Functional properties of *Lactobacillus plantarum* strains: A multivariate screening study

Angela Guidone [a,*], Teresa Zotta [b], Reynolds P. Ross [c,d], Catherine Stanton [c,d], Mary C. Rea [c,d], Eugenio Parente [a,b], Annamaria Ricciardi [a,b]

[a] *Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy*
[b] *Istituto di Scienze dell'Alimentazione, CNR, Avellino, Italy*
[c] *Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland*
[d] *Alimentary Pharmabiotics Centre, University College Cork, Ireland*

ARTICLE INFO

ABSTRACT

Thirty-two *Lactobacillus plantarum* strains isolated from different sources were genetically characterized at subspecies level with *recA* gene based multiplex PCR and pulsed-field electrophoresis.

All the strains were tested *in vitro* for functional properties (ability to form biofilms, agglutination of yeast cells, bile salt hydrolase activity, β-galactosidase activity, surface hydrophobicity, resistance to lysozyme, gastric juice and bile salts), for antimicrobial activity and for antibiotic resistance. The presence of *bsh* and *msa* genes and of the *pln* bacteriocin loci were also evaluated.

Hierarchical cluster analysis was used to identify eight different plantaritypes sharing similar patterns of *pln* loci. A global functional score was calculated by transforming values for continuous *in vitro* functional properties in an ordinal scale by cluster analysis, while a nominal scale was used for the other properties. Multidimensional scaling was used to evaluate the similarity in functional properties among the isolates and to evaluate the relationships between source of isolation and functional properties.

Nine strains showed the best *in vitro* functional potential and a significant relationship was found between source of isolation and functional score.

This study confirmed a high heterogeneity in functional properties among *L. plantarum* strains and provides insight for optimal screening strategies.

1. Introduction

Probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2006). The health benefits and clinical effects of strains belonging to the genera *Bifidobacterium* and *Lactobacillus* which are frequently used as probiotics (Rauch & Lynch, 2012) have recently been reviewed (Jonkers, Penders, Masclee, & Pierik, 2012). Within the latter genus several species are currently used as probiotics, including *Lactobacillus acidophilus*, *Lactobacillus casei/paracasei*, *Lactobacillus fermentum*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus salivarius* (Upadrasta, Stanton, Hill, Fitzgerald, & Ross, 2011).

Several functional properties are important to confer health benefits to the host. According to the FAO/WHO guidelines (2006), *in vitro* tests should include the evaluation of resistance to gastric acidity and bile salts, adherence to mucus and/or human epithelial cells and cell lines, the antimicrobial activity against potentially pathogenic bacteria and the bile salt hydrolase activity.

L. plantarum is a versatile and industrially important lactic acid bacterium which can be found in fermented foods (Siezen, Johan, & van Hylckama Vlieg, 2011), in probiotic, functional and therapeutic foods (De Vries, Vaughan, Kleerebezem, & de Vos, 2006). A large body of knowledge is available on the probiotic properties of *L. plantarum* (Bosh et al., 2010; Georgieva et al., 2008; Zago et al., 2011). The probiotic properties most frequently tested are the resistance to biological barriers, the antimicrobial activity, the antibiotic susceptibility, while *in vivo* trials in mice such as the assay cholesterol-lowering effect and clinical trials on the effects on human healthy volunteers or on disease are rare (De Vries et al., 2006). Univariate statistical analysis tests have generally been used to evaluate the results and this may reduce the ability to identify strains with favorable combination of functional properties. In

* Corresponding author. Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano, 10, 85100 Potenza, Italy. Tel.: +39 0971205552; fax: +39 0971205503.

E-mail address: angela.guidone@unibas.it (A. Guidone).

addition the *in vitro* screening of probiotic properties is labor intensive (Zago et al., 2011) and simplified approaches may be useful in the search of new strains for functional and probiotic foods (Vinderola et al., 2008). Therefore, the aim of this work was to carry on a multivariate screening study of *in vitro* functional properties which could be related to probiotic properties in 32 *L. plantarum* strains isolated from different sources, to find possible associations between functional properties and source of isolation and to identify rapid tests to simplify the screening procedures.

2. Materials and methods

2.1. Bacterial strains, media and reagents

The strains used in this study and their sources of isolation are listed in Table 1. All strains were maintained as frozen stocks at −20 °C in 25% (vol vol^{-1}) glycerol and routinely propagated in MRS (de Man, Rogosa, & Sharpe, 1960) broth, pH 6.8, for 16 h at 30 °C.

Unless otherwise stated all reagents were obtained from Sigma−Aldrich (Milan, Italy) while culture media and ingredients were obtained from Oxoid Ltd. (Basingstoke, Hampshire, UK).

2.2. Genotypic characterization and analysis of genes supporting in vitro functional action

Genomic DNA extraction was performed on stationary phase cells using GenElute™ Bacterial Genomic DNA Kit (Sigma−Aldrich). A multiplex PCR assay was performed with the *recA* gene-based primers (Parente et al., 2010) for species identification.

Pulsed field gel electrophoresis (PFGE) was performed as described previously (Brennan et al., 2002) with minor

Table 1
List of bacterial strains belonging to *Lactobacillus plantarum* species in this study, functional properties and results of PCR assays.

