

Preliminary evaluation of probiotic potential of *Lactobacillus plantarum* strains isolated from Italian food products

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Abstract The aim of this study was to investigate some probiotic properties of 42 wild *Lactobacillus plantarum* strains isolated from different Italian foods of animal origin. The strains were first screened for their antibiotic resistance profile (chloramphenicol, erythromycin, gentamicin, and tetracycline), subsequently they were tested for their in vitro resistance to lysozyme (100 mg L⁻¹), low pH (3.0, 2.5 and 2.0) and bile salts (0.3, 0.5 and 1.0 %). Moreover, agglutination property was studied (adhesion to *Saccharomyces cerevisiae* cells), as well as the presence of *bsh* and *msa* genes. The strains with the best characteristics were subjected to a further trial in order to evaluate their ability to survive to multiple stresses over time (lysozyme, low pH and bile salts) and the effect of these treatments on adhesion to yeast cells. All the strains were susceptible to chloramphenicol, erythromycin and gentamicin, while 6 strains were excluded from further evaluation because of their resistant phenotype against tetracycline. All the strains were able to grow in presence of lysozyme, as well as in MRS broth at pH 3.0. Only 4 strains showed a growth rate lower than 80 % when grown in MRS broth at pH 2.5, while a relevant growth rate decrease was observed after exposure to pH 2.0. Bile salts didn't affect the viability of the *L. plantarum* cells. Twenty-one strains out of 33 tested strains were able to adhere to *S. cerevisiae* cells. Presence of both *bsh* and *msa* genes was detected in 6 strains. The strains resistant to all the stresses, positive to agglutination

with *S. cerevisiae* and showing *bsh* and *msa* genes were selected for further evaluation and subjected to different stress treatments over time. The assessment of growth rates showed that exposure to lysozyme significantly increased low pH resistance in *L. plantarum*. This increase ranged from 2.35 to 15.57 %. The consequential lysozyme and low pH exposures didn't affect the growth rate values after bile salts treatment, as well as the ability of the strains to adhere to yeast cells wasn't modified by previous treatments (lysozyme, low pH and bile salts). The present work allows to increase knowledge about non starter lactic acid bacteria from Italian food products. The studied *L. plantarum* strains showed a good potential for their use as probiotic cultures. However, more in vivo tests are necessary to confirm this potentiality.

Keywords Food isolates · *Lactobacillus plantarum* · Probiotic activity · Multiple stresses

Introduction

The intestinal microbial population is a dynamic ecosystem characterized by high complexity and an elevated number of microorganisms including more than 400 bacterial species (Luckey and Floch 1972). This ecosystem develops numerous functions of great importance for the human organism. It plays a vital role by providing the host with enzymes necessary for assimilation and synthesis of some nutrients, as well as in detoxification of harmful dietary compounds (Heneghan 1988). Gastrointestinal microbiota represent a natural barrier against pathogens, opportunist microorganisms (Hentges 1992) and furthermore is able to stimulate bowel motility and the immune system (Holzapfel et al. 1998).

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According to FAO/WHO (FAO/WHO 2001), probiotics are non-pathogenic microorganisms that, when ingested in adequate amounts, exert a positive influence on their host's health. For these reasons, the consumption of probiotic food is very popular worldwide.

A large variety of microorganisms, overall belonging to the lactic acid bacteria group (LAB), have been evaluated for their probiotic potential ability and are applied as adjunct cultures in several types of food preparations or in pharmaceutical products (Rodgers 2008). Generally, LAB and bifidobacteria are the most common types of microbes used as probiotics. As not all lactic acid bacteria possess the ability to confer health benefits for the host, it becomes necessary to screen and characterize numerous strains to obtain an ideal probiotic culture. The colonization and survival rate in the digestive tract are considered critical to ensure optimal functionality and expression of health promoting physiological functions by probiotics. To survive in the gut, microorganisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract. For colonization, they should exhibit good surface hydrophobicity and aggregation properties (Collado et al. 2007; Del Re et al. 2000). These microorganisms may neutralize the effect of pathogens by inhibiting the toxin action, the binding of pathogens with mucosal surface or producing bacteriocins. They may also show antioxidative and immunomodulatory activities.

In particular, many strains of *Lactobacillus* spp. naturally colonize human intestine and, for this reason, such "intestinal strains" are preferentially developed for commercial employ as probiotics. Within the genus *Lactobacillus*, *Lactobacillus plantarum* is a heterogeneous and versatile species that is encountered in a variety of fermented foods, including dairy, meat, fish and many vegetable or plant products. Recent literature shows an increased interest as concern the use of *L. plantarum* strains as probiotic cultures (Zhang et al. 2012; Zago et al. 2011).

