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EXPERIMENTAL ARTICLES

Structural Characterization of the Extracellular Peptide Metabolites of *Luteococcus japonicus* subsp. *casei* and Their Protective Effect on Probiotic Bacteria

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Abstract—Protective effect of *Luteococcus japonicus* subsp. *casei* exometabolites on the cells of transient probiotic *Propionibacterium* strains, *Enterococcus faecium*, and the yeasts *Saccharomyces cerevisiae* Boulardii under exposure to bile salts (BS) and acid stress was studied. The extracellular peptide reactivating factor (RF) and the peptide component of the culture liquid (CL) after RF removal possessed a protective effect. Protective (preventive) and reactivating (after stress impact) application of RF resulted in 1.5- to 2.0-fold increased survival of the human transient probiotics *P. freudenreichii, P. acidipropionici* and *E. faecium* subjected to BS treatment of acid stress. The CL peptide fraction had a stronger protective effect. Its application for preincubation of *P. acidipropionici* cells resulted in 14-fold (BS treatment) and 8-fold (acid stress) increased survival compared to the control. Yeasts exhibited very high resistance to the stress factors used. Two active glycopeptide fractions with molecular masses of 1.8 and 2.4 kDa were found in RF. Analysis of their amino acid composition revealed the residues of glycine, leucine, proline, arginine, and aspartate/asparagine, while no residues of aromatic and sulfur-containing amino acids, or the disulfide bridge-type posttranslational modifications, were found. The possible mechanisms of reactivating and the protective effect of RF are discussed.

Keywords: microbial extracellular metabolites, stress, protective and reactivating action, transient bacteria, probiotics, prebiotics

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A human body is known to contain ~10^{14} symbiotic microbial cells, with ~1 kg of bacteria belonging to over 500 species present in the gastrointestinal tract (GIT) [1]. The complex of all microbiocenoses in a human or animal organism is considered as an extracorporal organ, the number of cells in which is 10 to 100 times higher than the total cell number in all tissues and organs of the host organism [2]. Disorders in intestinal microbiocenoses are closely associated with organic and functional pathology not only of GIT, but also of other tissues and organs of the organism. In the case of impaired microbiocenosis, probiotics are usually applied either orally in lyophilized form or as components of dairy and some other products. However, after oral introduction, viable probiotic microorganisms in the GIT of experimental animals constitute only 0.001% of their initial number [3]. The reasons for poor survival of probiotic bacteria during their transition through GIT include the detrimental effect of an aggressive environment, such as a high acidity of gastric juice which is followed by the alkaline medium of the small intestine, the presence of bile, enzymes, immunoglobulins, increased osmotic pressure, nutrient limitation, etc.

Dysbioses may be prevented by not only peroral introduction of probiotics but also by regeneration of intrinsic microflora. This approach is based on the assumption that metabolites of bacteria play an even more important role in efficient recovery of natural microflora than bacterial cells, although some metabolites can inhibit this process [4].

Prebiotics include materials of different origin and structure (dietary fibers, oligosaccharides, polysaccharides, monosaccharides, alcohols, peptides, some vitamins with antioxidant properties, and a number of other compounds), which have a selective effect on the development of beneficial microbiota. Their ability to regulate pH and redox potential and serve as receptors of "friendly" microorganisms or as the receptor inhibitors of undesirable microorganisms in certain GIT ecological niches may be favorable for the host health

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[2]. The study of such compounds is of great theoretical and practical interest.

From the culture liquid (CL) of *L. casei*, the peptide reactivating factor (RF) had been earlier isolated, which showed both reactivating and protective effects on bacteria, archaea, and lower and higher eukaryotes subjected to the lethal stress impact [5]. We revealed that RF was a complex containing two active peptide fractions with glycoside bonds [6]. However, CL of *L. casei* was found to contain also another active peptide fraction which showed a stress-protective effect on the cells.

Both RF and the peptide fraction (isolated from CL after the RF removal) increased resistance of the resident microflora (lactic acid bacteria and bifidobacteria) to bile salts (BS), acidity, and lyophilization [7]. Bifidobacteria and lactobacilli belong to typical probiotics, based on the strains isolated from human intestines where they dominated from the first days of the host life. Probiotic properties (albeit transient ones) were also revealed in other representatives of the normal human microflora inhabiting not only GIT, but also fermented milk foods and some other natural substrates.

Transient probiotics are characterized by the colonization resistance; they carry out immunogenic and antimutagenic functions, promote selective reproduction of representatives of the normal microflora, and inhibit growth and the adhesion capability of potentially pathogenic microorganisms. Transient probiotics include representatives of the genera *Enterococcus, Propionibacterium, Saccharomyces, Streptococcus, Lactococcus*, and *Leiconostoc*, of which the first three genera are the most important.