Species	Strain	Source	References	Isolation source (IS)	Short IS	Probiotic score	A	B	C	D	E	F	G	H	I	bsh	msa	plnJK	plnEF	plnC8aβ	orf345	plnA	plnC8IF	plnN
	895	PCC	Parente et al., 2010	Sourdough, Carasau bread, Italy	FS	15										1	0	1	1	0	0	0	0	0
	947	PCC	Parente et al., 2010	Sourdough, Moddizzosu bread, Italy	FS	10										0	0	1	1	0	0	0	0	0
	1069	PCC	Parente et al., 2010	Sourdough, Carasau bread, Italy	FS	13										1	1	1	1	0	0	0	1	1
	1505	PCC	Parente et al., 2010	Sourdough, Zichi bread, Italy	FS	15										1	1	1	1	0	0	1	0	0
	38AA	DBVR	Parente et al., 2010	Cassava, Colombia	FS	11										0	1	1	1	0	0	1	0	1
	C17	DBPZ	Parente et al., 2010	Caciocavallo cheese, Italy	CH	17										1	1*	1	1	0	0	1	0	1
	DCU101	DBVR	Parente et al., 2010	Silage	FV	19										1	1	1	0	0	0	0	0	0
	DPC1115	TDPCM	This paper	Unknown, Ireland	UN	10										1	1	1	1	0	0	1	0	0
	DPC1121	TDPCM	This paper	Unknown, Ireland	UN	13										0	1	0	0	0	0	0	0	0
	DPC1122	TDPCM	This paper	Cheese, Ireland	CH	11										0	1	1	0	0	0	0	0	0
	DPC2120	TDPCM	This paper	Cheddar cheese, Ireland	CH	16										1	0	1	0	0	0	0	0	0
	DPC2127	TDPCM	This paper	Cheddar cheese, Ireland	CH	17										0	1	1	1	0	1	0	0	0
Llp	DPC2159	TDPCM	This paper	Cheddar cheese, Ireland	CH	12										0	1*	1	1	0	0	0	0	0
	DPC2183	TDPCM	This paper	Cheddar cheese, Ireland	CH	19										1	1*	1	0	0	0	0	0	0
	DPC2190	TDPCM	This paper	Cheddar cheese, Ireland	CH	18										1	0	1	0	0	0	0	0	0
	DPC4229	TDPCM	This paper	Cheddar cheese, Ireland	CH	13										0	1	1	1	0	0	0	0	0
	DPC6421	TDPCM	This paper	Bovine faeces, Ireland	AN	15										1	0	1	1	0	1	0	0	0
	DPC6429	TDPCM	This paper	Hands of dairy workers, Ireland	HU	19										1	1	1	1	0	0	1	0	0
	DPC6430	TDPCM	This paper	Water of milking, Ireland	EN	12										1	1	1	0	0	0	0	0	0
	FSM170	TDPCM	Parente et al., 2010	Cheese, Italy	CH	13										0	1*	1	0	0	1	0	0	0
	MTD2S	DBPZ	Parente et al., 2010	Sourdough, Cornetto di Matera, Italy	FS	15										1	1	1	1	0	0	1	0	1
	MTF1L	DBPZ	Parente et al., 2010	Sourdough, Cornetto di Matera, Italy	FS	13										1	0	1	1	0	0	1	0	1
	NCFB340	NCIMB	Parente et al., 2010	Silage, United Kingdom	FV	16										1	1	1	1	0	0	0	0	0
	P1.5	DOFATA	Parente et al., 2010	Olives brine, Italy	FV	18										1	1*	1	1	0	0	1	0	0
	S12	DBPZ	Parente et al., 2010	Cheese, Italy	CH	13										1	1	1	1	0	0	1	0	0
	S85	DBVR	Parente et al., 2010	Fresh vegetable products, Italy	FV	17										1	0	1	0	0	0	0	0	0
	UBS3	DOFATA	Parente et al., 2010	Wine, Italy	FV	14										1	1	1	1	0	0	1	0	0
	UT2.1	DOFATA	Parente et al., 2010	Wine, Italy	FV	16										1	0	1	0	0	0	1	0	1
	WFCS1	DSANA	Zotta et al., 2012	Human saliva, unknown	HU	18										1	1	1	1	0	0	1	0	1
	DK36	DBVR	Parente et al., 2010	Tapioca, Nigeria	FS	13										1	0	1	0	0	0	0	0	0
Lpla	DKO22	DBVR	Parente et al., 2010	Sour cassava, Nigeria	FS	14										0	0	1	0	0	0	0	0	0
	NCIMB12120	NCIMB	Parente et al., 2010	Fermented cereals (ogi), Nigeria	FS	13										0	0	1	0	0	1	0	0	0

Lpl, *L. plantarum* subsp. *plantarum*; *Lpla*, *L. plantarum* subsp. *argentoratensis*; T, type strain. DBPZ, our culture collection; DBVR, Prof. S. Torriani, Department of Biotecnology, University of Verona, Italy; DOFATA, Prof. C. Caggia, University of Catania; DSANA, Dipartimento di Scienze Ambientali, 2° Università di Napoli, Italy; NCIMB, National Collection of Industrial, Marine and Food Bacteria, UK; PCC, Porto Conte Ricerche, Italy, TDPCM, Teagasc, Dairy Products Research Centre, Moorepark, Fermoy Co. Cork, Ireland. A, survival in simulated saliva with 100 mg/L lysozyme; B, bile resistance; C, resistance to simulated gastric juice; D, BSH activity; E, yeast agglutination without methyl-α-D-mannopyranoside; F, yeast agglutination in the presence of methyl-α-D-mannopyranoside; G, β-galactosidase activity; H, surface hydrophobicity; I, biofilm formation. Semi-quantitative scale for functional properties (A, B, C, G, H, I): 1, light gray cell; 2, gray cell; 3, black cell. For PCR assays 0, 1, 1* indicate the absence, presence and presence with aspecific bands of expected amplicons, respectively.

modifications. *L. plantarum* strains were grown in MRS broth with 20 mmol l^{-1} threonine to facilitate lysis. The restriction enzyme used was AscI (New England BioLabs, Hertfordshire, UK). Electrophoresis was carried out with CHEF-DR II pulsed-field apparatus (Bio-Rad Laboratories) in 1% w/v SeaKem Gold Agarose for PFGE (LONZA, USA) with 0.5× Tris-borate buffer at 14 °C for 18 h at 6 V cm^{-1} and an angle of 120° with pulse times ramping linearly from 1 to 15 s. The MidRange I PFG Marker (NEB) was used as a molecular weight marker. Gels were stained with 0.5 μg ml^{-1} ethidium bromide and gel images were digitized using Gel Doc XR (Bio-Rad Laboratories). DNA fingerprints were analyzed and normalized using FPQuest™ II software (Bio-Rad Laboratories). Similarity was calculated using Dice's correlation coefficient clustering and was performed with the unweighted pair group method with arithmetic averages (UPGMA). The discriminatory power of the technique was evaluated by using the index of diversity (ID) described by Hunter and Gaston (1988).

Primers developed by Sáenz et al. (2009) were used to amplify 7 genes (*orf345, plnA, plnC8αβ, plnC8IF, plnEF, plnJK, plnN*) of the *pln* locus using KOD Hot Start DNA Polymerase (Novagen) following the manufacturer's recommendations. Clustering of presence/absence of plantaricin genes was performed with Dice's coefficient and clustering with UPGMA.

The presence of *bsh* and *msa* genes encoding for the bile salt hydrolase (BSH) and for the mannose-specific adhesion (MSA) were also evaluated by using methods described by Zago et al. (2011).

2.3. Evaluation of functional properties

2.3.1. In vitro resistance to lysozyme, bile and simulated gastric juice (SGJ)

The lysozyme resistance was tested in simulated saliva (a sterile electrolyte solution, SES, containing 100 mg l^{-1} of lysozyme) according to the method of Zago et al. (2011). Reduction in viability was calculated after 30 min and 120 min of incubation as log $N\,N_0^{-1}$ where N and N_0 are CFU ml^{-1} enumerated in presence and absence of lysozyme respectively. Each experiment was done in duplicate.

