelatedwithstrain growth, acidification capacity and proteolytic activity. The total peptide amount released by *L. helveticus* NK1 whole cellswas significantly higher than that released by *L. rhamnosus*F и*L. reuteri* LR1 (Figure 1), which

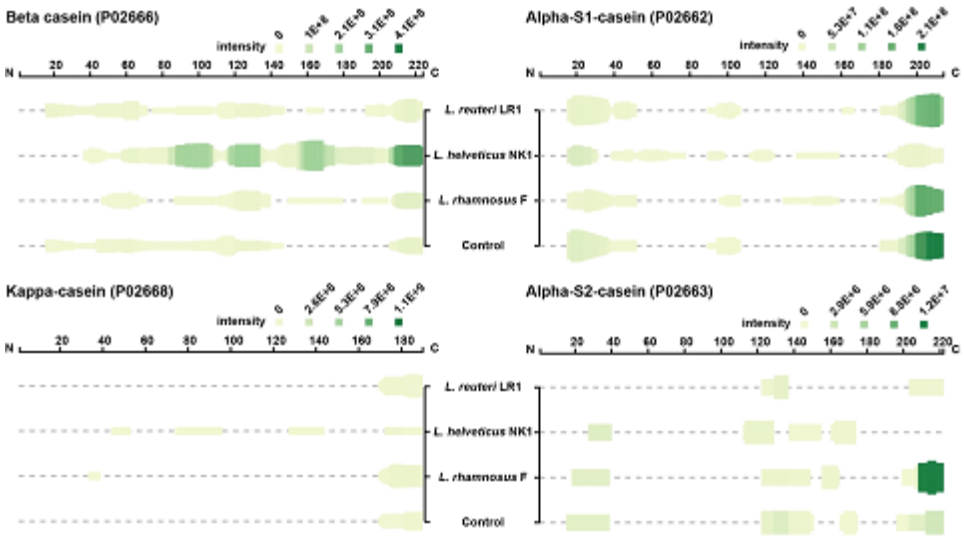

Figure 1: Heat maps of peptide profiles for Lactobacillus strains.

corresponds to the highest value of proteolytic activity – 12.2 mmol/LL-Leu equivalents versus 6.0 and 3.7 mmol/Lfor *L. rhamnosus*F и*L. reuteri* LR1.

The identified bioactive peptide composition of the milk samplesfermented by *L.helveticus*NK1, *L. rhamnosus*F, and *L.reuteri* LR1confirms their biological activities*in vitro* assay.By searching on the databases, allof the identified peptides in our study have previously beendescribed as ACE inhibitory, antioxidant ormulti-function peptides and their biological activities are summarized in Table 3.

As can be seen from datapresented in Table 2, *L.helveticus*NK1 exhibited higher ACE-I activity comparedwith other Lactobacillus during the 24 h fermentation. This finding can be probablyattributed to the high content ofidentifiedantihypertensive peptides (Table 3).*L. helveticus*strains are themost capable of producing bioactive peptides, including ACE-I peptides[2].Among the peptides with ACE-I activity, YPFPGPIPN (found in WSE of *L.reuteri*LR1),KVLPVPQ (found in WSE of *L.helveticus*NK1) and RPKHPIKHQ (found in WSE of *L.reuteri*LR1 and *L.helveticus*NK1) showed *in vivo* antihypertensiveactivity in spontaneously hypertensive rat.

4. Conclusion

The ability of *L.helveticus*NK1,*L. rhamnosus*F and *L.reuteri*LR1 to produce antioxidantand ACE inhibitory peptides during milk fermentation was found to be a strain specific characteristic which was closely connectedto the bacterial growth and proteolysis. *L. helveticus* was the most proteolytic among the LAB studied in this work, and it

TABLE 3: Bioactive peptides identified in milk samples fermented by *Lactobacillus* spp. strains after 24 h milk fermentation.