Lactobacillus plantarum strains have been found in many Italian cheese varieties (Pisano et al. 2010; De Angelis et al. 2001). Moreover, strains of *L. plantarum* have proven ability to survive to gastric transit and colonize the intestinal tract of humans and other mammals (Georgieva et al. 2009; Mathara et al. 2008; De Vries et al. 2006). *Lactobacillus* and other beneficial strains originally isolated from dairy products are probably the most suitable candidates for inclusion into several types of food as probiotics, because they are well adapted to the conditions and may therefore be more competitive than probiotics from other sources.

The aim of this study was to evaluate some in vitro probiotic properties of 42 wild *L. plantarum* strains isolated from several foods of animal origin, in order to select good candidates for further in vivo analysis.

Materials and methods

As an essential prerequisite, the antibiotic resistance profiles of the strains were first assessed. In particular resistance against chloramphenicol, erythromycin, gentamicin and tetracycline was investigated. Susceptible strains were then screened for some probiotics features: resistance to lysozyme, low pH, bile salts and agglutination property. Moreover, the presence of *bsh* and *msa* genes, codifying for bile salts hydrolase and mannose-specific adhesion was evaluated, as well as the effect of multiple consequential stresses on growth rate.

Bacterial and yeast strains

Forty-two *Lactobacillus plantarum* strains isolated from different Italian food sources (milk, cheese, fermented meat products) were studied (Table 1). The strains were previously identified on the basis of their metabolic profiles by API 50 CH (bioMérieux, Inc., Marcy l'Etoile, France) and by specie-specific PCR, according to Berthier and Ehrlich (1998). The type strains ATCC14917 (*L. plantarum*) and ATCC26106 (*Saccharomyces cerevisiae*) were obtained from the American Type Culture Collection (Rockville, MD). *L. plantarum* strains were grown at 37 °C in MRS broth or agar (Oxoid, Milan, Italy). *S. cerevisiae* strain was grown at 25 °C in Yeast Peptone Dextrose broth (Oxoid).

Antibiotic resistance

Antibiotic resistance was first screened by agar disk diffusion method to determine resistance profiles against four antibiotics (chloramphenicol, erythromycin, gentamicin and tetracycline) belonging to the clinically most relevant antibiotic classes. Bacterial suspensions with a turbidity equivalent to McFarland Standard 1 (approx 3.0×10^8 CFU•mL⁻¹) were swabbed on Lactic acid bacteria Susceptibility test Medium (LSM) (Klare et al. 2005) agar plates with a sterile cotton swab. Antibiotic disks containing 2 µg chloramphenicol, 15 µg erythromycin, 10 µg gentamicin and 30 µg tetracycline (Oxoid) were placed on the plates. Inhibition Diameter Zones (IDZ), including the diameter of the 6 mm disks, were measured after incubation under anaerobic conditions for 24 h at 30 °C. Strains were considered resistant, moderately susceptible or susceptible based on IDZ according to Charteris et al. (1998).

Strains considered resistant and moderately susceptible by agar disk diffusion method were further evaluated to determine the Minimum Inhibitory Concentration (MIC). MIC values were determined using a broth microdilution method with standardized LSM broth formulation. MIC microtiter test plates containing different antibiotics

Table 1 *Lactobacillus plantarum* strains: source, ripening time and provenience

Strains	Source	Ripening time (days)	Provenience
Lp1	ewes' raw milk cheese	60	Siena
Lp2	ewes' raw milk cheese	45	Siena
Lp3	ewes' raw milk cheese	7	Siena
Lp4	ewes' raw milk cheese	28	Siena
Lp5	ewes' raw milk cheese	60	Siena
Lp19	ewes' raw milk cheese	28	Pisa
Lp20	ewes' raw milk cheese	7	Pisa
Lp21	ewes' raw milk cheese	14	Pisa
Lp22	ewes' raw milk cheese	7	Pisa
Lp23	ewes' raw milk cheese	nd	Massa-Carrara
Lp24	ewes' raw milk cheese	nd	Massa-Carrara
Lp25	ewes' raw milk cheese	nd	Massa-Carrara
Lp26	ewes' raw milk cheese	nd	Massa-Carrara
Lp27	ewes' raw milk cheese	nd	Massa-Carrara
Lp28	ewes' raw milk cheese	nd	Massa-Carrara
Lp29	ewes' raw milk cheese	nd	Massa-Carrara
Lp30	ewes' raw milk cheese	28	Pisa
Lp31	ewes' raw milk cheese	28	Pisa
Lp32	ewes' raw milk cheese	42	Pisa
Lp33	ewes' raw milk cheese	42	Pisa
Lp34	ewes' raw milk cheese	42	Pisa
Lp35	ewes' raw milk cheese	42	Pisa
Lp36	ewes' raw milk cheese	42	Pisa
Lp37	ewes' raw milk cheese	42	Pisa
Lp38	ewes' raw milk cheese	60	Pisa
Lp39	ewes' raw milk cheese	60	Pisa
Lp40	ewes' raw milk cheese	60	Pisa
Lp41	ewes' raw milk cheese	60	Pisa
Lp42	ewes' raw milk cheese	2	Pisa
Lp43	ewes' raw milk cheese	2	Pisa
Lp44	ewes' raw milk cheese	2	Pisa
Lp45	ewes' raw milk cheese	7	Pisa
Lp46	ewes' raw milk cheese	7	Pisa
Lp47	ewes' raw milk cheese	28	Pisa
Lp48	ewes' raw milk cheese	28	Pisa
Lp49	ewe raw milk	nd	Pisa
Lp50	salami	nd	Tuscany
Lp51	salami	nd	Tuscany
Lp52	salami	nd	Tuscany
Lp53	salami	nd	Tuscany
Lp54	salami	nd	Tuscany
Lp55	donkey raw milk	nd	Grosseto