The bile resistance was determined on overnight cultures inoculated in duplicate in microwell plates containing MRS broth supplemented with 0, 0.25, 0.5 or 1% (wt vol^{-1}) bile salts at an initial OD$_{600}$ of 1.0. The plates were covered with a sterile flexible plastic tape and incubated at 37 °C in Synergy HT Multi-Mode Microplate Reader (Biotek). Growth was monitored for 24 h and was modeled using the dynamic model of Baranyi and Roberts (1994) with DMFit v. 2.0 (Baranyi & Le Marc, 1996). The bile resistance was estimated by dividing the maximum specific growth rate values (μ_{max}) in presence of 1% (wt vol^{-1}) bile salts by the μ_{max} in absence of bile salts.

The tolerance to simulated gastric juice (SGJ) was evaluated using a semi quantitative method. Overnight cultures were harvested by centrifugation at 12,000× g for 5 min, washed twice with 0.85% (wt vol^{-1}) NaCl and re-suspended in the same solution. Microwell plates containing 180 μl of 20 mmol l^{-1} potassium phosphate buffer pH 7 (PB7) or SGJ (0.3% wt vol^{-1} pepsin, 0.5% wt vol^{-1} NaCl, pH 2.0) were inoculated with 20 μl of washed cells and incubated for 30 min at 37 °C. Serial dilutions were carried out in PB7 and 10 μl of the treated and untreated cell suspensions were spotted on the surface of MRS agar plates. Plates were incubated for 48 h at 30 °C under anaerobic conditions in Generbox jars (bioMérieux SA, Marcy-l'Etoile, France), with AneroGen bags.

2.3.2. Bile salt hydrolase activity

Overnight cultures were spotted on MRS agar plates containing 0.37 g l^{-1} CaCl$_2$ and 0.5% (wt vol^{-1}) sodium

taurodeoxycholate hydrate, TDCA. Plates were incubated anaerobically at 37 °C for 72 h, as described by Nguyen, Kang, and Lee (2007). *Enterococcus faecalis* ATCC 14433 was used as BSH-positive strain. Strains grown in MRS without supplementation were used as negative controls.

2.3.3. Evaluation of surface properties: yeast agglutination, biofilm and hydrophobicity assay, exopolysaccharides (EPS) production

Mannose-adherence ability of *L. plantarum* strains was evaluated on the basis of the agglutination of *Saccharomyces cerevisiae* NCYC 1006 using the protocol described previously by Pretzer et al. (2005). Dark field images were captured using a Nikon Eclispe 80i microscope equipped with a 5 mega-pixels color cooled digital camera (DS-5Mc, Nikon). Images were processed using an ImageJ modified macro (Zotta, Guidone, Tremonte, Parente, & Ricciardi, 2012) to obtain measurements of cells and aggregates.

Measurements were processed using Systat 13 to test the null hypothesis that *L. plantarum* strains were able to aggregate yeast cells (by comparing the distribution of area of yeast cells aggregates and yeast cells with and without methyl-α-D-mannopyranoside) and that the aggregation was mannose dependent (by comparing the distribution of area of yeast cell aggregates with and without methyl-α-D-mannopyranoside).

The ability to form stable biofilms was monitored by growing cells in a 96-well (flat-bottom) cell culture plates (Costar, Corning, NY) following the method described by O'Toole et al. (1999) with minor modifications. Bacterial strains were cultured in MRS for 24 h. The supernatant from each well was poured off by inverting the plates and the biofilms were stained with 0.2 ml 0.05% (wt vol^{-1}) crystal violet for 45 min. The biofilms were rinsed three times with PB7, the dye was remobilized with 96% ethanol and absorbance at 595 nm was measured. Two replicates were used for each trial.

To evaluate the ability of cells to adhere to partition in hydrophobic solvents the method described by Vinderola and Reinheimer (2003) was performed. Two replicates were used for each trial. *L. plantarum* strains were also screened for EPS production by using a pick test (Zotta, Piraino, Parente, Salzano, & Ricciardi, 2008).

2.3.4. Ability to ferment lactose and β-galactosidase activity

Bromocresol Purple Lactose Agar (BCP agar, bioMérieux) was used to test the ability of each strain to use lactose as a carbon source, while β-galactosidase activity was measured in whole cells as described by Vinderola and Reinheimer (2003). Two replicates were used for each trial.

2.3.5. Antimicrobial activity

Inhibitory activity against *Listeria innocua* BL86/26, *Lactobacillus pentosus* 5TP and *L. plantarum* DPC1121, a *pln* negative strain, was tested using a deferred antagonism assay as described in Parente, Grieco, and Crudele (2001). *Lis. innocua* was propagated in Tryptone Soy broth supplemented with 0.6% (wt vol^{-1}) yeast extract for 16 h at 30 °C while the two lactobacilli were propagated in MRS broth at 37 °C for 16 h.

2.3.6. Antibiotic resistance

For the assessment of the susceptibility to gentamicin, tetracycline, erythromycin and chloramphenicol, serial two-fold dilutions ranging from 0 up to 128 μg ml^{-1}, were added to MRS broth supplemented with 0.16 g l^{-1} bromocresol purple in microwell plates. The wells were inoculated with the strains at a final concentration of 10^6 bacteria ml^{-1}. The plates were incubated anaerobically at 30 °C for 16 h and 24 h. The strains were categorized as susceptible

or resistant to antibiotics according to the guidelines reported in EFSA (2008).

2.4. Statistical analysis

Statistical analyses were performed using Systat 13 (Systat Inc., Chicago, IL). For each of the quantitative functional (survival in simulated saliva, bile resistance, resistance to SGJ, β-galactosidase activity, surface hydrophobicity, biofilm formation), a semi quantitative score (1–3; 1 weak − 3 strong) was calculated using k-means clustering on the Euclidean distance matrix to identify reasonable cut-points for clusters. For each feature, clusters were ordered in such a way that strains belonging to cluster 3 had the most and cluster 1 the least desirable properties. For BSH activity, for which only a nominal scale was possible 0 or 1 were used for absence or presence respectively. The cluster membership for each functional property was summed to obtain a functional score and in order to divide strains in different groups on the basis of their functional score a k-means clustering was also used. To evaluate the relationship between factors affecting functional properties and to obtain a graphical representation of the results, a multidimensional scaling (MDS) was performed using the Spearman rank order correlation coefficient. Contingency tables analysis was carried out on *bsh* gene, BSH activity, *msa* gene, mannose-adherence ability and on the association of these properties or isolation source with the functional score.