Propionic acid bacteria (PAB), which have been used in the food industry and for production of vitamins and fodder [8, 9], are now considered as promising probiotics [10]. The PAB due to a complex of probiotic properties represent an alternative to typical probiotics (bifidobacteria), although their adhesion in the GIT is as low as 0.2–0.6% of the introduced bacteria, whereas the adhesion level of lactobacilli and bifidobacteria is considerably higher (from 1.3 to 24%) [12]. However, it was shown that adhesion of PAB in the intestinal tract (IT) could be considerably increased by their preadhesion on the cells of probiotic strains of lactobacilli and bifidobacteria [13].

The tolerance of PAB to digestive stresses determines the efficiency of their application as living probiotics. Preadaptation of *P. freudenreichii* to bile salts [13] and acidity [14] increased their resistance to these factors, which was accompanied by synthesis of adaptive proteins [13, 14]; the capability of PAB for adaptation was strain-dependent.

Among enterobacteria inhabiting human and animal digestive tracts, *E. faecium* and *E. faecalis* occur the most frequently [2]. Clinical and probiotic strains of *E. faecium* are known [15]. All strains of *E. faecium* in probiotic products are resistant to vancomycin and produce no toxins [16]. *E. faecium* is applied in pediatrics as a component of various probiotic preparations [17], including its combinations with lactobacilli [18]. Many strains of *E. faecium* exhibit antimutagenic activity [19]. The cells of *E. faecium* isolated from cow's milk produce a great deal of acids; they are highly resistant to acidic stress and BS [20] and carry out barrier functions in the intestinal mucus layer due to their own adhesion, as well as to inhibition of adhesion of enteropathogenic bacteria [21]. Thus, it can be stated that bacteria *E. faecium* possess pronounced probiotic properties.

Among yeasts of the genus *Saccharomyces*, only strains of *S. cerevisiae* Boulardii belong to probiotics; they inhibited growth of *Vibrio cholerae, Escherichia coli, Salmonella typhi*, and *Clostridium difficile* [22]. Yeasts promoted growth and survival of probiotic bacteria due to production of peptides, amino acids, and vitamins [23]; they increased stability and the life duration of probiotic bacteria in fermented milk and decreased the risk and duration of antibiotic-dependent diarrhea [18].

The goal of the present work was to carry out structural analysis of active fractions of RF isolated from *L. casei* and to study protective effects of both RF fractions and the CL peptide fraction on transient probiotic microorganisms, namely bacteria of the genus *Propionibacterium* and *Enterococcus faecium* and yeasts *Saccharomyces cerevisiae* Boulardii.

MATERIALS AND METHODS

Microorganisms and their cultivation. Propionic acid bacteria *Propionibacterium freudereichii* 1857, *P. acidipropionici* 1859, *P. acnes* 3322, *P. technicum* 1864, *P. thoenii* 1865, and *P. globosum* 3323 were obtained from the Culture Collection of the Czech Republic (CCM); *Enterococcus faecium* M Z185 was obtained from the All-Russian Collection of Microorganisms (VKM); *Bifidobacterium bifidum* KM-23 and yeasts *Saccharomyces cerevisiae* Boulardii KM-13 were obtained from the culture collection of the Department of Microbiology, Moscow State University.

Bacteria *Luteococcus japonicus* subsp. *casei* were grown under static conditions in 100-mL flasks at 32°C in glucose–mineral medium containing the following (%): glucose, 1.0; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.1; Na_2HPO_4, 0.2; $MgSO_4$, 0.002; $CaCl_2$, 0.002; NaCl, 0.002; yeast extract, 0.1; pH 7.0 was maintained by addition of a 5% solution of NaOH. Reactivating factor (RF) was isolated from the culture liquid of *L. casei*.

Propionic acid bacteria as a test object were grown to the late exponential phase under static conditions in 10-mL tubes (48 h, 32°C) in the medium containing

the following (%): corn steep, 2.0; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.1; Na_2HPO_4, 0.2; $CoCl_2$, 1 mg/L; pH 7.2.

E. faecium was grown under static conditions in tubes with nutrient broth (Himedia, India) at 35°C.

Yeasts as a test object were cultivated aerobically to the end of the exponential growth phase in 500-mL flasks with 100 mL of brewing wort (4° Balling, pH 6.5) on a shaker (200 rpm) at 28°C.

The cells of test microorganisms were separated from the medium by centrifugation (10000 *g*, 20 min), washed with physiological saline, and then resuspended in saline up to optical density (OD) of 0.4–0.5 U as determined in a FEK-56 PM photoelectrocolorimeter (Russia); (2-cm cuvette; λ = 600 nm). The obtained cell suspensions were used as text objects in the studies on the protection of probiotic cells from the stress impact.