nd not determined

(test ranges from 0.015 to 256 ppm) diluted in LSM medium were inoculated at a final density of 10^8 CFU•mL⁻¹. After incubation for 48 h at 37 °C, MIC

values of each antibiotic were determined visually as the lowest concentrations at which no growth was observed. Cut-off values considered to discriminate susceptible and resistant strains were 8, 1, 16 and 32 ppm for chloramphenicol, erythromycin, gentamicin and tetracycline, respectively, according to EFSA guidelines (2012). Resistant strains have been excluded from further evaluation.

Lysozyme resistance

Evaluation of lysozyme resistance (i.e. saliva enzyme) was performed to assess the in vitro ability of the strains to survive to the oral cavity transit. Cells of *L. plantarum* strains grown overnight in 6.0 mL MRS broth at 37 °C, were pelleted by centrifugation (10 min at 3,500 rpm) and resuspended in 10.0 mL of sterile saline solution in presence of 100 mg L⁻¹ of lysozyme (Sigma-Aldrich) according to Vizoso-Pinto et al. (2006). Bacterial suspensions in sterile saline solution without lysozyme were included as controls. Samples were incubated at 37 °C and microbial counts after 30 and 90 min were carried out on MRS agar (48 h; 37 °C; anaerobiose). Survival rate was calculated as percentage of the growth after 30 and 90 min compared to the growth of control samples. Strains that maintained at least 80 % of their growth rate after treatment for 90 min with lysozyme were considered for further evaluation.

Acidity tolerance

Evaluation of low pH resistance was performed to assess the in vitro ability of the strains to survive to the gastric cavity passage. Cells of *L. plantarum* strains grown overnight were pelleted by centrifugation (10 min at 3,500 rpm), resuspended in 10.0 mL of sterile saline solution and serially diluted to enumerate bacterial cells for control reference (MRS agar, 48 h; 37 °C; anaerobiose). Suspensions were pelleted again (10 min at 3,500 rpm), resuspended in 6.0 mL of MRS broth at pH 3.0 and incubated at 37 °C for 30 min. After incubation, the suspensions were pelleted, resuspended in 10.0 mL of sterile saline solution and serially diluted to enumerate bacterial cells after treatment at pH 3.0. The same cellular pellets were then treated with MRS broth at pH 2.5 and pH 2.0 for 30 min following the same protocol. Survival rate was calculated as percentage of bacterial growth after treatments at pH 3.0, 2.5 and 2.0 compared to the growth of controls. Strains that maintained at least 80 % of their growth rate after treatment at pH 2.5 were considered for further evaluations.

Bile resistance

Evaluation of bile salts resistance was performed to assess the in vitro ability of the strains to survive to the intestinal transit. The ability of the strains to grow in the presence of

0.3, 0.5 and 1.0 % of bile salts (% w/v) (Sigma-Aldrich) for 24 h at 37 °C was determined according to Vinderola and Reinheimer (2003). The results were expressed as the percentage of growth compared to controls. Strains that maintained at least 80 % of their growth rate in presence of 1.0 % of bile salts were considered for further evaluations.

