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University College Cork, Ireland Coláiste na hOllscoile Corcaigh Genotypic and phenotypic characterization of food-associated *Lactobacillus plantarum* isolates for potential probiotic activities

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# Keywords: *Lactobacillus plantarum*, Fermented foods, Multilocus Sequence Typing (MLST), Antibiotic resistance, Probiotic properties

**One sentence summary:** This study supports a specific preliminary platform to characterize *Lactobacillus plantarum* intended for fermentation purposes and for probiotic consumption on the basis of genotype and phenotype.

## ABSTRACT

Lactic acid bacterium, *Lactobacillus plantarum*, has been applied, for centuries, for food and drink fermentations. Given the benefits associated with fermented products, *Lb. plantarum* strains have captured considerable industrial and scientific interest, so that they are included as fundamental components of functional foods. Indeed, some strains are marketed as probiotics. In the present

study, food and gut associated *Lb. plantarum* isolates were genetically characterized by Multilocus Sequence Typing (MLST) and phenotypically characterized for properties that could influence their probiotic potential. MLST and phylogenetic analysis stratified 22 *Lb. plantarum* isolates into six lineages. The isolates were further phenotypically characterized by *in vitro* assay to assess their potential gut community influence *via* a limited number of assays including acidification activity, strain displacement activity and their intrinsic range of antibiotic resistance. Given growing recognition of the benefits of fermented foods, and the prevalence of *Lb. plantarum* in these applications, this study highlights analysis of a subset of preliminary important strain specific features. These features are of interest to all stakeholders, to inform isolate comparison and selection for current functional food associations and that can serve as a basis for future strain and food-microbe fermentation product development.

## INTRODUCTION

*Lactobacillus plantarum*, a natural inhabitant of the human gastro-intestinal tract (GIT) is a highly versatile species of lactic acid bacteria (LAB) that is encountered in a wide range of different ecological niches including food (Siezen *et al.* 2011; Corsetti *et al.* 2018). Recognition of their importance in food fermentation processes has led to their use as starter cultures to support a wide range of fermented food products, including those obtained from vegetable fermentations (Gardner et al. 2001; Wouters *et al.* 2013; Zago et al. 2013, Benincasa *et al.* 2015), fermented dairy products (Pisano *et al.* 2011; Zago *et al.* 2011) and fermented meat products (Rubio *et al.* 2013; Zhang *et al.* 2013). In many of these fermentations, *Lb. plantarum* appears to be dominant, particularly in the later stages of fermentation. This enrichment may well be due to its ability to tolerate low pH (Luxananil *et al.* 2009) as well as the metabolic capability to ferment a broad spectrum of carbohydrates (Xu *et al.* 2015).

Indeed, the ecological flexibility and environmental adaptation of Lb. plantarum could be related to its genome size (average 3.3 Mb). It represents one of the largest detected within the Lactobacillus genus (Kleerebezem et al. 2003; Molenaar et al. 2005). These considerations along with their ease of culture, genetic malleability and their relative representation in different niches including the human gut (de Vries 2006; Molin et al. 2014) further support Lb plantarum for probiotic applications. Accordingly, the diverse range of properties associated with individual Lb. plantarum strains and an expanded interest in Lb. plantarum strain application as probiotics necessitates a reliable molecular method to delimit *Lb. plantarum* to the strain level. Relating strain specific molecular identification combined with beneficial strain properties could inform and optimize LAB selection for functional foods and fermentations. Multilocus sequence typing (MLST) represents a reliable molecular method for both identification and typing of closely related isolates and strains within and outside a species (Maiden et al. 1998). This method is widely used to investigate relatedness in predicting degree of pathogenicity among important human and food borne pathogens (Ragon et al. 2008; Achtman et al. 2012). More recently, it has gained credence for typing LAB, facilitating precise comparative identification to strain level (de Las Riva et al. 2006; Oh et al. 2010; Xu et al. 2015).