3. Results and discussion

3.1. Genotypic characterization and genes relevant for in-vitro functional action

PFGE is a powerful method for strain typing and is frequently used as a tool for the identification at strain level or to evaluate the relationships between group of biotypes (Singh, Goswami, Singh, & Heller, 2009). Thirty two strains were identified by multiplex-PCR as *L. plantarum* subsp. *plantarum* (29) and *L. plantarum* subsp. *argentoratensis* (3). The dendrogram of the PFGE patterns is shown in Fig. 1. PFGE separated *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis* strains which were classified in 5 different clusters (at a level of similarity of 50%). The percentage of similarity between unrelated and closely related profiles varied from 34 to 96%. The polymorphism observed with PFGE was high as indicated by the index of diversity, ID = 0.796 and it is consistent with the different origin of the isolates. No correlation was found between clusters and source of isolation. To our knowledge this is the first report in which PFGE analysis was carried out for *L. plantarum* subsp. *argentoratensis* strains and for *L. plantarum* subsp. *plantarum* strains isolated from almost 12 different sources. In two studies using PFGE on comparable number of strains from a single source (sourdoughs, Pepe et al., 2004; grape musts and wines, López, Torres, & Ruiz-Larrea, 2008) also reported a high intraspecific diversity in *L. plantarum*, thus confirming the
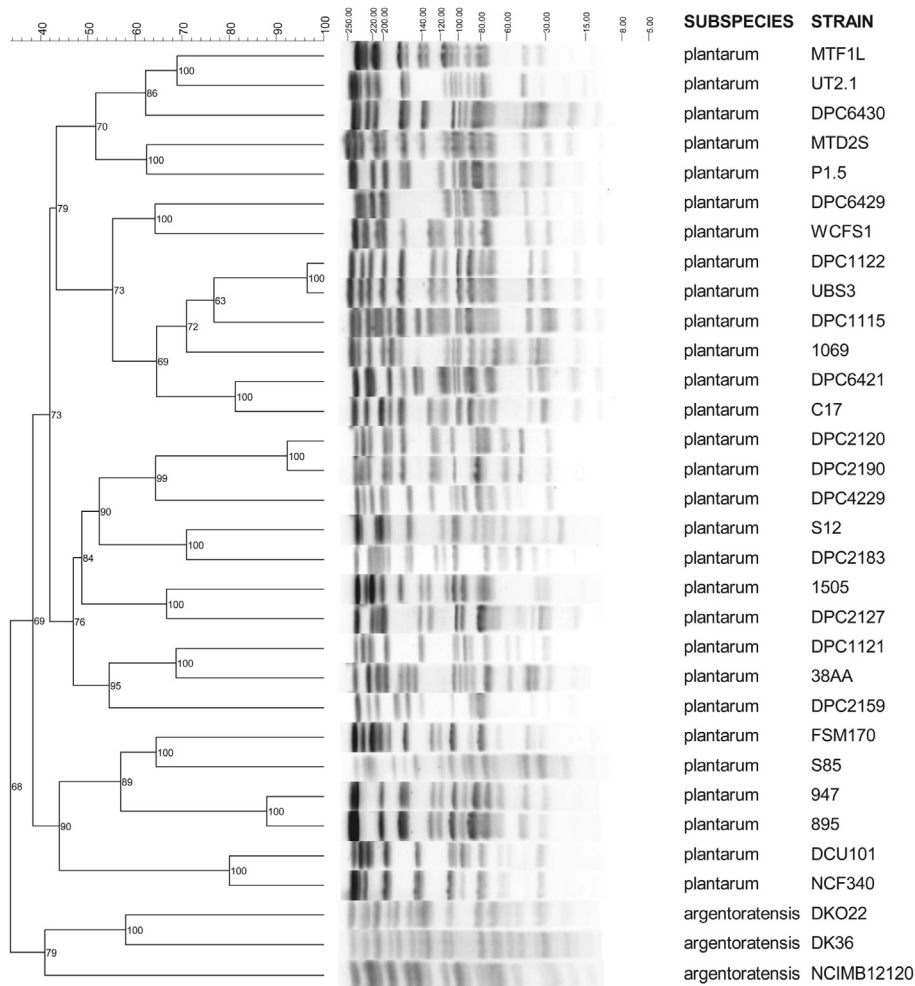
Fig. 1. AscI PFGE patterns and similarity dendrogram of 32 *L. plantarum* isolates. Similarity was calculated using Dice's correlation coefficient clustering and was performed with the unweighted pair group method with arithmetic averages (UPGMA).

difficulties in finding associations between source of isolation and PFGE clusters.

The production of bacteriocins may contribute to probiotic functionality via different mechanisms: bacteriocins may function as colonizing, killing or signaling peptides in bacterial consortia (Dobson, Cotter, & Ross, 2012). The presence of 7 genes of the *pln* loci responsible for bacteriocin biosynthesis was investigated and results are presented in Table 1. Cluster analysis (Fig. 2) revealed 8 genetic groups (plantaritypes) with the same combination of plantaricins (from none to 4, in different combinations) while *L. plantarum* subsp. *plantarum* 1069 and FSM170 had unique combinations of genes, and DPC1121 had none of the plantaricins genes tested in this study. Genes/operons encoding for plantaricin JK, EF, and A were found in several strains (31, 21 and 12, respectively) while genes for the other plantaricins were found in 6 *(plnN)* to 1 strains (NC8 and NC(-IF)). In *L. plantarum* several *pln* loci have been identified and several authors have demonstrated the mosaic aspect of *pln* loci (Diep, Straume, Kjos, Torres, & Nes, 2009; Maldonado, Jiménez Díaz, & Ruiz-Barba, 2004; Sáenz et al., 2009). We also found a remarkable plasticity in the presence of different type of *pln* loci among the 32 *L. plantarum* strains used in this study. However none of the strains was capable of inhibiting *Lis. innocua* BL86/26, *L. pentosus* or *L. plantarum* strains, in liquid or solid media. Plantaricin production is known to be controlled by autoinduction (Maldonado et al., 2004) or to be induced by the presence of target bacteria (Gong, Meng, & Wang, 2010); moreover, plantaricin production is known to be activated at low pH values (Vrancken, De Vuyst, Rimaux, Allemeersch, & Weckx, 2011), while, in the present study, a buffered medium was used to test antimicrobial activity to rule out the effect of acid production. Further work is needed to clarify if bacteriocin production occurs at least under some conditions.

