Characterization of *Lactococcus Lactis* for Probiotic Properties *in Vitro*

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Abstract. *The aim of the study was to investigate some of the probiotic characteristics and safety aspects of selected Lactococcus lactis strains intended to be used in food or feed and isolated from raw cow milk samples. Antibacterial, hemolytic, gelatinase and enzymatic activities, resistance towards seven antibiotic as well as acid and bile salt were examined. In general, all strains were acid and bile salt tolerant, expressed good antibacterial activity against tested food spoilage and pathogenic bacteria such as Listeria monocytogenes, Escherichia coli, Brochotix thermosphacta and others. Tested L. lactis strains expressed acid resistance up to 80%, whereas the highest resistance was observed in strain 8 where after 3 h of incubation at 30°C under an acidic condition the growth of the isolate decreased from 7.47 ± 0.02 to 6.71 ± 0.16 $\log_{10}$CFU/mL expressing resistance of 90%. All isolates were resistant at 0.3% bile salt with resistance of more than 50%, whereas strains 8 and 25 expressed a growth decrease from 6.81 ± 0.03 to 6.74 ± 0.02 $\log_{10}$CFU/mL and from 7.06 ± 0.03 to 6.56 ± 0.04 $\log_{10}$CFU/mL showing resistance of 99% and 93%, respectively. The highest antibacterial activity was expressed by L. lactis strains 24 and 25 against spoilage bacteria Brochothrix thermosphacta. With regard to hemolytic activity, one strain showed α-hemolysis; thus, this strain could not be used as a probiotic culture. Moreover, one strain (L. lactis 25) expressed strong activity of harmful enzyme α-chymotrypsin; thus, this strain also could not be applied as a probiotic strain. Only L. lactis strain 8 exhibited probiotic characteristics in vitro and was evaluated as safe.*

Introduction

According to the definition by the World Health Organization (WHO), probiotics are defined as live microorganisms which, when administered in adequate amounts, provide a health benefit to the host (FAO/WHO, 2001). Some of the potential benefits are maintenance or improvement of the intestinal microbiota, prevention of various gastrointestinal disorders, protection against mucosal infections, and regulation of lactose intolerance (Zhang et al., 2020). Products containing probiotics are beneficial for human nutrition and as animal feed supplements (Duc, Hong, Barbosa, Henriques, Cutting, 2004). For this reason, probiotics have been receiving special attention from farmers that search for alternatives to the use of traditional antibiotics as growth promoters (Sandes et al., 2017; Schofield et al., 2017) and from the food industry for functional food production. The interest is reasonable as numerous conducted studies show that probiotics have increased milk yields and meat production (García-Hernández et al., 2016; Schofield et al., 2017) as well as could have health benefits for humans (Sandes et al., 2017).

In recent years, a tendency of increased use of probiotic bacteria in various food products like cheese and yoghurts has been observed (Kumar and Kumar, 2015). Food products containing probiotics, the so-called functional foods, have several therapeutic benefits like anticancer, hypoglycemic properties, antioxidant, and immunomodulatory effects; therefore, isolation of new probiotic strains with health promoting benefits is of big interest (Abushelaibi, Al-Mahadin, El-Tarabily, Shah, Ayyash, 2017).

The main criteria used for a strain to be used as probiotic is generally recognized as safe (GRAS) status, ability to survive under unfavorable conditions such as low pH and bile salt condition (Zhang et al., 2020), antimicrobial activity against pathogenic bacteria and antibiotic resistance (Abouloifa et al., 2019).

A large group of probiotic microorganisms used in medicine and food production belongs to the lactic acid bacteria (LAB) (Das, Khowala, Biswas, 2016; Han, Kong, Chen, Sun, Zhang, 2017; Kumar, Kumar, 2015). LAB are usually employed in food manufacturing and preservation processes being generally recognized as safe to their host's health (Sandes et al., 2017). Among the LAB group, *Lactococcus lactis* is included in the Qualified Presumption of Safety (QPS) list and authorized for use in the food and feed chain within the European Union (EFSA, 2012). The source of *L. lactis* is diverse and, although *L. lactis* may naturally be found in different environments, it is most widely known for its association with the milk environment (Cavanagh, Fitzgerald, McAuliffe, 2015).