In selecting bacterial strains intended for use in the food industry, identification and safety for use are of the utmost importance in establishing Generally Recognized as Safe (GRAS) or Qualified Presumption of Safety (QPS) status. Guidelines issued by both the Food and Agriculture Organization and the World Health Organization (FAO/WHO) and the European Food Safety Authority (EFSA) have recommended a range of criteria that should be examined for any potential probiotic strain in respect of this. One criterion includes assessment of inherent antibiotic resistances. Genetic resistance to antibiotics could be transferred from LAB to other commensal microorganisms on plasmids or through conjugative transposons, thereby increasing opportunities for transmission of antibiotic resistance. Such transmission has the potential to increase virulence and resistance to other strains, including pathogens with the added possibility to increase antibiotic resistance through the food chain (Teuber *et al.* 1999). Therefore, assessing *Lb. plantarum* isolate specific antibiotic strains. Moreover, it is universally recognized as an important consideration for putative probiotic strains. Moreover, it is universally recognized that probiotics should be capable of transit to the digestive tract to elicit their beneficial effects (Papadimitriou *et al.* 2015). In doing so, inherent gut health and microbial populations should be preserved, if not enhanced.

The current study was initiated to provide preliminary characterization of new probiotic candidates among a collection of *Lb. plantarum* isolates of fermented food origin. Applying genetic and phenotypic approaches, this work provides a preliminary platform that may be applied for strain specific identification and characterization in order to initially characterize and optimize strain selection, to facilitate recognition of redundancy in fermentation processes and in probiotic selection for human consumption.

## MATERIALS AND METHODS

## Bacterial isolates applied in this study

Food-borne *Lb. plantarum* isolates investigated in this study were originally isolated from fermented foods or are of human origin and are part of the University of Teramo collection (Table S1). All the isolates were identified as *Lb. plantarum* species and characterized by this group for other functional properties (Prete *et al.* 2017; Garcia *et al.* 2018; Prete *et al.* 2020). Type strain *Lb. plantarum* ATCC14917<sup>TM</sup>, WCFS1 and two probiotics IMC510 and IMC513 (Synbiotec srl, Camerino, Italy) were included in the study as reference strains (Table 3).

## DNA isolation and genetic characterization

Total DNA was prepared individually for all isolates following overnight bacterial MRS broth cultures using GenElute<sup>TM</sup> Bacterial Genomic DNA kit (Sigma-Aldrich) following their instructions.

Six housekeeping genes were chosen for MLST analysis: *pgm* (encodes phosphoglucomutase), *ddl* (encodes for D-alanine-D-alanine ligase), *gyrB* (encodes the B subunit of DNA gyrase), *purK1* 

(encodes the ATPase subunit of phosphoribosylaminoimidazole carboxylase), *gdh* (encodes glutamate dehydrogenase) and *mutS* (encodes one DNA mismatch repair protein). Homologous DNA sequences of these candidate loci are available from GenBank. These candidate genes were selected based on MLST performed by de las Rivas *et al.* (2006) and on the criterion that they are widely separated on the chromosome, with the smallest distance recorded for *purK*1 and *gdh* which are 28.5 kb apart (Kleerebezem *et al.* 2003). Primer sequences are indicated in Table S2 and PCR was performed as described by de las Rivas *et al.* 2006.

## MLST data analysis

Sequence alignment, analysis and the identification of polymorphic sites were performed using the MEGA 7.0 software package (http://www.megasoftware.net). For each of the six loci, the sequences obtained for all isolates were compared and unique nucleotide sequences defined on the basis of deviation in sequence for each allele. Each isolate was defined by a unique allele profile or sequence type (ST) derived from the combination of alleles obtained at each locus. Isolates that shared the same allelic profile could belong to the same ST, while sequences with deviations, at a single nucleotide site or more were considered distinct alleles. The sequences of the six housekeeping genes of 22 *Lb. plantarum* isolates were concatenated. A phylogenetic tree based on concatenated sequences were constructed using the Neighbor-Joining method, with a Kimura two-parameter distance model in the MEGA 7.0 software package. The percentage of bootstrap confidence levels, for internal branches, was calculated from 1000 random resamplings as defined by the MEGA program (Kumar *et al.* 2016). The tree was rooted using *Bacillus subtilis* as an outgroup and an optimal tree was constructed.