Typical PCR products for *bsh* (919 bp of length) and for *msa* genes (1740 bp) were obtained for 22 and 21 strains respectively, but non-specific amplicons were also observed for 5 strains amplified with *msa* primers (Table 1). Non-specific *msa* amplicons could due to *msa* gene sequence variation of *L. plantarum* different strains (Gross, Snel, Boekhorst, Smits, & Kleerebezem, 2010).

3.2. In vitro resistance to biological barriers

To reach the GI tract in a viable form, probiotic strains have to overcome several biological barriers including the presence of lysozyme in the saliva, low pH in gastric juice and bile salts in the upper GI tract (Rauch & Lynch, 2012).

All *L. plantarum* strains tested in this study showed a high resistance to lysozyme (100 mg l^{-1}) in simulated saliva with a reduction of 0.02–1.21 log after 120 min of incubation (Table 1).

Treatment with simulated gastric juice resulted in a decrease of viable cells of 5.0 log (*L. plantarum* subsp. *plantarum* WCFS1) to 8 log (*L. plantarum* subsp. *plantarum* DPC6429).

In vitro simulation of gastric transit showed a strain-dependent tolerance to low pH and bile salts, in agreement with previous studies (Georgieva et al., 2008; Zago et al., 2011). Bile salts are organic compounds with strong antimicrobial activity. The amphipathic property confers a detergent action which may damage or severely impair membrane functionality. Furthermore, bile salts cause oxidative stress, DNA damage and protein misfolding (Margolles & Yokota, 2011).

Although bile salt tolerance is considered an important property in candidate probiotics, there is still no consensus about the concentration to which the strain should be tolerant. The physiological concentration of bile acids in the small intestine is between 5 and 20 mmol l^{-1} (Vizoso-Pinto, Franz, Schillinger, & Holzapfel, 2006) and in this study we used a maximum concentration of 1% (w/v), equivalent to 24.5 mmol l^{-1}. We found that the increase of bile salts concentration (from 0.25 to 1% w v^{-1}) caused an increase in the duration of the lag phase and a reduction of the estimated
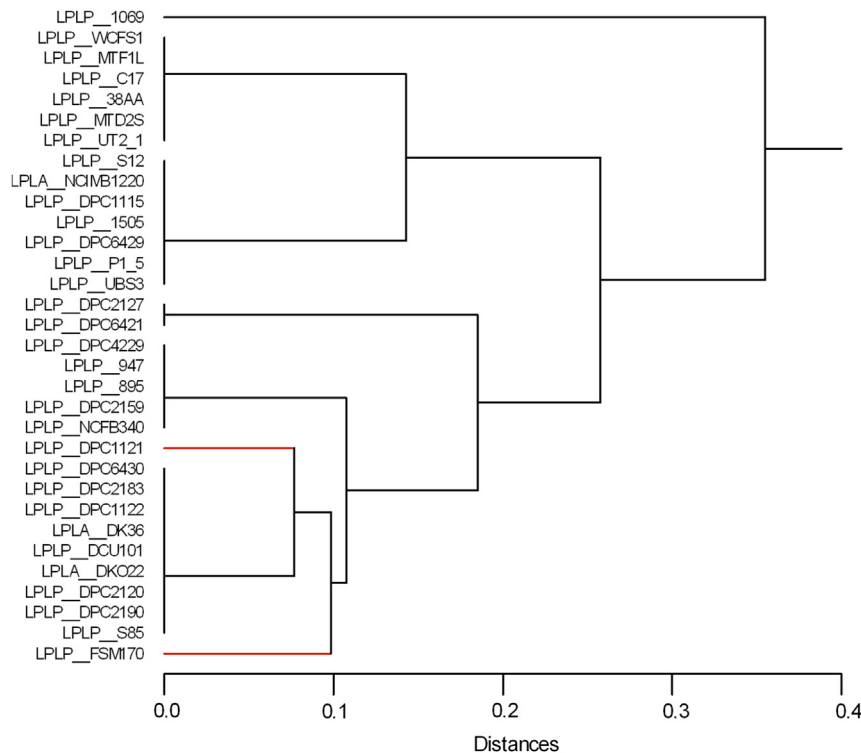

Fig. 2. Cluster analysis on *plnJK, plnEF, plnC8αβ, orf345, plnA, plnC8IF, plnN* genes/operons encoding for the plantaricins: JK, EF, NC8, J51; for the pheromones: A, NC8I–F and for the peptide PlnN.

maximum specific growth rate but the most resistant strains were able to grow at the highest concentration, even at a slower rate.

Deconjugation of bile salts, carried out by BSH, protects gut microbiota and probiotic bacteria from the toxicity of conjugated bile salts and may have has a significant impact on the physiology of the host (Margolles & Yokota, 2011). Eighteen strains of *L. plantarum* subsp. *plantarum* and one strain of *L. plantarum* subsp. *argentoratensis* were able to weakly hydrolyze TDCA (Table 1), as shown by faint halos visible when colonies were removed from agar surface. BSH activity is a widespread trait in *L. plantarum* (Vizoso-Pinto et al., 2006). We found a significant (χ^2 test, $p < 0.001$) association between BSH activity and *bsh* gene detection. Some strains were able to grow in the presence of bile salts though they were not able to deconjugate them; this is in accordance with the hypothesis that the capacity to express BSH is not related to resistance to conjugated bile salts (Moser & Savage, 2001).

3.3. Surface properties

The association with the epithelial surface or the mucus layer is essential for probiotic strain persistence. One mechanism of bacterial adhesion is based on the binding to mannose-containing receptor sites on epithelial cells and the gene encoding the mannose-specific adhesion (*msa*) of *L. plantarum* has been identified (Pretzer et al., 2005). We found that 13 *L. plantarum* strains were unable to agglutinate *S. cerevisiae* cells, while the other 19 strains showed different levels of agglutination, with different levels of intensity depending on the presence or absence of methyl-α-D-mannopyranoside (Table 1). The occurrence of *msa* gene and the mannose-adherence ability were not significantly associated and this is in agreement with Gross et al. (2010) which explained that genetic diversity of *msa* among *L. plantarum* strains lead to different *msa* expression levels or variable domain composition within Msa protein with consequently varying mannose-adhesion capacity. Of the 11 *msa* negative strains, 64% were able to induce yeast cell aggregation and further work is needed to clarify the involvement of other gene products in mannose dependent and independent aggregation. Our results partially confirm those of Zago et al. (2011) who used PCR detection of *msa* and *bsh* as a preliminary screening tool, although *bsh* may be a better indication of the probiotic potential of a strain.