Determination of the stress-protective effect. The following stress factors were used: (1) bile salts (sodium cholate and sodium deoxycholate) and (2) superoptimal acidification of medium. In the control variants, 1 mL of cell suspension of test strains in saline was incubated with an equal volume of (1) BS solution with the end concentration of 2 g/L (pH 7.0) for 30 min at 33°C or (2) 0.1 M citrate–phosphate buffer (pH 2.0) for 15 min. In experimental variants, to study the protective effect, 1 mL of RF solution or CL was added to 1 mL of cell suspension of the test strain 10 min before the stress impact; then BS solution or citrate–phosphate buffer (2 mL of each) was added, and the mixture was incubated as indicated above. To determine the reactivating effect, 1 mL of cell suspension was supplemented with 1 mL of citrate–phosphate buffer or 1 mL of BS solution, and the mixture was incubated at 33°C for 15 or 30 min, respectively; then RF or CL solution (2 mL of each) was added, and the mixture was incubated at the same temperature for 15 min. The number of viable cells was determined by the number of colony-forming units (CFU/mL) after plating 5 μL of the serial tenfold dilutions of each sample (in six replicates for each dilution) onto Petri dishes with agar medium.

In experiments on the study of stress impact on propionic acid bacteria, the cell number was determined on solid media containing 1.5–2.0% agar and 1% $CaCO_3$. The inoculated plates were incubated under anaerobic conditions in a Genbox 96124 thermostat (bioMerieux, France) for 7 days.

The efficiency of protective or reactivating effects of the *L. casei* extracellular metabolites was expressed as a division index (DI) of test objects and evaluated as a ratio of the CFU titer of cell suspensions incubated with RF or with the peptide fraction of CL before (protective effect) or after (reactivating effect) the stress impact to the CFU titer of the control cell suspension exposed to the stress impact.

Isolation of the fractions of stress-protective extracellular metabolites from *L. casei*. To obtain RF, the *L. casei* culture grown to the end of the exponential phase (48 h) was centrifuged (10000 *g*, 20 min), and the stress-protective metabolites were isolated from the supernatant by the method [7]: 100 mL of CL was passed through a 0.22-μm nitrocellulose membrane filter (Millipore, United States); the adsorbed components were eluted with 3 mL of a 3% solution of NaCl and filtered through a membrane filter with low affinity for protein (Pall, United States) for complete cell removal. The obtained peptide solution was indicated as RF and used as the antistress agent.

It was found that CL remaining after the RF removal showed protective and reactivating effects, which were considerably decreased after its treatment with 2 mg/mL of trypsin (Merck, Germany) for 60 min at 37°C. This fact indirectly evidenced the peptide nature of the active metabolites, which are further referred to as the peptide fraction of CL. The absence of bacterial cells in the preparation was controlled by plating of the sample onto agar medium.

Determination of bactericidal activity of extracellular stress-protective peptide metabolites of *L. casei*. Test culture and CL (1 mL of each) were applied into test tubes with 10 mL of appropriate medium. In the control, 1 mL of saline was added instead of CL. Test cultures were grown for 48 h at 32°C. Bactericidal effect of CL was determined from a change in optical density (OD) of cell suspension measured on a FEK-56 PM photoelectrocolorimeter (Russia) (2-cm cuvette; $\lambda = 600$ nm).

Structural analysis of the RF active fractions. The RF was isolated from 135 L of CL. Two active fractions of RF were obtained by a combination of methods of liquid chromatography [6].

Structural analysis of active RF fractions was carried out by the MALDI MS TOF/TOF method of tandem mass spectrometry in the regime of positive ions with the use of the laser high-energy ionization. Molecular masses of the peptides were measured with a Ultraflex mass spectrometer (Bruker Daltonics, Germany) equipped with a 337-nm UV laser using 2,5-dihydroxybenzoic acid as a matrix. Equal volumes (0.7 μL) of the sample and matrix (15 mg of matrix per 1 mL of 80% solution of CH_3CN supplemented with 0.1% solution of trifluoroacetic acid (TFA) in MQ water) were mixed on a target. The mixture was applied by a drop method with an automatic dispenser on a steel target plate and air-dried. Mass spectra were analyzed using the Bruker DataAnalysis for TOF software package. The error of these measurements was 0.015%.

To analyze amino acid composition, the fractions were hydrolyzed in a mixture of concentrated hydrochloric acid and propionic acid (1 : 1, vol/vol) at 140°C under vacuum for 2.5 h; the hydrolysate was evaporated on a vacuum concentrator; then water was added to the hydrolysate, and it was repeatedly evapo-

rated. Amino acid analysis was performed on a LC3000 analyzer (Biotronik, Germany).

For peptide reduction and alkylation, about 1 nmol of peptides was diluted in 35 μL of solution containing 6 M guanidine hydrochloride, 3 mM EDTA, and 0.5 M Tris-HCl buffer (pH 7.8); then 4 μL of 1.4 M solution of dithioerythritol (DTET) in 2-propanol was added, and the reaction mixture was sparged with argon for 2 min. Reduction of the peptides was carried out at 40°C for 4 h, then the reaction mixture was supplemented with 4 μL of a 50% (vol/vol) solution of 4-vinylpyridine in 2-propanol and incubated in the dark for 20 min. Finally, the incubation mixture was diluted with 40 μL of 0.1% TFA, and the products were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Luna C_{18} column (4.0 × 150 mm) using linear gradient of solvent B (80% CH_3CN and 0.1% TFA) from 10 to 50% for 40 min at a flow rate of 0.75 mL/min. Peptides were detected at 214 nm. To determine the presence of free SH groups, the peptides were alkylated without preliminary reduction.