## In vitro antimicrobial susceptibility assay

The degree of susceptibility of *Lb. plantarum* isolates to a range of antibiotics was assessed and taken as a readout of antibiotic resistance for each isolate. Each isolate was exposed to a range of different classes of antibiotics following the EFSA guidelines (EFSA 2008). Antimicrobial susceptibility was determined by disc diffusion using the agar overlay diffusion assay described by the National Committee for Clinical Laboratory Standards (Figure 1S). MRS agar was used for testing *Lb. plantarum* isolates and TSA medium was applied for benchmarking strains: *Escherichia coli* (Migula) Castellani and Chalmers (ATCC<sup>®</sup>25922<sup>TM</sup>) and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC<sup>®</sup>25923<sup>TM</sup>). Briefly, overnight cultures were inoculated into soft agar media, which was applied to overlay solid agar embedded with antibiotic containing discs at recommended concentrations (Table S3). Assays were incubated anaerobically at 37°C for 48 hours (Figure 1S). Growth inhibition. The average from three independent applications were expressed in terms of resistance, moderate susceptibility, or susceptibility, according to interpretative standards described by Charteris *et al.* 2008 (see Table S3).

## Lb. plantarum isolate specific influence on GI resident bacteria

An *in vitro* growth-inhibition assessment was applied to examine food-borne *Lb. plantarum* isolates interaction with each and with gut isolated *Lb.* microbes (Figure 1) including representative species isolated from the human microbiota, including as *Lb. reuteri* Cardioviva<sup>TM</sup> and *Lb. rhamnosus* GG, from UCC collection (Table S4).

## Acidification activity

Acidification is an important consideration regionally in GI tract. Strain specific acidification activity was assessed by monitoring the pH at 37°C during growth in MRS broth media. The pH was monitored at 0, 12, 24 and 48 hours and in triplicate at each time point.

## Data analysis

Each experiment was performed independently and in triplicate and the data are reported as average  $\pm$  standard deviation (SD). Acidification activity was analyzed using one-way analysis of variance. Differences between strain means were tested for significance by Bonferroni's multiple comparison test using Prism 5.0 (GraphPad Software Inc., La Jolla, CA).

## RESULTS

## MLST

## MLST loci selection and genetic diversity among Lb. plantarum isolates

Six widely distributed housekeeping gene loci were selected from *Lb. plantarum* genome following the system established by de las Rivas *et al.* (2006) see Table 1 and Table S2. For each locus: *pgm* (656 bp), *ddl* (702 bp), *gyrB* (626 bp), *purK*1 (525 bp), *gdh* (441 bp), *mutS* (594) sequence variation is presented in Table 1 below. For each locus comparisons were made relative to type strain *Lb. plantarum* WCFS1 genome sequence (Kleerebezem *et al.* 2003), allele numbers were assigned to each unique sequence and sequence type (ST) was assigned to each strain. The genetic diversity within each of the six housekeeping genes for each isolate is reported in Table 2. The MLST analysis revealed between 5 (*purK*1) and 74 (*gyrB*) polymorphic sites for each gene totalling 170 small nuclear polymorphisms (SNPs) among the loci. The DNA G+C percentage content of the different gene fragments ranged from 41.9 (*ddl*) to 48.3 (*gdh*) percentage. Each isolate examined showed a different sequence type (ST) so that 22 unique STs with different allelic combinations were resolved (Table 2).