Cell surface hydrophobicity estimated by the hexadecane partition has been used as an indirect test for ability to adhere to epithelial cells (Vinderola et al., 2008; Zago et al., 2011). Strains tested in this study showed a variable hydrophobicity, with values ranging from 2.1% to 71.6%, and differing ability to form stable biofilms with average values of the absorbance of the culture at 595 nm from 1.18 to 4. In agreement with Zago et al. (2011) we found no significant association between hydrophobicity and adhesion properties, as well as between presence of the *msa* gene and ability to form stable biofilms. However, other assays, including the adhesion to Caco-2 cells, to intestinal pig mucosa or to mucus secreting HT29-MTX cells (Turpin, Humblot, Noordine, Thomas, & Guyot, 2012) are needed to confirm that the strains show ability to adhere to epithelial cells.

Adhesion to human intestinal mucus and biofilm formation are also mediated by EPS whose production has been credited with health promoting properties such as cholesterol lowering, immunomodulation, antitumorogenic effects, and prebiotic effects (Jolly, Vincent, Duboc, & Neeser, 2002). *L. plantarum* subsp. *plantarum* MTD2S and MTF1L were able to produce EPS from different sugars (glucose, maltose, sucrose) in solid media but this trait is rarely tested when assessing potential *L. plantarum* probiotic strains.

3.4. Ability to ferment lactose and β-galactosidase activity

β-galactosidase positive strains may improve lactose tolerance (De Vrese et al., 2001). All strains showed the ability to use lactose, as shown by the change of the color of BCP agar and showed different levels of β-galactosidase activity. Except for four strains that showed a β-galactosidase activity less than 10 Miller units, the average value was ca 30 Miller units and DPC2120 displayed the highest activity (100 Miller units). These values were smaller than to those found in other studies (Zago et al., 2011) in which only *L. plantarum* strains isolated from cheese were used. However, in our study, strains isolated from cheese did not necessarily have the highest activity.

3.5. Antibiotic susceptibility test

Probiotics must be safe for human consumption and should not harbor transmissible antibiotic resistance determinants. According to the guidelines reported in EFSA (2008), *L. plantarum* strains can be categorized as susceptible or resistant to antimicrobials when they are inhibited or not at breakpoint levels for a specific antimicrobial.

We tested all the strains for susceptibility to 4 antibiotics belonging to clinically relevant antibiotic classes and we found that only the *L. plantarum* subsp. *plantarum* C17 and S85 strains were both resistant to tetracycline and C17 was also resistant to erythromycin. Tetracycline-resistant *L. plantarum* strains were also detected in isolates from italian fermented dry sausages and from italian and argentinean cheeses (Zago et al., 2011; Zonenschain, Rebecchi, & Morelli, 2009).

3.6. Functional score and multidimensional scaling (MDS)

Studies on screening for functional properties related to probiotic potential (Bosh et al., 2010; Georgieva et al., 2008; Zago et al., 2011), rarely include multivariate statistical analysis. This is surprising since the probiotic potential of a strain is related to many different properties. We used a combination of *K*-means clustering and MDS analysis to analyze our results.

K-means clustering allowed the division of the strains in three clearly separated clusters on the basis of several functional properties (Fig. 3). The functional scores, calculated as described in Section 2.4, ranged from 10 to 19 (Table 1) and *k*-means clustering identified the strains with the best, intermediate and poor *in vitro* functional scores showed a score from 16 to19, 15 to13 and 12 and 10, respectively.

The distribution of strains in MDS plots was significantly affected in the first dimension by bile tolerance and aggregation, while surface hydrophobicity, biofilm and tolerance to gastric juice affected the distribution in the second dimension and bile resistance was the main factor affecting functional properties in the third dimension (Fig. 4). As a consequence, strains with the best combination of functional properties (DCU101, DPC2183, DPC6429, DPC2190, P1.5, WCFS1, C17, DPC2127 and S85) were located towards the left and the back of the plot.

We found a significant association between functional score and isolation source. The majority of strains were isolated from 3 isolation sources (CH, FS, FV, see Table 1); the mean values of functional score for these sources were 14.9, 13.2 and 16.7, respectively. A χ^2 test was carried out to test the null hypothesis that the frequency of strains with functional scores >16 was equal among isolation sources; the null hypothesis was discarded with $p < 0.01$. A Kolgomorov–Smirnov two-sample test confirmed that the functional score is significantly higher ($p < 0.01$) in strains isolated from fermented vegetables compared to those isolated from FS. To our knowledge, this is the first study in the functional
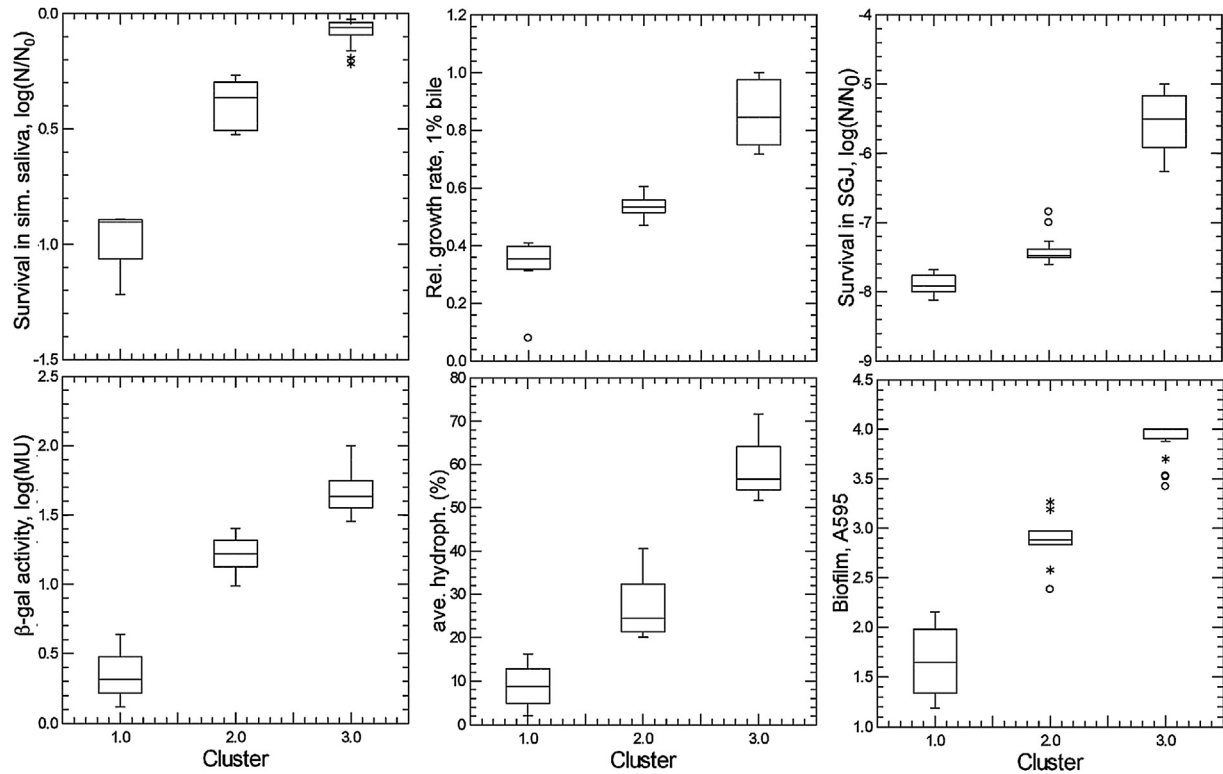
Fig. 3. Box-plots showing the strains splitting in three cluster for functional properties: survival in simulated saliva, bile resistance, resistance to gastric juice, β-galactosidase activity, surface hydrophobicity and biofilm formation.