Yeast agglutination

The agglutination test between yeasts and bacteria was performed to evaluate the mannose-adhesion capacity of the strains. This assay was conducted as described by Pretzer et al. (2005) with modifications. Bacterial strains were grown overnight, pelleted by centrifugation (10 min at 3,500 rpm) and suspended in 0.1 mL of sterile saline solution. One hundred μ L were suspended in 4 mL of sterile saline solution. Fifty μ L were transferred to microtiter plates. Fifty μ L of sterile saline solution were added to each well, as well as 100 μ L of 1.0 % (w/v) *S. cerevisiae* suspended cells in sterile saline solution. The microtiter plates were shaken for 10 min at room temperature, then were incubated for 20 min at 25 °C. The ability of each strain to induce visible yeast cell agglutination was evaluated by optical microscope (magnification 40 \times , Leica Microscope DM2000, Leica Microsystems, Wetzlar, Germany) after staining with crystal violet. To evaluate the involvement of mannose receptors in the adhesion mechanism, agglutination with the addition of 50 μ L of methyl- α -D-mannopyranoside (final concentration 100 mM) (Sigma-Aldrich) was performed.

Detection of *bsh* and *msa* genes by PCR amplification

The *L. plantarum* strains were tested by PCR to detect the presence of *bsh* and *msa* genes encoding for the bile salts hydrolase (BSH) and the mannose-specific adhesin (MSA). DNA was extracted by GenElute TM Bacterial Genomic DNA Kits (Sigma-Aldrich) according to the supplier's procedures. Primers pairs and conditions were chosen according to Zago et al. (2011). Forward and reverse primers sequences for the detection of *bsh* gene were 5'-CGTATCCAA GTGCTCATGGTTTAA-3' and 5'-ATGTGTACTGCCAT AACTTATCAATCTT-3', respectively. Forward and reverse primer sequences for the detection of *msa* gene were 5'-GCTATTATGGGGATTACGTTG-3' and 5'-CTGTCT TGACAATAGCCATATA-3', respectively. The expected PCR products lengths were of 919 bp (for *bsh*) and 1,740 bp (for *msa*). PCR amplifications were performed in 25 μ L volumes in a GeneAmp[®] PCR SYSTEM 2700 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) and were set as follows: 4 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 64 °C (*bsh*) or 52 °C (*msa*), and 1 min at 72 °C; the final extension step consisted of 7 min at 72 °C. The PCR products were separated on a 1.5 % agarose gel in Tris-Acetate EDTA buffer

(TAE: 40 mM Tris-Acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide, and visualized under UV light.

Effect of multiple consequential stresses on growth rate

Strains susceptible to antibiotics, able to survive to the previous treatments (lysozyme, pH, bile salts), to adhere to yeast cells and showing both *msa* and *bsh* genes were selected for a combined assay. The strains were first treated with lysozyme (100 mg L⁻¹), then with MRS broth at pH 3.0 and 2.5, MRS broth with 1.0 % bile salts subsequently and finally checked for their ability to adhere to the yeast cells (with and without methyl- α -D-mannopyranoside), as previously described. The aim of this test was to evaluate whether the sequence of different stress treatments over time could vary the ability of the strains to survive.

Statistical analysis

Paired samples t test was performed using Microsoft[®] Excel 2007 to verify the statistical significance of different growth rates in single and consequential stress treatments. For each tested strain growth rates of control and treated samples were compared. The observed differences between trials (single and consequential stress) were statistically analyzed ($P < 0.05$).

Results

Antibiotic resistance

Antimicrobial disk diffusion susceptibility of the 43 strains of lactic acid bacteria is summarized in Table 2. All the strains were susceptible to chloramphenicol and erythromycin. Only six strains were moderately susceptible to tetracycline and nine strains were resistant to gentamicin. No multiple drug resistance was detected. MIC value was determined for all the resistant or moderately susceptible strains to confirm the results of disk diffusion. All the tested strains showed gentamicin MIC values lower than the EFSA cut-off (16 ppm), ranging from 2 to 8 ppm. On the contrary all the strains moderately susceptible for tetracycline by disk diffusion method were then excluded from further analysis because of their MIC values higher than EFSA cut-off (32 ppm) (Table 3). Thus, six strains (Lp1, Lp2, Lp3, Lp5, Lp20 and Lp47) were excluded for further analysis.

Lysozyme resistance

The overall resistance of the 37 strains to lysozyme, expressed as percentage of survival, ranged from a minimum mean value of 95.15 % to a maximum mean value of

Table 2 Inhibition zone diameters (mm) against chloramphenicol (C) and erythromycin (E), gentamicin (CN), tetracycline (TE) of 42 *L. plantarum* strains and ATCC14917