#### Genetic relationships among food borne Lb. plantarum isolates based on MLST analysis

The allelic profile of all 22 isolates and type strain WCFS1 was applied for phylogenetic clustering analysis to uncover genetic relationship among *Lb. plantarum* isolates investigated in this study (Figure 2). The evolutionary distance was established using MEGA 7.0 (Kumar *et al.* 2016) by the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in Figure 2 (Felsenstein *et al.* 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004). Gaps and missing data were eliminated from analysis. Although all 22 isolates showed unique STs, they could be assigned to one of six different clades or clusters (Table 2, Figure 2). Clade 4 (specifically LT53, LAB49, LAB62 and LT100) clade 5 (LT52 and LAB40) and Clade 6 (LT99, LAB30 and LT21) represented the majority of *Lb. plantarum* isolates of raw cheese origin. Clade 3 included reference strains *Lb. plantarum* ATCC14917<sup>TM</sup> and *Lb. plantarum* WCFS1 and isolates of all origins with the exception of those isolated from raw cheeses. Clade 1 was dominated by *Lb. plantarum* isolates of table olives origin.

#### **Phenotypic tests**

#### Lb. plantarum isolate antibiotic resistance and susceptibility

Antibiotic disc and isolate overlay assay performed and analysed according to proposed EFSA guidelines (EFSA 2008) revealed that all isolates were susceptible to ampicillin, erythromycin, chloramphenicol and the streptogramins (quinopristin/dalfopristin). Partial or moderate susceptibility was evident in the presence of each of tetracycline, clindamycin, rifampicin and novobiocin. All of the isolates tested showed resistance to vancomycin and ciprofloxacin; streptomycin and kanamycin could not inhibit the growth of any *Lb. plantarum* isolates tested. The majority of isolates proved resistant to gentamycin, with the exception of four isolates (O5, O13 both from table olives; LT99 and LT100 both from raw cheeses), only partially inhibited by the tested concentrations. Nine isolates were resistant to Penicillin (2 units), whereas the others were moderately susceptible with the exception of *Lb. plantarum* WCFS1, this strain showed complete susceptibility to this antibiotic. Overall, irrespective of isolate origin and clade assignment, antibiotic resistance and susceptibility appears sporadic among isolates and is not related to origin or clade genetic assignment.

## Influence of Lb. plantarum isolates on growth in vitro

*In vitro* growth assessment to assess *Lb. plantarum* inter-isolate influence on growth *in vitro* was applied. The aim was to determine the potential of food-borne *Lb. plantarum* isolates to act against other microbes as displacement strains. The isolates were benchmarked against a background of known beneficial probiotic strains, including representative species isolated from the human microbiota, including *Lb. reuteri* Cardioviva<sup>TM</sup> and *Lb. rhamnosus* GG. Cross-compatibility for

growth indicated that all *Lb. plantarum* isolates permitted growth of each other over 48 hours without any inhibition (data not shown). Moreover, when tests were performed against 9 established probiotic strains representing different species, including two *Lb. plantarum* strains (299v and CSUR P691), no growth inhibition of probiotics was detected (Table S5). These results indicate, *in vitro*, that food-borne isolates of *Lb. plantarum* may not disturb the *Lb.* representative species of the human microbiota en route through the GIT.

#### Lb. plantarum isolates acidification activity

Acidification activity was assessed in order to indirectly assess lactic acid production and potential environment pH alteration during growth in MRS broth over a 48-hour time frame (Table S6). All isolates produced detectible lactic acid within 12 hours, with pH changes detected from an initial pH of  $6.18 \pm 0.01$  to within a range from  $4.30 \pm 0.02$  (21B) to  $3.74 \pm 0.01$  (WCFS1) (see Figure 3). The media acidification continued to reach an average value of  $3.55 \pm 0.02$  after 48 h of growth. All the isolates showed similar pH range and acidification behaviour with no significant differences between isolates (p>0.05) by ANOVA Bonferroni's test.