properties of *L. plantarum* strains isolated from a large variety of sources was evaluated and in which *L. plantarum* subsp. *argentoratensis* strains were studied: all exhibited low scores (13–14) showing a poor agglutination and a lower resistance to bile, gastric
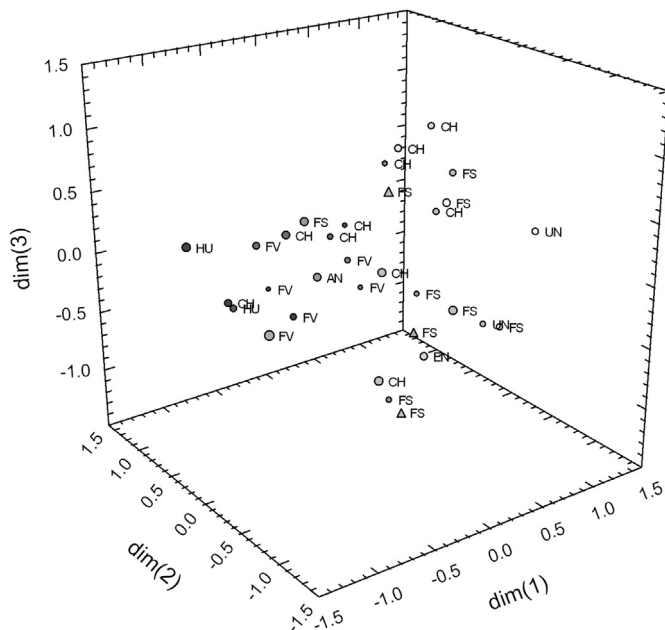

Fig. 4. MDS carried out using the Spearman rank order correlation coefficient. AN, animal isolation source; CH, cheese isolates; EN, environmental isolation source; FS, fermented starch products included cassava, cereals and sourdough; FV, fermented vegetables included silage, olives and wine; HU, human isolates; UN, unknown isolates. O, *L. plantarum* subsp. *plantarum*; Δ, *L. plantarum* subsp. *argentoratensis*. Empty symbols, functional score from 10 to 12; light gray symbols, functional score from 13 to 15; dark gray symbols, functional score from 16 to 19.

juice and hydrophobicity but good β-galactosidase activity and ability to form a stable biofilm. This species may therefore be a poor source of potential probiotic strains. A significant association (χ^2 test, $p < 0.001$) was also found between the occurrence of *bsh* gene and a high functional scores (>13).

4. Conclusions

We found a large heterogeneity in functional properties related to potential probiotic traits in *L. plantarum* strains confirming that they are strain dependent. Although simplified sets of *in vitro* tests are not novel (Vinderola et al., 2008), the functional score developed in this study is a simple way to summarize the potential of a strain in probiotic applications. We showed for the first time that *L. plantarum* subsp. *argentoratensis* strains or strains isolated from fermented starchy products and that strains lacking the *bsh* gene rarely have desirable functional properties: exclusion of strains on the basis of their taxonomic position or of their source of isolation and the use of simple molecular tests (detection of *bsh* by PCR) early in the screening may increase the success of screening programs and reduce their costs.

References

Baranyi, J., & Le Marc, Y. (1996). *Dmfit manual, Version 2.0*. Norwich: UK: Institute of Food Research.

Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology, 23*, 277–294.

Bosh, M., Rodriguez, M., Garcia, F., Fernández, E., Fuentes, M. C., & Cuñé, J. (2010). Probiotic properties of *Lactobacillus plantarum* CECT 7315 and CECT 7316 isolated from faeces of healthy children. *Letters in Applied Microbiology, 54*, 240–246.

Brennan, N. M., Ward, A. C., Beresford, T. P., Fox, P. F., Goodfellow, M., & Cogan, T. M. (2002). Biodiversity of the bacterial flora on the surface of a smear cheese. *Applied and Environmental Microbiology, 68*, 820–830.

De Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., & Schrezenmeie, J. (2001). Probiotics compensation of lactase insufficiency? *The American Journal of Clinical Nutrition, 73*, 421S–429S.

De Vries, M. C., Vaughan, E. E., Kleerebezem, M., & de Vos, W. M. (2006). *Lactobacillus plantarum* – survival, functional and potential probiotic properties in the human gastrointestinal tract. *International Dairy Journal, 16*, 1018–1028.

Diep, D. B., Straume, D., Kjos, M., Torres, C., & Nes, I. F. (2009). An overview of the mosaic bacteriocin pln loci from *Lactobacillus plantarum*. *Peptides, 30*, 1562–1574.

Dobson, A., Cotter, P. D., & Ross, R. P. (2012). Bacteriocin production as a probiotic trait? *Applied and Environmental Microbiology, 78*, 1–6.

European Food Safety Authority. (2008). Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *The EFSA Journal, 732*, 1–15.

FAO/WHO. (2006). Probiotics in food. Health and nutritional properties and guidelines for evaluation. In *FAO Food and nutrition paper 85 Roma*.

Georgieva, R. N., Iliev, I. N., Chipeva, V. A., Dimitonova, S. P., Samelis, J., & Danova, S. T. (2008). Identification and in vitro characterisation of *Lactobacillus plantarum* strains from artisanal Bulgarian white brined cheeses. *Journal of Basic Microbiology, 48*, 234–244.