New LAB isolates have to express several properties including tolerance to bile and acid conditions to be considered as probiotic. Moreover, to ensure the safe use of strains as probiotic cultures, it is necessary

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to evaluate their safety properties like resistance to antibiotic, antibacterial activity, hemolytic and enzymatic activities. Therefore, the aim of the study was to evaluate bile and acid tolerance of potential probiotic *L. lactis* strains, as well as to evaluate their safety aspects.

Materials and Methods

Lactococcus lactis strains

Three tested *Lactococcus lactis* strains were previously isolated and identified from raw cow milk samples (Kondrotiene et al., 2018). Before conducting any experiments, strains were revitalized in MRS broth (Biolife, Milano, Italy) by growing for 18 h at 30°C.

Antibacterial activity of L. lactis strains

Antibacterial activity of *L. lactis* strains was evaluated using an agar spot test (Schillinger, Lücke, 1989). 3 µL of revitalized strains were spotted on the surface of MRS agar (Biolife) and incubated anaerobically in a jar with Anaerogen (Oxoid) for the generation of anaerobic conditions for 24 h at 30°C. Plates were then overlaid with 7 mL soft agar (0.7%) inoculated with 100 µL of the indicator strain and incubated for 24 h at an optimal growth temperature and atmosphere for the indicator strain. All indicator strains used in the study (Table 1) were revitalized before the experiment in the appropriate medium and temperature. Antibacterial activity was evaluated by measuring a clear inhibition zone diameter around the colony of the tested strain.

Antibiotic resistance evaluation

Antibiotic susceptibility was evaluated using MIC Test Strips (Liofilchem) following the manufacturer's instructions. Tested *L. lactis* strains were revitalized on MRS agar plates (Biolife) by growing for 48 h at 37°C. The inoculum suspension of the tested *L. lactis* strains was prepared by selecting a couple of well-isolated *L. lactis* colonies and preparing McFarland 0.5 standard suspension. Each Mueller-Hinton agar (Oxoid, England) plate was streaked with a sterile swab that was previously soaked in the inoculum suspension. Etest strips of tested antibiotics were placed on a dried plate and incubated for 20 h at 37°C. Minimum inhibitory concentrations (MIC) were determined from the MIC reading scale and expressed in µg/mL.

Enzymatic profile evaluation

Enzymatic profiles of three *L. lactis* (8, 24 and 25) strains were assessed using the API ZYM kit (bioMerieux, Marcy-l'Étoile, France). Each well of the API ZYM strip was inoculated with 65 µL of the McFarland 5 standard suspension of overnight cultures of the strains and incubated at 30°C for 4 h. After incubation, ZYM-A and ZYM-B reagents were added to each well and then incubated at 30°C for 5 min. Results were interpreted according to the manufacturer's instructions. Changes of color were scored from 0 to 5. Color reaction grade 0 was interpreted to correspond to a negative reaction, grades 1 and 2 corresponded to a weak reaction, and grades 3, 4, and 5 corresponded to a strong reaction.

Bile and acid tolerance

For evaluation of bile salt tolerance, tested strains were revitalized and 1 mL of culture was transferred into 9 mL of MRS broth containing 0.3% bile salt. Incubation was carried out at 30°C, and the number of viable bacteria counts was determined after 0 h and 24 h incubation on MRS agar plates. Acid tolerance was evaluated using 1 mL of a revitalized strain that was transferred to 9 mL of PBS adjusted to pH 2.5 (with 5M HCl) and incubated at 30°C. The number of viable bacteria counts was evaluated after 0 h and 3 h incubation periods on MRS agar plates (Thirabunyanon, Boonprasom, Niamsup, 2009).