## DISCUSSION

Here, a collection of food-associated *Lb. plantarum* isolates were characterized genetically and phenotypically to include potential probiotic properties. According to the FAO/WHO guidelines in evaluating potential probiotic isolates for food applications, the genus and species of any potential probiotic bacteria must be established. Following from this, current evidence suggests that strain-specific effects should also be considered among bacterial isolates (FAO/WHO 2002). Intraspecific and interspecific discrimination is considered a fundamental preliminary step in the selection process of probiotics candidates, in order to link isolates both to species and to the strain levels. These efforts would allow monitoring of specific strain associated health effects and enable accurate surveillance and epidemiological studies (EFSA 2008).

The current taxonomy of the *Lb. plantarum* group identifies five closely related species among *Lactobacillus*: *Lb. plantarum* (subsp. *plantarum* and subsp. *argentoratensis*), *Lb. paraplantarum*, *Lb. pentosus*, *Lb. fabifermentans* and *Lb. xiangfangensis* (Siezen *et al.* 2011; Miyashita *et al.* 2015). In order to discriminate very closely related species of *Lb. plantarum* isolates and strains, several molecular methods have been developed combining comparative analysis based on phylogenetic molecular markers (16S rRNA, *recA*) and with molecular typing techniques such as RAPD-PCR, RFLP-PFGE, 16S ARDRA, ribotyping and repetitive element PCR (Corsetti *et al.* 2018). While discriminatory, these techniques have not delimited relative phylogenetic relationships among strains within the same species. We therefore investigated whether such relationships could be inferred from MLST analysis, a system based on differential molecular clock (or rate of mutation accumulation) of multiple loci, was developed for *Lb. plantarum* according to de la Rivas *et al.* (2006). We had previously identified all 18 isolates of food origin as *Lb. plantarum* species by performing molecular analysis such as 16S rRNA gene

sequencing (Corsetti *et al.* 2008), *rec*A gene multiplex PCR and RAPD-PCR (Prete *et al.* 2017), but this data did not infer phylogenetic analysis among the isolates.

The internal fragments of six loci (pgm, ddl, gyrB, purK1, gdh and mutS) were amplified from the DNA isolated from 22 Lb. plantarum isolates (Table 2). Analysis of the primary sequence confirmed the genetic heterogeneity of all isolates within this species. Each isolate represented a different sequence type (ST). Phylogenetic analysis recognized six ancestral sources of polymorphism within the Lb. plantarum isolates (Figure 2). However, only half groups or clades could be related to the different food sources of origin. Indeed three clades (Clade 4, Clade 5 and Clade 6) were dominated by isolates of raw cheese origin while those of table olive origin, were spread within three different clades (Clade 1, Clade 2, and Clade 3). Curiously, the type strain of *Lb. plantarum*, ATCC14917<sup>TM</sup> originally isolated from pickled cabbage, clustered with both sourdough and human-associated isolates and type strains within clade 3. Xu et al. (2015) reported a similar food origin relationship among their food borne isolates (179 in total) relative to reference strains (7 in total) analysed in their study. Our findings were not similar to that reported by de las Rivas and co-workers (2006), where they observed little relationship with the sources of just 16 isolates. Molenaar et al. (2005) substantiated our data; the relatively high level of genetic diversity among their 20 Lb. plantarum strains using microarray analysis was attributed to increased genome plasticity and integral rearrangements. The absence of an association to origin was proposed due to the versatility of individual Lb. plantarum strain, which can survive or even grow in different environments. However, it may be that the diverse range and origin of isolates applied to their study may not have held sufficient power to uncover these relationships. In this study, the vast majority of isolates were of raw cheese origin and they clustered into three different clades (Figure 2).