Strains	C (2 µg)	E (15 µg)	CN (10 µg)	TE (30 µg)
Lp1	26	20	18	15 ^{MS}
Lp2	25	24	21	15 ^{MS}
Lp3	25	25	18	15 ^{MS}
Lp4	25	29	17	20
Lp5	25	30	16	15 ^{MS}
Lp19	35	30	20	23
Lp20	27	28	23	15 ^{MS}
Lp21	30	29	16	19
Lp22	30	30	20	23
Lp23	30	33	24	20
Lp24	30	30	15	20
Lp25	29	30	15	20
Lp26	29	30	15	20
Lp27	27	30	15	20
Lp28	32	38	24	23
Lp29	34	35	25	24
Lp30	30	28	11 ^R	25
Lp31	30	35	22	20
Lp32	30	33	21	24
Lp33	34	34	23	23
Lp34	35	35	23	25
Lp35	30	31	13	26
Lp36	27	25	0 ^R	25
Lp37	32	30	13	25
Lp38	28	27	8 ^R	20
Lp39	29	27	8 ^R	24
Lp40	30	28	8 ^R	25
Lp41	32	29	9 ^R	25
Lp42	30	29	13	24
Lp43	32	30	11 ^R	26
Lp44	32	30	15	25
Lp45	30	29	10 ^R	25
Lp46	29	34	23	20
Lp47	29	35	19	18 ^{MS}
Lp48	30	34	27	24
Lp49	30	35	26	20
Lp50	34	30	23	25
Lp51	32	32	22	24
Lp52	34	32	23	25
Lp53	32	33	22	24
Lp54	32	32	22	25
Lp55	31	34	23	24
ATCC14917	28	25	12 ^R	25

R resistant strains, MS moderately susceptible strains

Inhibition zone diameter ranges (mm)

Resistant: C ≤ 13; E ≤ 13; CN ≤ 12; TE ≤ 14

Moderately susceptible: C 14–17; E 14–17; CN -; TE 15–18

Susceptible: C ≥ 18; E ≥ 18; CN ≥ 13; TE ≥ 19

Table 3 MIC values (ppm) of resistant and moderately susceptible strains according to disk diffusion method

Strains	CN	TE
Lp1	nd	64 ^R
Lp2	nd	64 ^R
Lp3	nd	128 ^R
Lp5	nd	64 ^R
Lp20	nd	64 ^R
Lp30	2	nd
Lp36	8	nd
Lp38	4	nd
Lp39	4	nd
Lp40	4	nd
Lp41	4	nd
Lp43	4	nd
Lp45	8	nd
Lp47	nd	64 ^R
ATCC14917	2	nd

CN gentamicin, TE tetracycline, R resistant strains, nd not determined

102.09 %. All the strains showed a high lysozyme resistance, both after 30 and 90 min of treatment, with a survival rate always >80 % (Table 4). In some cases after the incubation with lysozyme the number of cells per ml was higher than those of control samples. After 30 min of treatment only one strain (Lp43) showed a decrease of the growth rate under 98 %. After 90 min five strains demonstrated the same trend (Lp27, Lp32, Lp35, Lp37 and Lp43) with a decreased growth rate ranging from 95.15 to 97.59 %. Twelve strains in particular, demonstrated a high lysozyme resistance, with a survival rate >100 % at both the incubation times. All the 37 strains were considered for further analysis.

Acidity tolerance

The resistance to low pH was evaluated for 37 strains. The effect of pH 3.0, 2.5 and 2.0 on the survival rate of the 37 *Lactobacillus* strains is shown in Table 5. It was found that all the strains were able to survive after 30 min at pH 3.0, with growth rate values ranging from 96.98 to 102.87 %. After 30 min at pH 2.5 only 4 strains (Lp34, Lp42, Lp48 and Lp49) showed a decremented growth rate with values below 80 %. After treatment at pH 2.0 we observed a strains viability loss, 68 % of the strains showed a growth rate value below 30 % compared to the control samples. Only Lp20 and Lp22 maintained a growth rate over 50 %. The 33 strains able to survive at pH 2.5 with a growth rate higher than 80 % were considered for the evaluation of bile salts resistance.

Bile resistance

Thirty-three strains out of 43 were tested for their ability to grow in presence of bile salts (0.3, 0.5 and 1.0 %). The highest reduction of bacterial survival occurred after incubation at

Table 4 *L. plantarum* strains growth rate (%) after treatment with lysozyme (100 mg L⁻¹) for 30 and 90 min

Strains	Time 30 min	Time 90 min
Lp4	100.37	98.57
Lp19	99.35	99.17
Lp21	99.59	99.59
Lp22	102.01	100.86
Lp23	100.90	101.60
Lp24	99.02	99.70
Lp25	101.06	99.33
Lp26	101.84	100.00
Lp27	100.92	95.15
Lp28	100.07	100.43
Lp29	100.09	99.82
Lp30	100.62	99.01
Lp31	100.99	99.56
Lp32	99.96	97.37
Lp33	101.82	100.47
Lp34	99.11	98.05
Lp35	100.28	95.91
Lp36	100.25	100.37
Lp37	98.82	96.84
Lp38	99.83	99.55
Lp39	99.68	99.34
Lp40	100.76	99.92
Lp41	100.69	101.76
Lp42	100.32	101.02
Lp43	97.72	97.59
Lp44	100.81	101.28
Lp45	98.81	100.23
Lp46	100.00	101.93
Lp48	100.72	98.92
Lp49	100.74	100.43
Lp50	99.59	102.09
Lp51	100.16	100.35
Lp52	100.98	98.91
Lp53	100.07	99.44
Lp54	99.72	99.88
Lp55	99.18	98.65
ATCC14917	98.39	98.18