Hemolytic activity

Hemolytic activity was evaluated using plates containing sheep blood agar. After incubation for 48 h at

Table 1. Food spoilage and pathogenic strains used in the study and their revitalization conditions

Strains	Growth Media	Incubation Temperature (°C)	Incubation Conditions
Listeria monocytogenes ATCC 35152	BHI	37	Aerobic
Staphylococcus aureus ATCC 9144	BHI	37	Aerobic
Escherichia coli ATCC 8739	BHI	37	Aerobic
Pseudomonas aeruginosa NCTC 6750	BHI	37	Aerobic
Bacillus cereus ATCC 11778	BHI	30	Aerobic
Salmonella Typhimurium ATCC 13311	BHI	37	Aerobic
Pseudomonas florescens ATCC 13525	BHI	30	Aerobic
Brochotix thermosphacta ATCC 11509	BHI	25	Aerobic

ATCC – American Type Culture Collection; NCTC – National Collection of Type Cultures, a Culture Collection of Public Health England; BHI – Brain Heart Infusion medium.

30°C, hemolytic activity was recorded as β-hemolysis, α-hemolysis and γ-hemolysis (considered as negative hemolysis) represented as clear zones, green zones or halos around the colonies, respectively (Maragkoudakis et al., 2009).

Gelatinase production

Gelatinase production was evaluated using Luria Bertani agar (Liofilchem). Of each revitalized strain, 1 µL was spotted on the surface of LB agar (Liofilchem) supplemented with 3% (w/v) gelatin (Sigma) and incubated at 37°C and 42°C for 48 h, 25°C for 72 h, and 10°C and 15°C for 10 days. After incubation, the plates were kept at 4°C for 4 h, and the hydrolysis of gelatin was indicated by the formation of opaque halos around the colonies (Perin, Miranda, Todorov, Franco, Nero, 2014).

Results

Enzymatic activity evaluation is presented in Table 2. The evaluation revealed that all tested *L. lactis* strains (8, 2 and 25) had strong activities of esterase (C4) and leucine arylamidase. Besides, all tested *L. lactis* strains had weak activity of valine arylamidase. Strain 8 produced high activities of esterase lipase (C8), cystine arylamidase, acid phosphatase and Naphthol-AS-BI-phosphohydrolase. Other *L. lactis* strains had weak or no activities of these enzymes. No or weak activities were determined for alkaline phosphatase, lipase (C14), valine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. *L. lactis* strain 25 produced high activity of α-chymotrypsin, while other strains produced no activity of this enzyme.

Table 2 presents results of selected *L. lactis* strains to acid and bile salts. The survival of LAB in low pH of the stomach is important for tolerating the initial acid stress (Kumar, Kumar, 2015). All *L. lactis* strains were resistant to acid pH value 2.5. Strains 8, 24 and 25 expressed acid resistance up to 80%. The highest resistance was observed in strain 8 where after 3 h of incubation at 30°C under the acidic condition the growth of the isolate decreased from 7.47 ± 0.02 to 6.71 ± 0.16 $\log_{10}$CFU/mL expressing resistance of 90%. Strains 24 and 25 expressed a growth decrease from 7.55 ± 0.01 to 6.64 ± 0.04 $\log_{10}$CFU/mL and from 7.96 ± 0.00 to 6.71 ± 0.07 $\log_{10}$CFU/mL showing resistance of 88% and 84%, respectively.

Tolerance to bile salts is an important property for any potential probiotic bacteria and is one of the criteria for a strain to be used as a probiotic culture (Kumar, Kumar, 2015). All isolates were resistant at 0.3% bile salt with resistance more than 50%. Minimum resistance was observed in strain 24 where after 24 h of incubation at 30°C the growth of isolate decreased from 7.15 ± 0.05 to 3.64 ± 0.04 $\log_{10}$CFU/mL expressing resistance of 51%. Strains 8 and 25 expressed a growth decrease from 6.81 ± 0.03 to 6.74 ± 0.02 $\log_{10}$CFU/mL and from 7.06 ± 0.03 to 6.56 ± 0.04

Table 2. Enzymatic activities of *L. lactis* strains evaluated by the API-ZYM test