Statistics. The paper presents the mean arithmetic values and their standard deviations from three independent experiments.

RESULTS

Characteristics of active fractions of RF. Two active fractions with reactivating properties have been earlier isolated from RF of *L. casei* by combination of the liquid chromatography methods [6]. Fractions 12 and 25 were obtained by cation-exchange/reverse-phase and anion-exchange/reverse-phase chromatography, respectively. In this study, by using method of MALDI MS TOF/TOF tandem mass spectrometry in a regime of positive ions, monoisotope masses of fractions 12 and 25 were found to be 1.8 and 2.4 kDa, respectively, and their standard isotope distribution was revealed. Amino acid analysis revealed in these fractions residues of glycine, leucine, proline, arginine, and asparagine/aspartate, whereas sulfur-containing cysteine residues were absent. Alkylation of the molecules without preliminary addition of reducing agent (DTET), as well as after preliminary reduction, showed no increase in molecular masses, which also indicated the absence of Cys residues in the molecule structure.

Thus, the molecules probably had unordered or spiral (possibly with polyproline helix) spatial conformation without posttranslational modifications of the type of the disulfide bridge formation. The application of the MALDI MS TOF/TOF method using high-energy laser ionization revealed a decrease in molecular masses, which was indicative of the presence of glycoside bonds. Based on a difference in the masses of molecular ions, the presence of carbohydrate residues (hexose and fructose) was identified.

By using tandem mass spectrometry for both compounds, a large number of derived fragments formed as a result of hydrolysis of labile peptide bonds (e.g., Asp-Pro) were obtained. For both fractions (using small volumes), absorption spectra in the UV range of 200–340 nm were recorded, with the maximum at less than 230 nm, whereas no absorption was revealed in a range of 260–340 nm. The obtained results indicate that these structures contain no residues of aromatic amino acids (phenylalanine, tyrosine, or tryptophan).

Thus, it can be concluded that active fractions of RF are glycopeptides with molecular masses of 1.8 and 2.4 kDa, which are composed of residues of glycine, leucine, proline, arginine, and asparagine/aspartate but contain no residues of aromatic amino acids and cysteine; they form no posttranslational modifications of the type of the disulfide bridge formation.

Bactericidal effect of the *L. casei* exometabolites. It was found that peptide exometabolites (RF and peptide fraction of CL) produced by strain of *L. casei* and belonging to the family *Propionibacteriacea* contained no bacteriocins since they showed no bactericidal effects towards either bifido- and lactobacteria or representatives of various species of propionic acid bacteria, such as *P. acnes, P. freudenreichii, P. globosum, P. technicum, P. acidipropionici*, and *P. thoenii*. Thus, it can be concluded that RF and peptide fraction of CL are not bacteriocidal.

Protective and reactivating effects of the *L. casei* exometabolites. The effects of RF or CL (remaining after the RF removal) on the transient probiotic bacteria as the subjects for the GIT stress impact were studied. To determine the protective or reactivating effects of metabolites 10 min before or 15–20 min after exposure to the stress impact, cell suspensions of test microorganisms were incubated with equal volumes of solutions of RF or the CL peptide fraction.

It was found that both RF and the CL peptides showed a protective effect on probiotic propionic acid bacteria (PAB), which were used as models, in the presence of bile salts (BS) (Table 1). The survival of *P. freudenreichii* and *P. acidipropionici* cells was increased twofold after their preincubation with RF.

It should be noted that the peptide fraction of CL was more active than RF; preincubation with it increased survival of *P. acidipropionici* cells 14-fold. The initial cultivation medium had no protective effect.

Exometabolites of *L. casei* showed a similar protective effect on bacterial cells in the case of acidic stress (Table 2). In experiments with *P. acidipropionici*, the CL peptide fraction had a stronger protective effect (DI = 8.0) than RF. The protective effect of the CL peptides on *P. freudenreichii* cells was almost four times lower than that on *P. acidipropionici* cells, but still higher than that of RF (Table 2). The reactivating effect of RF on cells exposed to bile salts (Table 3) was similar to that in the case of the acid stress (Table 4).