Table 5 *L. plantarum* strains growth rate (%) after treatment at different low pH values

Strains	pH 3.0	pH 2.5	pH 2.0
Lp4	99.17	93.24	40.58
Lp19	100.94	99.80	41.36
Lp21	99.67	100.08	57.47
Lp22	100.61	97.30	54.85
Lp23	99.26	98.38	43.95
Lp24	101.06	86.65	44.16
Lp25	101.15	92.68	41.13
Lp26	100.64	92.14	21.13
Lp27	101.11	90.02	32.66
Lp28	99.89	90.73	31.14
Lp29	99.33	91.48	20.92
Lp30	99.02	83.11	24.21
Lp31	98.93	87.12	21.13
Lp32	99.28	81.73	20.64
Lp33	100.22	82.36	19.60
Lp34	98.79	71.43	19.89
Lp35	97.94	90.20	0.00
Lp36	97.21	91.29	0.00
Lp37	97.86	87.83	4.92
Lp38	97.89	87.25	3.11
Lp39	97.59	86.22	4.90
Lp40	97.58	85.29	0.00
Lp41	100.46	96.29	17.22
Lp42	98.52	60.69	12.70
Lp43	96.98	96.39	24.67
Lp44	100.81	86.66	29.20
Lp45	102.87	85.20	3.24
Lp46	101.06	96.42	19.22
Lp48	99.72	77.87	0.00
Lp49	99.45	60.69	3.04
Lp50	99.32	97.18	0.00
Lp51	99.26	96.48	33.88
Lp52	99.42	96.99	45.80
Lp53	99.84	95.26	35.10
Lp54	99.26	98.18	22.93
Lp55	99.61	93.33	14.02
ATCC14917	99.53	82.80	20.92

37 °C for 24 h in MRS broth added with 1.0 % of bile salts, with mean values ranging from 85.24 to 97.85 % compared to control samples (Table 6). The strains always maintained a growth rate percentage higher than 80 % and they were all considered suitable for further analysis.

Yeast agglutination

Thirty-three strains were evaluated for their ability to agglutinate *S. cerevisiae* cells. Twenty-one strains were

considered positive to the test. All the strains did not agglutinate *S. cerevisiae* cells after addition of methyl- α -D-mannopyranoside.

Detection of *bsh* and *msa* genes by PCR amplification

Thirty-three strains were screened for the presence of *msa* and *bsh* genes, that are considered to be associated with probiotic features. Twelve strains showed the presence of

Table 6 *L. plantarum* strains growth rate (%) after treatment with bile salts at different concentration

Strains	0.3 %	0.5 %	1.0 %
Lp4	95.73	93.67	91.46
Lp19	99.31	96.06	90.47
Lp21	97.87	96.48	90.37
Lp22	96.89	95.51	88.51
Lp23	96.00	89.95	89.55
Lp24	100.51	90.00	88.96
Lp25	97.57	90.26	89.28
Lp26	97.99	90.33	89.62
Lp27	96.53	93.42	89.91
Lp28	97.47	95.35	89.37
Lp29	98.49	93.48	89.85
Lp30	96.13	96.91	90.58
Lp31	99.24	98.18	93.33
Lp32	98.74	99.18	93.05
Lp33	100.29	101.80	96.63
Lp35	101.12	100.00	97.85
Lp36	96.87	94.80	89.19
Lp37	97.43	99.23	95.55
Lp38	97.82	94.12	88.90
Lp39	95.32	93.72	87.34
Lp40	97.42	95.30	89.35
Lp41	97.30	96.58	92.39
Lp43	98.88	93.31	87.50
Lp44	98.04	94.19	87.00
Lp45	96.91	93.63	87.40
Lp46	96.33	94.67	85.24
Lp50	98.82	92.86	89.29
Lp51	98.76	96.57	90.93
Lp52	97.97	96.31	90.19
Lp53	97.84	95.42	90.38
Lp54	97.68	95.25	89.59
Lp55	97.85	97.20	91.40
ATCC14917	93.72	92.60	89.65

bsh gene (ATCC14917, Lp4, Lp30, Lp31, Lp33, Lp36, Lp37, Lp38, Lp39, Lp40, Lp41 and Lp55), while seven strains were positive for *msa* gene (ATCC14917, Lp36, Lp38, Lp39, Lp40, Lp41 and Lp55). Thus, only 7 strains out of the 43 strains tested showed the expected amplicons for both genes.