Enzyme	Strains		
	8	24	25
Alkaline phosphatase	1	0	1
Esterase (C4)	4	3	3
Esterase lipase (C8)	3	2	2
Lipase (C14)	0	0	0
Leucine arylamidase	4	3	4
Valine arylamidase	2	2	2
Cystine arylamidase	3	2	2
Trypsin	0	0	0
α-chymotrypsin	0	0	3
Acid phosphatase	4	1	2
Naphthol-AS-BI-phosphohydrolase	3	2	0
α-galactosidase	0	0	0
β- galactosidase	0	0	0
β- glucuronidase	0	0	0
α-glucosidase	0	0	0
β-glucosidase	0	0	0
N-acetyl-β-glucosaminidase	0	0	0
α-mannosidase	0	0	0
α-fucosidase	0	0	0

Table 3. Tolerance of *L. lactis* strains to acid and bile salt

L. lactis strains	Media			
	MRS	MRS+0.3% bile salt	MRS	PBS pH 2.5
		$\log_{10}$CFU/mL		$\log_{10}$CFU/mL
8	6.81 ± 0.03	6.74 ± 0.02	7.47 ± 0.02	6.71 ± 0.16
24	7.15 ± 0.05	3.64 ± 0.04	7.55 ± 0.01	6.64 ± 0.04
25	7.06 ± 0.03	6.56 ± 0.04	7.96 ± 0.00	6.71 ± 0.07

The presented values are means of three replicates ± standard deviation.
*%: final (CFU/mL)/control (CFU/mL) × 100.

$\log_{10}$CFU/mL showing resistance of 99% and 93%, respectively.

Antibacterial activity evaluation of *L. lactis* strains using the agar spot test method is presented in Table 4 and Fig.1 a. The strains showed an antagonistic capacity against all tested food spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Brochotix thermosphacta* and others. All the diameters of the inhibition zones were higher than 10 mm, except for strains 8 and 25. Strain 8 showed the smallest zone of inhibition against *Pseudomonas aeruginosa* and *Bacillus cereus* with diameters of 4 and 7 mm, respectively. Strain 25 showed the smallest zone of inhibition against *Salmonella* Typhimurium and *Pseudomonas florescens* with diameters of 6 and 9 mm, respectively. The highest antibacterial activity was expressed by strains 24 and 25 against spoilage bacteria *Brochothrix thermosphacta* with inhibition zones being 22 and 23 mm, respectively.

Table 5 and Fig. 1 b present results of antibiotic resistance of the tested *L. lactis* strains. None of the tested strains showed resistance to tested antibiotics such as chloramphenicol, clindamycin, streptomycin, gentamicin, tetracycline, erythromycin and ampicillin above the breakpoints provided by the European Food Safety Authority (European Food Safety Authority, 2012).

Table 6 shows the results of hemolytic activity (also see Fig. 1 c) and gelatinase production. In this study, two tested *L. lactis* strains displayed γ-hemolysis, and in contrast one strain (*L. lactis* strain 24) displayed harmful α-hemolysis; therefore, this strain could not be used as a probiotic culture. Phenotypic testing of gelatinase production revealed that none of the tested *L. lactis* strains presented this activity.

Discussion

Regardless of the interest to examine LAB as starter cultures or biopreservatives for their technological properties, there is a growing tendency to evaluate them for probiotic properties (Perin et al., 2014).

Table 4. Antibacterial activity of *Lactococcus lactis* strains

Indicator strains	Source	*L. lactis* strains		
		8	24	25
		The diameters of the inhibition zones around the colonies on agar plate, mm		
Listeria monocytogenes	ATCC 35152	13 ± 1.41	12 ± 0.00	16 ± 1.41
Staphylococcus aureus	ATCC 9144	15 ± 1.41	14 ± 0.00	11 ± 1.41
Escherichia coli	ATCC 8739	12 ± 0.00	11 ± 1.41	12 ± 0.00
Pseudomonas aeruginosa	NCTC 6750	4 ± 0.00	10 ± 0.00	10 ± 0.00
Bacillus cereus	ATCC 11778	7 ± 0.00	10 ± 0.00	12 ± 0.00
Salmonella Typhimurium	ATCC 13311	10 ± 0.00	17 ± 1.41	6 ± 0.00
Pseudomonas fbrescens	ATCC 13525	10 ± 0.00	10 ± 0.00	9 ± 1.41
Brochothrix thermosphacta	ATCC 11509	17 ± 1.41	22 ± 1.41	23 ± 1.41