Table 1. Protective effects of RF and the CL peptide fraction from *L. casei* on cells of propionic acid bacteria exposed to the bile salt mixture (final concentration, 2 g/L)

Experimental conditions	Viable cells, $\times 10^6$ CFU/mL	Survival, %	Division index (DI)
	P. freudenreichii		
Intact cells	130.0 ± 7.42	100	–
Cells preincubated with:			
NaCl and then treated with BS	0.014 ± 0.0015	0.011	1.0
RF and then treated with BS	0.032 ± 0.0017	0.025	2.3
	P. acnes		
Intact cells	260.0 ± 18.21	100	–
Cells were incubated:			
NaCl and then treated with BS	0.212 ± 0.0522	0.082	1.0
RF and then treated with BS	0.228 ± 0.0337	0.088	1.1
	P. acidipropionici		
Intact cells	100.0 ± 4.47	100	–
Cells were incubated:			
NaCl and then treated with BS	0.040 ± 0.0022	0.040	1.0
CL (without RF fraction) and then treated with BS	0.560 ± 0.0379	0.560	14.0
inactivated CL* (without RF fraction) and then treated with BS	0.267 ± 0.0221	0.267	6.7
RF and then treated with BS	0.064 ± 0.0036	0.064	2.1

* CL was preincubated with trypsin (2 mg/mL, 60 min, 37°C).

The reactivating effects of the CL peptides on *P. acidipropionici* cells exposed to BS or acidic stress (DI = 1.9 and 2.8, respectively) were stronger than those of RF (DI = 1.5 and 1.6, respectively).

Thus, high biological activity of the CL peptides was not determined by possible residual RF contamination, but was due to the action of other protective metabolites which also had peptide nature.

Preincubation of *P. acnes* (which usually inhabits human skin and animal rumen but not GIT) with RF resulted in a twofold increase in survival of the cells exposed to acidic stress but had no effect in the case of BS impact.

Our results confirm rather high resistance of *E. faecium* under lethal impact of both BS and increased acidity (Tables 5 and 6). Preincubation of *E. faecium* cells with RF before their exposure to acidic stress or BS increased cell survival by 1.6 and 2.1 times, respectively, whereas the reactivating effect of RF was less pronounced (Tables 5 and 6).

Yeasts *S. cerevisiae* Boulardii were extremely resistant to both BS and increased acidity; under a high-intensity impact of these factors, the number of survived cells was 10 and 30%, respectively (data not shown).

DISCUSSION

Inverse correlation between efficiency of the RF treatment and the level of cell survival after the stress impact has been shown earlier [24, 25]. Therefore, the RF effect is of particular importance in the case of stresses of lethal intensity, which we used in this study.

Transient probiotics, unlike the classical ones, were more resistant to BS and increased acidity; they exhibited a less pronounced response to protective and reactivating effects of the *L. casei* exometabolites. While postincubation and preincubation with RF of the cell suspension of a classical probiotic *Bifidobacterium bifidum* increased cell survival after BS treatment by 5.6 and 2.6 times, respectively [6], survival of transient bacteria under the same conditions was increased only by 2.3 and 1.6 times, respectively; reactivation of propionic acid bacteria and *E. faecium* was increased by 1.5 and 1.6 times, respectively. It is known that the cells of *S. cerevisiae* Boulardii and especially of *E. faecium* may be preserved in the GIT for a long time [2], unlike propionic acid bacteria, which possess unique probiotic properties but are unable to colonize the GIT. However, the results of this study provide a foundation for hope for intensification of these properties and increasing resistance of these probiotics to BS and acidity of the gastric juice. It should be noted that although PAB have been adapted to BS and acid-

Table 2. Protective effects of RF and the CL peptide fraction from *L. casei* on cells of propionic acid bacteria exposed to the acid stress

Experimental conditions	Viable cells, $\times 10^6$ CFU/mL	Survival, %	Division index (DI)
P. freudenreichii			
Intact cells	190.0 ± 8.65	100	–
Cells were incubated:			
NaCl and then treated with acid	0.084 ± 0.0031	0.044	1.0
CL (without RF fraction) and then treated with acid	0.144 ± 0.0063	0.076	2.0
RF and then treated with acid	0.124 ± 0.0073	0.065	1.5
P. acnes			
Intact cells	80.0 ± 5.48	100	–
Cells were incubated:			
NaCl and then treated with acid	0.020 ± 0.0018	0.025	1.0
RF and then treated with acid	0.040 ± 0.0028	0.050	2.0
P. acidipropionici			
Intact cells	150.0 ± 7.42	100	–
Cells were incubated:			
NaCl and then treated with acid	0.020 ± 0.0021	0.013	1.0
CL (without RF fraction) and then treated with acid	0.164 ± 0.0286	0.109	8.2
RF and then treated with acid	0.040 ± 0.0033	0.026	2.0

Final pH of the mixture of the cell suspension and citrate–phosphate buffer (1 : 1, vol/vol) was 2.0.

ity [9, 13, 14], probably due to development of more resistant intrapopulation phase variants, the acquired properties could be lost as a result of reversion to the dominant phenotype.