Effect of multiple consequential stresses on growth rate

Strains susceptible to antibiotics, able to survive to the single stress treatments (lysozyme, pH, bile salts), to adhere to yeast cells and showing both *msa* and *bsh* genes were selected for a combined assay. These strains (ATCC14917, Lp36, Lp39, Lp41 and Lp55) were subjected to the same previous stress treatments with the only difference of the consequentiality in time to better evaluate the stress response of the strains in physiological conditions. After exposure to consequential stresses all strains presented an incremented pH resistance (Table 7). The strains showed a significant increment in Log viable counts of treated samples compared with control samples ($P < 0.05$). The increase observed ranged from 2.35 to 15.57 %, with a mean value of 9.0 %. Low pH exposure didn't affect the growth rates values after bile salts treatment (Table 8), nor the ability of the strains to adhere to yeast cells.

Discussion

An important requirement for probiotic strains is that they should not carry transmissible antibiotic resistance genes. The use in food industry of bacteria harboring these genes is undesirable (EFSA guidelines 2012). Horizontal gene transfer to different bacteria in the gut could lead to the development of new antibiotic resistant microorganisms. In the present study 43 strains (42 wild type strains and 1 ATCC strain) were tested for antibiotic susceptibility

Table 7 Mean values of difference in viable counts (Log) between control and treated samples and growth rate (%) after multiple consequential (lysozyme and low pH) stresses and single stresses (low pH)

Strains	Δ Log pH 2.5 ^a	Δ Log pH 2.5 ^b	Growth rate (%) ^a	Growth rate (%) ^b
Lp36	0.6653 \pm 0.0002*	0.0359 \pm 0.0054*	99.41	91.29
Lp39	0.8096 \pm 0.0027*	0.0624 \pm 0.0146*	99.21	86.22
Lp41	1.3424 \pm 0.0066*	0.0855 \pm 0.0044*	98.64	96.29
Lp55	0.3732 \pm 0.0033*	0.1450 \pm 0.0234*	99.66	93.33
ATCC14917	1.7242 \pm 0.0034*	0.1684 \pm 0.0125*	98.37	82.80

Δ Log: difference in Log viable counts between control and treated samples (mean \pm SD)

^a consequential stresses

^b single stress

* significant difference between treatments ($P < 0.05$)

Table 8 Mean values of difference in viable counts (Log) between control and treated samples and growth rate (%) after multiple consequential (lysozyme, low pH and bile salts) stresses and single stresses (bile salts)

Strains	Δ Log Bile 1 % ^a	Δ Log Bile 1 % ^b	Growth rate (%) ^a	Growth rate (%) ^b
Lp36	0.8920 \pm 0.0024	0.9962 \pm 0.0118	90.39	89.19
Lp39	1.1198 \pm 0.0167	1.4898 \pm 0.0333	90.02	87.34
Lp41	1.3455 \pm 0.0148	1.0629 \pm 0.0131	91.06	92.39
Lp55	0.7918 \pm 0.0113	0.9393 \pm 0.0028	90.55	91.40
ATCC14917	1.0638 \pm 0.0188	1.0187 \pm 0.0267	90.01	89.65

Δ Log: difference in Log viable counts between control and treated samples (mean \pm SD)

^a consequential stresses

^b single stress

* significant difference between treatments ($P < 0.05$)

against 4 antimicrobial compounds (chloramphenicol, erythromycin, gentamicin and tetracycline). Agar disk diffusion method allowed to detect resistant phenotypes for gentamicin (20.9 %), while moderately susceptible strains were detected for tetracycline (13.9 %). These strains were then subjected to the determination of their MIC values. The results obtained showed a low accuracy of the agar disk diffusion method in detecting resistant strains. In fact, the gentamicin resistant strains showed a MIC value lower than the EFSA cut-off (16 ppm), while the tetracycline moderately susceptible strains were identified as resistant because of their MIC values (>32 ppm). This confirms the necessity of more specific inhibition zone range values for interpretation of data from strains belonging to the species *L. plantarum*. Tetracycline resistance in *L. plantarum* strains has been recently reported by several authors and the ability of *L. plantarum* isolates to transfer *tet* genes to other bacteria has been also observed (Egervärn et al. 2009; Mathara et al. 2008; Gevers et al. 2003).