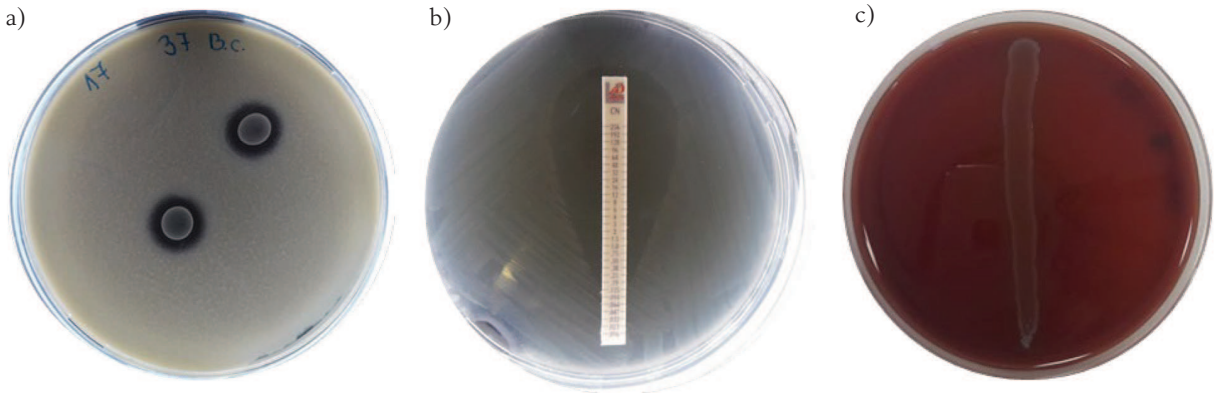

Fig. 1. a) Antibacterial activity evaluation of *L. lactis* strains. Clear zones around the colonies show antibacterial activity against tested microorganisms; b) Antibiotic susceptibility testing of *L. lactis* strains using MIC test strips. Clear zone around the strip show minimum inhibitory concentration; c) Hemolytic activity of *L. lactis* strains (showing γ-hemolysis, which is considered as negative hemolysis).

Table 5. Antibiotic susceptibility of *L. lactis* strains

Antibiotics	Strains			Breakpoints*
	8	24	25	
	Minimum inhibitoryconcentration (MIC), μg/ml			
Chloramphenicol	8	3	3	8
Clindamycin	0.047	0.047	0.064	1
Streptomycin	12	3	0.38	32
Gentamicin	0.25	0.50	0.094	32
Tetracycline	0.19	0.19	0.38	4
Erythromycin	0.094	0.094	0.094	1
Ampicillin	0.094	0.094	0.19	2

*Breakpoints provided by EFSA (2012).

Table 6. Results of hemolytic activity and gelatinase production of *L. lactis* strains

Strains	Hemolytic activity*	Gelatinase production (°C)				
		10	15	25	37	42
8	γ-hemolysis	-	-	-	-	-
24	α-hemolysis	-	-	-	-	-
25	γ-hemolysis	-	-	-	-	-

*α hemolysis means partial hemolysis, γ-hemolysis means absence of hemolysis.
– negative result; + positive result.

The effectiveness of a probiotic strain is species or strain dependent; thus, it is necessary to evaluate each candidate for safety (isolation from suitable habitats, correct identification and antimicrobial susceptibility), functional (resistance to gastrointestinal environment) and beneficial (antagonism against pathogens) properties (FAO/WHO, 2002; García-Hernández et al., 2016). In this study, the probiotic properties of three *L. lactis* strains, previously isolated and identified from raw cow milk samples, were evaluated by *in vitro* tests.