We applied another approach, namely protection and, especially importantly, reactivation of PAB cells after the stress impact by using exometabolites of *L. casei*. According to modern medical concepts, restoring the proprietary probiotic organisms with low or even lost activity is more reasonable than introduction of novel bacteria, unknown to the host organism [4]. For this purpose, the CL peptide fraction and RF isolated from *L. casei* can be used; their reactivating and protective properties have been earlier demonstrated for organisms of different domains [5]; therefore, they may be considered as a new type of natural prebiotics.

Prebiotics are mainly represented by short-chain carbohydrates with the polymerization level of 2 and higher. The effect of probiotics on the cell growth depends on the prebiotic concentration; however, efficiency of prebiotics is diminished if they cannot entirely reach targets [26]. The RF and the CL peptide fraction are devoid of this shortcoming; they are rather stable and exhibit a protective effect on the studied probiotics before the latter enter the GIT and, therefore, preserve the cells from the stress impact. The cells of probiotics incubated with extremely low concentrations of RF (20–40 μg/mL) for 10 min acquire the capability for stress resistance and rehabilitation after the stress impact.

This work showed RF to include two active fractions of glycopeptides with molecular masses of 1.8 and 2.4 kDa, which contained no residues of sulfur-containing cysteine and aromatic amino acids (phenylalanine, tyrosine, or tryptophan). Interestingly, the absence of these amino acids is typical of bacterial flagella [27]. A lack of cysteine prevents the flagellum proteins from deaggregation in the case of a change in the ambient redox potential. The absence of sulfur-containing amino acids, which is possibly typical of the organelles and molecules directly contacting with the environment, may increase their stability. The RF was also rather stable and retained high activity under heating, UV irradiation, prolonged storage, and lyophilization [7, 28].

The patterns of the action of RF and of the CL peptide fraction open new possibilities for the technology of probiotic production, based on short-term preincubation of the probiotics in weak solutions of RF and CL in order to increase cell resistance to the GIT stresses.

Table 3. Reactivating effects of RF and the CL peptide fraction from *L. casei* on cells of propionic acid bacteria inactivated by the mixture of bile salts (2 g/L)

Experimental conditions	Viable cells, $\times 10^6$ CFU/mL	Survival, %	Division index (DI)
	P. freudenreichii		
Intact cells	160.0 ± 6.32	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.101 ± 0.0012	0.063	1.0
with RF	0.142 ± 0.0014	0.088	1.4
	P. acnes		
Intact cells	190.0 ± 16.28	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.178 ± 0.0262	0.094	1.0
with RF	0.180 ± 0.0301	0.094	1.0
	P. acidipropionici		
Intact cells	100.0 ± 4.47	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.032 ± 0.0016	0.032	1.0
with CL (without RF fraction)	0.060 ± 0.0032	0.060	1.9
with RF	0.048 ± 0.0038	0.048	1.5

Table 4. Reactivating effects of RF and the CL peptide fraction from *L. casei* on cells of propionic acid bacteria exposed to acidic stress. The final pH of the mixture of cell suspension and citrate–phosphate buffer (1 : 1, vol/vol) was 2.0

Experimental conditions	Viable cells, $\times 10^6$ CFU/mL	Survival, %	Division index (DI)
	P. freudenreichii		
Intact cells	190.0 ± 8.65	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.084 ± 0.0031	0.044	1.0
with CL (without RF fraction)	0.096 ± 0.0066	0.051	1.2
with RF	0.124 ± 0.0047	0.065	1.5
	P. acnes		
Intact cells	80.0 ± 5.48	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.020 ± 0.0029	0.025	1.0
with RF	0.028 ± 0.0038	0.035	1.4
	P. acidipropionici		
Intact cells	150.0 ± 7.42	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.020 ± 0.0031	0.013	1.0
with CL (without RF fraction)	0.056 ± 0.0046	0.037	2.8
with RF	0.032 ± 0.0037	0.021	1.6

Table 5. Protective and reactivating effects of RF from *L. casei* on the cells of *E. faecium* inactivated by a mixture of bile salts (2 g/L)

Experimental conditions	Viable cells, $\times 10^6$ CFU/mL	Survival, %	Division index (DI)
Protection			
Intact cells	60.0 ± 7.42	100	–
Cells were incubated:			
with NaCl and then treated with BS	0.030 ± 0.0039	0.050	1.0
with RF and then treated with BS	0.062 ± 0.0074	0.103	2.1
Reactivation			
Intact cells	60.0 ± 7.42	100	–
Cells were treated with BS and then incubated:			
with NaCl	0.036 ± 0.0051	0.060	1.0
with RF	0.056 ± 0.0064	0.093	1.6

Table 6. Protective and reactivating effects of RF from *L. casei* on the cells of *E. faecium* exposed to acidic stress. The final pH of the mixture of cell suspension and citric acid (1 : 1, vol/vol) was 2.0

Experimental conditions	Viable cells, $\times 10^6$ CFU/mL	Survival, %	Division index (DI)
Protection			
Intact cells	70.0 ± 6.33	100	–
Cells were incubated:			
with NaCl and then treated with acid	0.028 ± 0.0014	0.050	1.0
with RF and then treated with acid	0.044 ± 0.0030	0.103	1.6
Reactivation			
Intact cells	70.0 ± 6.33	100	–
Cells were treated with acid and then incubated:			
with NaCl	0.024 ± 0.0018	0.060	1.0
with RF	0.032 ± 0.0038	0.093	1.3

What is the mechanism of the RF action? The RF properties, such as a type of the concentration dependence, requirement for an intact barrier membrane, high rate of reactivation of the treated cells, and resistance to the stress impact [7, 25], indicate a "cascade" mechanism of signal transduction to the transcriptional level with involvement of two-component systems [29].