FAO/WHO recommendations support preliminary assessment of the safety and functionality of putative probiotic strains (FAO/WHO, 2006). Phenotypical *in vitro* assays provide a fundamental basis in preliminary evaluation of probiotic candidate features (including antibiotic resistance, physico-chemical properties, potential displacement of gut flora), particularly when new strains and/or species are considered (Cozzolino *et al.* 2020). These data may align with genetic distance when considered in conjunction with MLST analysis. In applying these recommendations, in this study, all *Lb. plantarum* isolates were phenotypically assessed *via* a limited number of *in vitro* assays; including acidification activity, susceptibility to different antibiotic classes (Charteris *et al.* 1998), and their potential gut community influence. All isolates tested were similar in their acidification abilities or lactic acid production at the different time points examined (Figure 3). *In vitro* growth-inhibition assessment showed an overall lack of strain displacement potential *in vitro* indicating that beneficial gut microbes of the same species may not be altered through their introduction to the human GIT microbiota (Figure 1, Table S5).

Of paramount importance in the selection of probiotic strains is the level and classes of antibiotic resistances carried by particular isolates. The genus *Lactobacillus* is the largest group among LAB and likely, represents the most widely applied probiotic bacteria (Marco and Golomb, 2016). The antibiotic susceptibility of *Lactobacillus* species has received little attention and this reflects their status as nonpathogenic, commensal bacteria with a time-honored reputation as health promoters. However, greater caution should be exercised in the selection and characterization of potential probiotic bacteria, particularly in light of the current drive by the global food industry to

apply them, for use in functional foods. Therefore, while antibiotic resistance *per se* is not a safety issue; it becomes such when the risk of resistance transfer (plasmidic or transposable) is present.

With this perspective, all Lb. plantarum isolates were assayed for their susceptibility to different class of antibiotics. The resistance levels detected for the selected isolates are reported in Table 3. All isolates examined showed susceptibility to inhibitors of cell wall synthesis (ampicillin) and to the broad-spectrum antibiotics (tetracycline, chloramphenicol, clindamycin), including streptogramins and inhibitors of nucleic acid synthesis (rifampicin, novobiocin). In addition, complete growth inhibition was evident for all isolates in response to erythromycin, a Grampositive spectrum antibiotic, known for its effective inhibition of LAB (Zhou et al, 2005) (see Table 3). On the other hand, all *Lb. plantarum* isolates were resistant to five antibiotics: vancomycin, gentamycin, streptomycin, kanamycin and ciprofloxacin (Table 3). Interestingly, the vancomycin-resistant phenotype widespread in Lactobacillus is perhaps the best-characterized intrinsic resistance in LAB (Hollimann et al. 1988; Ruoff et al. 1988; Nicas et al. 1989) and is due to the presence of D-alanine: D-alanine ligase-related enzymes (Elisha and Courvalin 1995). Indeed, lactobacilli, *Pediococcus* spp. and *Leuconostoc* spp. have a high natural resistance to vancomycin, a property that proves useful to separate them from other Gram-positive bacteria (Hamilton-Miller and Shah, 1998; Simpson et al. 1988). In addition, resistance to aminoglycoside antibiotics, such as kanamycin and gentamicin had already been observed for Lb. plantarum strains isolated from fermented vegetables (Petrovic et al. 2012; Botta et al. 2014), this resistance could be considered as an intrinsic feature of LAB, lacking cytochrome-mediated electron transport, a system which normally mediates drug uptake in bacteria (Hummel et al. 2007). Our data show that little differences exist and that any difference detected was not clade or food origin related. Some probiotic strains with intrinsic antibiotic resistance could be useful for restoring the gut microbiota after antibiotic treatment (Gueimonde et al. 2013). However, specific antibiotic resistance determinants carried on mobile genetic elements, such as tetracycline resistance genes, are often detected in the typical probiotic genera, and constitute a reservoir of resistance for potential food or gut pathogens, thus representing a serious safety issue (Gueimonde et al. 2013). Indeed, in a QPS system the safety assessment of food LAB could be limited to the presence of transmissible antibiotic resistance markers as other tests are not relevant for LAB (Marthur et al. 2005). This study did not examine whether resistance detected was plasmidic or chromosomal in origin and therefore we did not determine potential transmissibility. It would be interesting to perform whole genome and DNA sequencing for these isolates in order to decipher antibiotic transmissibility as well as their genetic and metabolic potential. In doing so, isolate and strain-specific properties might be revealed.