Peptide	Protein fragment	Relativeamount			Activity	Ref.
		Lr LR1	Lrh F	Lhel NK1		
DKIHPF	β-CN f(47-52)	nd	1.34×10^{6}	3.33×10^{6}	ACE-I /Antioxidant	[12]
VVPPFLQPE	β-CN f(83-91)	nd	nd	1.44×10^{6}	ACE-I	[13]
YPFPGPIPN	β-CN f(60-68)	2.77×10^{5}	nd	nd	ACE-I	[14]
NIPPLTQTPV	β-CN f(73-82)	nd	nd	6.56×10^{6}	ACE-I	[12]
TQTPVVVPPFLQPE	β-CN f(78-91)	nd	nd	1.25×10^{6}	Antioxidant	[15]
DVENLHLPLPLLQSWM	β-CNf(129-144)	nd	nd	8.21×10^{5}	ACE-I	[16]
LHLPLPLLQSW	β-CN f(133-143)	nd	nd	1.29×10^{5}	ACE-I	[12, 13]
MHQPHQPLPPT	β-CNf(144-154)	nd	nd	2.71×10^{7}	Antirotaviral	[15]
SLSQSKVLPVPQK	β-CNf(164-176)	nd	nd	9.27×10^{6}	Antioxidant	[15]
KVLPVPQ	β-CNf(169-175)	nd	nd	2.17×10^{5}	ACE-I	[17]
LLYQEPVLGPVRGPFPIIV	β-CN f(191-209)	8.52×10^{5}	nd	1.28×10^{8}	ACE-I /Antioxidant Anticancer Immunomodulatory Antithrombin Antimicrobial	[15]
YQEPVLGPVRGPFP	β-CN f(193-206)	3.42×10^{6}	1.73×10^{5}	nd	ACE-I	[15]
YQEPVLGPVRGPFPIIV	β-CN f(193-209)	5.76×10^{6}	4.61×10^{7}	4.2×10^{6}	Immunomodulatory Antimicrobial	[15]
QEPVLGPVRGPFPIIV	β-CN f(194-209)	1.8×10^{6}	3.04×10^{7}	1.13×10^{8}	ACE-I /Antioxidant	[15]
EPVLGPVRGPFP	β-CN f(195-206)	4.17×10^{5}	nd	nd	ACE-I	[18]
GPVRGPFPIIV	β-CN f(199–209)	7.49×10^{6}	2.66×10^{4}	2.42×10^{7}	ACE-I	[13]
RPKHPIKHQ	α_{S1}-CN f(1-9)	4.09×10^{4}	nd	6.06×10^{6}	ACE-I	[14]
EVLNENLLRF	α_{S1}-CN f(14-23)	1.46×10^{5}	nd	nd	ACE-I	[15]
FVAPFPEVFGKE	α_{S1}-CN f(24-35)	nd	nd	3.72×10^{5}	ACE-I /Antioxidant	[13]
VAPFPEVFGKE	α_{S1}-CN f(25-35)	nd	nd	4.45×10^{5}	ACE-I	[15]
LYQGPIVLNPWDQVK	α_{S2}-CN f(99-113)	nd	nd	3.9×10^{5}	ACE-I	[15]
NAVPITPT	α_{S2}-CN f(115-122)	5.81×10^{5}	nd	nd	ACE-I	[15]
KYIPIQYVL	κ-CN f(30-38)	nd	nd	4.47×10^{5}	Antioxidant	[16]
VQVTSTAV	κ-CN f(162–169)	3.15×10^{5}	nd	nd	ACE-I	[13]

also showed the highest antioxidant and ACE-inhibitory activities*in vitro*. *L. reuteri* LR1 probiotic strain in our collection displayed only moderate proteolytic activities.

Finally, since *L.helveticus*NK1,*L. rhamnosus*F and *L.reuteri*LR1 strains released several potential bioactive peptides, they could be promisingfunctional starters or adjunct cultures for formulating dairyproducts with health promoting properties.

5. FUNDING

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Abbreviationsare: β-CN –β-casein; α_{S1}-CN –α_{S1}-casein; α_{S2}-CN –α_{S2}-casein; κ-CN – κ-casein.

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