It has been shown earlier that RF exhibits an antioxidant activity by "quenching" the organic radicals, which differ considerably in their reactivity [6]. These properties of RF indicate its possible role in the regulation of the cell antioxidant status and redox-signaling in realizing its biological effects, considering that oxidative stress is one of the most universal responses of organisms to different stress factors [30]. The peptides with low molecular masses (containing 5–16 amino acid residues) were shown to possess antioxidant properties [31]; these peptides have a number of advantages over enzymatic antioxidants, such as more simple structure, higher stability, and absence of negative immune reactions [32].

The goal of our further investigations is to isolate and identify the active components from the CL peptide fraction of CL, which show pronounced protective and reactivating effects on probiotics.

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REFERENCES

1. Tannock, G.W., *Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology*, Gibson, G.R. and McFarlane, G.T., Eds., Boca Raton: CRC, 1995, pp. 257–271.
2. Shenderov, B.A., *Meditsinskaya mikrobiologiya, ekologiya i funktsional'noe pitanie* (Medical Microbiology, Ecology, and Functional Nutrition), Moscow: Grant'', 2001, vol. 3, pp. 1–284.
3. Darmov, I.V., Chicherin, I.Yu., and Pogorel'skii, I.P., Survival of probiotic microorganisms under in vitro conditions simulating the process of human digestion, in *Kishechnaya Mikroflora: Tezisy o Glavnom* (Intestinal Microflora: Major Theses), 2013, no. 2, pp. 1–11.
4. Gracheva, N.M., Correction and prophylaxis of dysbacteriosis. New approaches to treatment of gastrointestinal diseases, in *Kishechnaya Mikroflora: Tezisy o Glavnom* (Intestinal Microflora: Major Theses), 2013, pp. 12–19.
5. Vorobjeva, L.I., Khodzhaev, E.Yu., Novikova, T.M., Mulyukin, A.L., Chudinova, E.M., Kozlova, A.N., and El'-Registan, G.I., Stress-protective and cross action of the extracellular reactivating factor of the microorganisms of the domains *Bacteria, Archaea*, and *Eukaryota, Microbiology* (Moscow), 2013, vol. 82, no. 5, pp. 594–599.
6. Vorobjeva, L.I., Rogozhin, E.A., Khodzhaev, E.Yu., Nikolaev, I.V., and Tourova, T.P., Biological effect of extracellular peptide factor from *Luteococcus japonicus* subsp. *casei* on probiotic bacteria, *Appl. Biochem. Microbiol.*, 2015, vol. 51, no. 1, pp. 44–51.
7. Vorobjeva, L.I., Khodzhaev, E.Yu., Kharchenko, N.V., Novikova, T.M., and Cherdyntseva, T.A., Biological effect of extracellular peptide factor from *Luteococcus japonicus* subsp. *casei* on probiotic bacteria, *Appl. Biochem. Microbiol.*, 2014, vol. 50, no. 4, pp. 346–352.
8. Ho, P.H., Luo, J.B., and Adams, M.C., Lactobacilli and dairy propionibacteria with potential as biopreservatives against food fungi and yeast contamination, *Appl. Biochem. Microbiol.*, 2009, vol. 45, no. 4, pp. 414–418.
9. Thierry, A., Falentin, H., Deutsch, S.-M., Dalmasso, M., Cousin, F.S., and Yan, G., New insights into physiology and metabolism of *Propionibacterium* freudenreichii, *Int. J. Food Microbiol*. 2011, vol. 149, no. 1, pp. 19–27.
10. Vorobjeva, L.I., Khodjaev, E.Yu., and Vorobjeva, N.V., Propionic acid bacteria as probiotics, *Microb. Ecol. Health Disease*, 2008, vol. 20, no. 2, pp. 109–112.
11. Perez, A.C., Zarate, G., and Oliver, G., The probiotic properties of propionibacteria, *Le Lait*, 1999, vol. 79, no. 1, pp. 175–185.
12. Ouwehand, A.C., Suomalainen, N., Tolko, A., and Salminen, S., In vitro adhesion of propionic acid bacteria to human intestinal mucus, *Le Lait*, 2002. vol. 82, no. 1, pp. 123–144.
13. Leverrier, P., Dimova, D., Pichereau, V., Aufray, Y., Boyaval, P., and Jan, G., Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii* and proteomic analysis, *Appl. Environ. Microbiol.*, 2003, vol. 69, no. 7, pp. 3809–3818.