Gong, H. S., Meng, X. C., & Wang, H. (2010). Mode of action of plantaricin MG, a bacteriocin active against *Salmonella typhimurium*. *Journal of Basic Microbiology, 50*, S37–S45.

Gross, G., Snel, J., Boekhorst, J., Smits, M. A., & Kleerebezem, M. (2010). Biodiversity of mannose-specific adhesion in *Lactobacillus plantarum* revisited: strain-specific domain composition of the mannose-adhesin. *Beneficial Microbes, 1*, 61–66.

Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology, 26*, 2465–2466.

Jolly, L., Vincent, S., Duboc, P., & Neeser, J. R. (2002). Exploiting exopolysaccharides from lactic acid bacteria. *Antonie Van Leeuwenhoek, 82*, 367–374.

Jonkers, D., Penders, J., Masclee, A., & Pierik, M. (2012). Probiotics in the management of inflammatory bowel disease: a systematic review of intervention studies in adult patients. *Drugs, 72*, 803–823.

López, I., Torres, C., & Ruiz-Larrea, F. (2008). Genetic typification by pulsed field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) of wild *Lactobacillus plantarum* and *Oenococcus oeni* wine strains. *European Food Research and Technology, 227*, 547–555.

de Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*, 130–135.

Maldonado, A., Jiménez Díaz, R., & Ruiz-Barba, J. L. (2004). Induction of plantaricin production in *Lactobacillus plantarum* NC8 after coculture with specific gram-positive bacteria is mediated by an autoinduction mechanism. *Journal of Bacteriology, 186*, 1556–1564.

Margolles, A., & Yokota, A. (2011). Bile acid stress in lactic acid bacteria and bifidobacteria. In K. Sonomoto, & A. Yokota (Eds.), *Lactic acid bacteria and bifidobacteria: Current progress in advanced research* (pp. 111–142). Norfolk, UK, Caister: Academic Press.

Moser, S. A., & Savage, D. C. (2001). Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Applied and Environmental Microbiology, 67*, 3476–3480.

Nguyen, T. D. T., Kang, J. H., & Lee, M. S. (2007). Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *International Journal of Food Microbiology, 113*, 358–361.

O'Toole, G. A., Pratt, L. A., Watnick, P. I., Newman, D. K., Weaver, V. B., & Kolter, R. (1999). Genetic approaches to study of biofilms. *Methods in Enzymology, 310*, 91–109.

Parente, E., Ciocia, F., Ricciardi, A., Zotta, T., Felis, G. E., & Torriani, S. (2010). Diversity of stress tolerance in *Lactobacillus plantarum, Lactobacillus pentosus* and *Lactobacillus paraplantarum*: a multivariate screening study. *International Journal of Food Microbiology, 144*, 270–279.

Parente, E., Grieco, S., & Crudele, M. A. (2001). Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). *Journal of Applied Microbiology, 90*, 943–952.

Pepe, O., Blaiotta, G., Anastasio, M., Moschetti, G., Ercolini, D., & Villani, F. (2004). Technological and molecular diversity of *Lactobacillus plantarum* strains isolated from naturally fermented sourdoughs. *Systematic and Applied Microbiology, 27*, 443–453.

Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, P. A., Lambert, J., et al. (2005). Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *Journal of Bacteriology, 187*, 6128–6136.

Rauch, M., & Lynch, S. (2012). The potential for probiotic manipulation of the gastrointestinal microbiome. *Current Opinion in Biotechnology, 23*, 192–201.

Sáenz, Y., Rojo-Bezares, B., Navarro, L., Díez, L., Somalo, S., Zarazaga, M., et al. (2009). Genetic diversity of the pln locus among oenological *Lactobacillus plantarum* strains. *International Journal of Food Microbiology, 134*, 176–183.

Siezen, R. J., Johan, E. T., & van Hylckama Vlieg, J. E. T. (2011). Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. *Microbial Cell Factories, 10*, S3.

Singh, S., Goswami, P., Singh, R., & Heller, K. J. (2009). Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: a review. *LWT – Food Science and Technology, 42*, 448–457.

Turpin, W., Humblot, C., Noordine, M. L., Thomas, M., & Guyot, J. P. (2012). *Lactobacillaceae* and cell adhesion: genomic and functional screening. *PLoS ONE, 7*, e38034. http://dx.doi.org/10.1371/journal.pone.0038034.

Upadrasta, A., Stanton, C., Hill, C., Fitzgerald, G. F., & Ross, R. P. (2011). Improving the stress tolerance of probiotic cultures: recent trends and future directions. In E. Tsakalidou, & K. Papadimitriou (Eds.), *Stress responses of lactic acid bacteria* (pp. 395–438). Springer.

Vinderola, G., Capellini, B., Villarreal, F., Suárez, V., Quiberoni, A., & Reinheimer, J. (2008). Usefulness of a set of simple in vitro tests for the screening and identification of probiotic candidate strains for dairy use. *Lwt-food Science and Technology, 41*, 1678–1688.

Vinderola, G., & Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria, a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Research International, 36*, 895–904.

Vizoso-Pinto, M. G., Franz, C. M. A. P., Schillinger, U., & Holzapfel, W. (2006). *Lactobacillus* spp. with in vitro probiotic properties from human faeces and traditional fermented products. *International Journal of Food Microbiology, 109*, 205–214.

Vrancken, G., De Vuyst, L., Rimaux, T., Allemeersch, J., & Weckx, S. (2011). Adaptation of *Lactobacillus plantarum* IMDO 130201, a wheat sourdough isolate, to growth in wheat sourdough simulation medium at different pH values through differential gene expression. *Applied and Environmental Microbiology, 77*, 3406–3412.

Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suarez, V., Vinderola, G., et al. (2011). Characterization and prebiotico potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology, 28*, 1033–1040.

Zonenschain, D., Rebecchi, A., & Morelli, L. (2009). Erythromycin- and tetracycline resistant lactobacilli in Italian fermented dry sausages. *Journal of Applied Microbiology, 107*, 1559–1568.

Zotta, T., Guidone, A., Tremonte, P., Parente, E., & Ricciardi, A. (2012). A comparison of fluorescent stains for the assessment of viability and metabolic activity of lactic acid bacteria. *World Journal of Microbiology and Biotechnology, 28*, 919–927.

Zotta, T., Piraino, P., Parente, E., Salzano, G., & Ricciardi, A. (2008). Characterization of lactic acid bacteria isolated from sourdoughs for Cornetto, a traditional bread produced in Basilicata (Southern Italy). *World Journal of Microbiology and Biotechnology, 24*, 1785–1795.