One of the main indicators for a strain to be used as probiotic is its ability to inhibit microbial pathogens (Kumar, Kumar, 2015). The highest antibacterial activity was expressed by two tested *L. lactis* strains 24 and 25 against spoilage bacteria *Brochothrix thermosphacta*. This spoilage organism is associated with spoilage characterized by cheesy, buttery, or sour odors and shares its environmental niche with a member of its sister taxon, *Listeria monocytogenes*, the foodborne pathogen and causative agent of listeriosis (Stanborough, Fegan, Powell, Tamplin, Chandry, 2017). All tested *L. lactis* strains showed good antibacterial activity against this pathogen.

Enzyme production is also one of the main indicators when selecting probiotics (Ji, Jang, Kim, 2015). Strains should not produce harmful enzymes like β-glucosidase, β-glucuronidase (Ji et al., 2015) α-chymotrypsin and N-acetyl-β-glucosaminidase (Abouloifa et al., 2019). Enzymes α-chymotrypsin, N-acetyl-β-glucosaminidase and β-glucuronidase are associated with intestinal diseases (Abouloifa et al., 2019). The possible presence of these enzymes was investigated in this study and strong activity of α-chymotrypsin in *L. lactis* 25 strain was detected; therefore, this strain could not be considered as probiotic. In contrast, β-galactosidase production would be favorable, as this enzyme is considered a beneficial enzyme for a probiotic strain, supporting the reduction of lactose intolerance and milk acidification (Leite et al., 2015); however, none of the tested *L. lactis* strains were producers of this enzyme.

The pH in the human stomach ranges from 1.5 to 4.5, and it has been reported before that acidity has the most negative effect on bacterial growth and viability (Ji et al., 2015). Our study showed that tested *L. lactis* strains 8, 24 and 25 expressed acid resistance up to 80%. These results demonstrate good acid resistance.

Moreover, strains must have good bile tolerance. Physiological concentrations of human bile range from 0.3% to 0.5% (García-Ruiz et al., 2014). It is known that bile salts dissolve membrane lipids leading to the cell's death because of the leakage of the cell contents (Choi, Chang, 2015); therefore, it is important to evaluate the ability of potential probiotic cultures to survive in the presence of bile in order for a probiotic strain to arrive alive in the small intestine or the colon (Kim, Kim, Lee, Kim, Kim, 2012). As it was stated before, all tested *L. lactis* strains were able to resist 0.3% bile salt with resistance above 50%, which reflects a good bile tolerance (Mathara et al., 2008). These results are in accordance with García-Ruiz et

al., 2014 and Kumar & Kumar, 2015. They detected good bile resistance to a variety of LAB strains.

In the antibiotic resistance test, all *L. lactis* strains were susceptible to tested antibiotics. This is a common feature of a probiotic strain (Kumar, Kumar, 2015). However, antibiotic resistance could be considered as an advantage if an antibiotic resistant strain is given during antibiotic treatment. On the other hand, if resistance genes are present on plasmids, they could be transferred to other bacteria including pathogens (Briggiler Marcó, Zacarías, Vinderola, Reinheimer, Quiberoni, 2014).

Hemolytic activity is a typical feature of pathogenic bacteria. This harmful effect may only happen if the ingested bacteria end up in the blood; however, this is an unlikely situation. Nevertheless, this test provides an important information about tested strain's pathogenicity (Miquel et al., 2015). In this study, two tested *L. lactis* strains desplayed γ-hemolysis, and in contrast one strain displayed harmful α-hemolysis; therefore, this strain could not be used as probiotics.

Conclusions

Although strain 24 expressed good antibacterial activity against tested food spoilage and pathogenic bacteria, was acid and bile salt resistant, susceptible to tested antibiotics and was not a producer of harmful enzymes, the strain displayed α-hemolysi; therefore, this strain could not be applied as a probiotic culture. *L. lactis* strain 25 was a producer of α-chymotrypsin; thus, this strain also could not be applied as a probiotic culture.

Only strain 8 exhibited probiotic characteristics *in vitro* and was evaluated as safe.

Results of this study showed that although LAB are generally recognized as safe organisms and are widely used as probiotics, newly isolated strains have to be properly characterized for safety aspects in order to be applied for food or feed production.

More studies are needed to examine probiotic potential of selected a strain and to evaluate its possible beneficial effect and application in food or feed.

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