14. Jan, G., Leverrier, P., Pichereau, V., and Boyaval, P., Changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii, Appl. Environ. Microbiol.*, 2001, vol. 67, no. 5, pp. 2029–2036.
15. Kim, E.B. and Marco, M.L., Non clinical and clinical *Enterococcus faecium* strains, but not *Enterococcus faecalis* strains, have distinct structural and functional genomic features, *Appl. Environ. Microbiol.*, 2014, vol. 80, no. 1, pp. 154–165.
16. Noguchi, N., Nakaminami, H., Nakase, K., and Sasatsu, M., Characterization of *Enterococcus* strains contained in probiotic products, *Biol. Pharm. Bull.*, 2011, vol. 34, no. 9, pp. 1469–1473.
17. Gonchar, N.V., Alekhina, L.A., and Suvorov, A.N., Probiotic enterococcal strains as a tool for treatment and prophylaxis of stomach disorders, *Eksper. Klin. Gastroenterolog.*, 2013, no. 1, pp. 74–78.
18. Smirnov, V.V., Kovalenko, N.K., Podgorskii, V.S., and Sarakulova, I.B., Probiotics based on living microbial cultures, *Mikrobiol. Zh.*, 2002, vol. 64, no. 4, pp. 62–80.
19. Vorob'eva, L.I. and Abilev, S.K., Antimutagenesis of bacteria, *Appl. Biochem. Microbiol.*, 2002, vol. 38, no. 2, pp. 97–107.
20. Banwo, K., Sanni, A., and Tan, H., Technological properties and probiotic potential of *Enterococcus faecium* strains isolated from cow milk, *J. Appl. Microbiol.*, 2013, vol. 114, no. 1, pp. 229–241.
21. Rao, S., Ihara, Y., Arakawa, F., Fujimura, T., Murakami, H., and Morimatsu, F., Characterization of probiotic properties of *Enterococcus faecium* NHRD IHARA isolated from porcine feces, *Biosci. Biotechnol. Biochem.*, 2013, vol. 77, no. 9, pp. 1967–1969.
22. Hatum, R., Labrie, S., and Fliss, I. Antimicrobial and probiotic properties of yeasts: from fundamental to novel applications, *Front. Microbiol.*, 2012, vol. 3, no. 3, pp. 421–428.
23. Katakura, Y., Sano, R., Hashimoto, T., Ninomiya, K., and Shioya, S., Lactic acid bacteria display on the cell surface cytozolic proteins that recognize yeast mannan, *Appl. Microbiol. Biotechnol.*, 2010, vol. 86, no. 1, pp. 319–326.
24. Vorobjeva, L.I., Khodzhaev, E.Yu., Ponomareva, G.M., and Bryukhanov, A.L., Extracellular protein metabolite of *Luteococcus japonicus* subsp. *casei* reactivates cells subjected to oxidative stress, *Appl. Biochem. Microbiol.*, 2003, vol. 39, no. 2, pp. 178–182.
25. Vorob'eva, L.I., Khodzhaev, E.Yu., Mulyukin, A.L., and Toropygin, I.Yu., The mechanism of action of reactivating factor from *Luteococcus japonicus* subsp. *casei, Appl. Biochem. Microbiol.*, 2009, vol. 45, no. 5, pp. 489–493.
26. Palframan, R., Gibson, G.R., and Rastall, R.A., Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides, *Lett. Appl. Microbiol.*, 2003, vol. 37, no. 4, pp. 281–284.

27. Meadow, P.M., The chemistry and composition of microorganisms, in *Essays in Microbiology*, Chicester: Wiley, 1978, pp. 6–10.
28. Vorobjeva, L.I., Khodzhaev, E.Yu., and Ponomareva, G.M., Extracellular protein of *Luteococcus japonicus* subsp. *casei* reactivates cells inactivated by irradiation or heat shock, *Microbiology* (Moscow), 2003, vol. 72, no. 4, pp. 428–433.
29. El-Sharoud, W.M., Two component signal transduction systems as key players in stress responses of lactic acid bacteria, *Sci. Prog.*, 2005, vol. 88, no. 4, pp. 203–228.
30. Rowbury, R.J., UV radiation-induced enterobacterial responses, other processes that influence UV tolerance and likely environmental significance, *Sci. Prog.*, 2003, vol. 86, nos. 1/2, pp. 313–332.
31. Chen, G.T., Zhao, L., Zhao, L.I., Cong, T., and Bao, S.F., In vitro study on antioxidant activities of peanut protein hydrolysate, *J. Sci. Food Agric.,* 2007, vol. 87, no. 2, pp. 357–362.
32. Bahareh, H.S. and Amin, I. Antioxidative peptides from food proteins: a review, *Peptides*, 2010, vol. 31, no. 10, pp. 1949–1956.

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