All the antibiotic susceptible strains were subjected to different stresses to simulate the transit in the gastrointestinal tract. Beneficial effects of probiotics bacteria are only achievable if microorganisms are able to survive to unfavorable conditions in the gastrointestinal tract, such as high concentration of lysozyme, low pH, as well as high concentration of bile salts. Lysozyme contained in human saliva is the first step to be passed. Resistance to lysozyme has been attributed to the peptidoglycan structure in the cell wall, physiological state of the cell and lysozyme structure in the medium (Cunningham et al. 1991). All the selected strains showed a high resistance to 100 mg L⁻¹ of lysozyme after 90 min of exposure as previously reported by other authors (Bosch et al. 2011; Zago et al. 2011). The studied strains also showed a good adaptation to exposure at low pH values, especially at pH 3.0 and 2.5. *L. plantarum* strains presented a mean growth rate of 99.5 % after treatment with pH 3.0, with a decrease to 88.83 % after treatment at pH 2.5. As concern the resistance at pH 2.0,

the detected growth rate ranged from 0 to 57.47 %, with a mean value of 22.4 %. These results suggest pH 2.0 as a strong discriminative pH for the selection of high acid-tolerant strains. However, in considering the potential use of *L. plantarum* strains as probiotic culture we should take into account that in the stomach the acid secretion is also buffered by the ingested food. Our findings are in accordance with Jensen et al. (2012), Zago et al. (2011), Bosch et al. (2011) and Nagata et al. (2009). On the contrary, Kaushik et al. (2009), Klayruang et al. (Klayruang et al. 2008) and Charteris et al. (1998) highlight the ability of *L. plantarum* strains to survive at pH 2.0 and 1.5. Also for bile salts resistance we observed a trend similar to those reported by Jensen et al. (2012) and Bosch et al. (2011). The mean growth rate percentages detected in our study after treatments were 90.3, 95 and 97.7 %, in MRS broths added with 1.0, 0.5 and 0.3 % of bile salts, respectively. A lower ability in surviving the exposure to bile salts was observed in strains studied by Klayruang et al. (2008), Nagata et al. (2009).

Comparison among data on resistance to low pH and bile salts of *L. plantarum* from different authors shows that these characteristics are strain-related. However, discrepancies in protocols from different authors in evaluating the strains response to stressful treatments must be taken into account.

The ability of *L. plantarum* to adhere to *S. cerevisiae* cells was compared to the presence of *msa* gene in the strains genome. We found out that the 63.6 % of the strains was able to in vitro aggregate to yeast cells, although only few strains presented the *msa* gene (21.2 %). Moreover, two strains (Lp38 and Lp40) presented the *msa* gene, but were unable to adhere yeast cells. This could be due to the *msa* gene sequence variations in *L. plantarum* strains. As a matter of fact, Gross et al. demonstrated in (2010) that mannose-adhesion capacity in *L. plantarum* is not only related to the simple presence or absence of the *msa* gene, but corresponds to strain specific *msa* gene sequences.

The selected isolates were screened for the presence of bile salts hydrolase (*bsh*) gene, since BSH enzyme is considered as an important probiotic marker that helps organisms resist toxic bile salts environment in the gastrointestinal tract. Only the 36.3 % of the strains able to survive to the 1.0 % of bile salts, also presented the *bsh* gene, which highlights that the resistance to bile is a complex interaction between different factors. The presence of *bsh* gene in *L. plantarum* genome could also play an important role in cholesterol lowering (Patel et al. 2010). This seems to be a promising feature for new potential probiotic candidates.

Finally, the *L. plantarum* strains with the best characteristics were subjected to lysozyme, low pH and bile salts treatments in an integrated assay in order to detect any change in growth rate values previously observed or modification in the ability to adhere to *S. cerevisiae*. A decremented cells viability was expected, on the contrary we observed a better response of *L. plantarum* strains to low pH after lysozyme treatment. The observed incremented cells viability was statistically significant ($P < 0.05$). This could be due to the effect of subliminal stress exposure, which could lead to modifications in the expression of proteins. Further analysis are required to understand the role of lysozyme treatment and to assess the strains dependence of this behavior.

Conclusion

The present work allows to increase knowledge about non starter lactic acid bacteria from Italian food products. The studied *L. plantarum* strains showed a good potential for their use as probiotic cultures. However, more in vivo tests are necessary to confirm this potentiality.

More phenotypic and genotypic characterizations are also required to increase the knowledge about resistance to bile salts and potential adherence with *S. cerevisiae* in relation with the presence of *bsh* and *msa* genes.

The incremented low pH resistance observed after lysozyme exposure (100 mg L^{-1}) of *L. plantarum* represents a valuable aspect. Further tests are required to better understand the mechanism behind this response. Lysozyme is already used as additive in different hard and semi-hard cheeses for its antibacterial activity. The effect on lactic acid bacteria resistance in the environment could be another important aspect to take into account.

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