Finally, this study did not confirm any evident correlation between phenotypic features and isolation source among the limited range of 22 isolates applied to this study. This lack of correlation may be due to the versatility of individual *Lb. plantarum* strains, they can persist and proliferate in a diverse range of different environments. The power of the study could be increased by expanding the range and number of isolates associated with these diverse food environments. In support of this, an association between food sources and bacteria functionality has been reported with larger isolate numbers (Cao *et al.* 2013; Tremonte *et al.* 2017). While probiotic features (genetic and phenotypic) are recognized as strain-specific (Morelli *et al.* 2007), collating

commonalities to identify important features that correlate with specific food fermentation ability would provide a valuable resource for purpose driven future strain and isolate selection.

#### CONCLUSIONS

This study highlights the importance of preliminary characterization of new probiotic strains by combining phenotypic and genotyping methods. Overall, our data support previously recognized plasticity of *Lb. plantarum* species, but among our range of isolates genetic correlation with food source was evident only for dairy strains. Phenotyping testing indicated similar promising properties and antibiotic resistance patterns among all *Lb. plantarum* isolates. The applied combined approach may be applied as a useful tool for the precise and unambiguous characterization of *Lb. plantarum* isolates. It also considers the safety features of the isolates. This study constitutes a preliminary screen of food-borne *Lb. plantarum* isolates for important strain specific features that can be expanded for future strain and food fermentation product development.

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Conflict of interest. None declared.

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**Figure 1.** Interaction assessment of *Lb. plantarum*. Here, shown ATCC14917<sup>TM</sup> (1) and *Lb. plantarum* WCFS1 (2) are applied as donor strains (centre line in each agar plate) against **A.** *Lb. reuteri* CARDIOVIVA<sup>TM</sup>, *Lb. rhamnosus* GG. **B.** *Lb. casei* NCDO161, *Lb. salivarius* UCC118. **C.** *Lb. rhamnosus* CSUR PS67, *Lb. sakei* CSURP1130 and *Lb. rhamnosus* CSUR P994 represented as perpendicular to the donor strain and in parallel to each other. Inhibition of growth was represented by a lack of growth in the region of strain crossover.



#### 005

**Figure 2.** Genetic relationship of the *Lb. plantarum* isolates characterized in this study. Neighbor-joining phylogenetic tree infers evolutionary distance using the Neighbor-Joining method, computed based on the concatenated nucleotide sequence of six MLST housekeeping loci. Bar scale indicates phylogenetic distances. Bootstrap values are reported for 1000 replicates. The tree was rooted using *Bacillus subtilis* as outgroup. Shaded colour-based blocks indicate the different *Lb. plantarum* phylogenetic clusters.



**Figure 3.** Acidification activity, over time, of *Lb. plantarum* isolates. **A.** Reference strains, **B.** Isolates from table olives, **C.** Isolates from sourdough, **D.** Isolates from raw-milk cheeses. Mean values from three biological replicates. All the isolates showed significant differences (p<0.05) compared to the control (MRS broth), but no significance were found among them, by ANOVA Bonferroni's test.

Gene	Fragment size	Mean GC content	No. of alleles	No. of polymorphic sites
	(bp)	(%)		
pgm	656	42.0	7	16
ddl	702	41.9	10	8
gyrB	626	44.3	4	74
purK1	525	46.5	5	5
gdh	441	48.3	19	38
mutS	594	46.7	21	29

**Table 1.** Sequence variation detected was at six different loci among *Lb. plantarum* strains and isolates.

				Allele no. at locus									
Isolates/Strains	Origin	MLST cluster	ST	pgm	ddl	gyrB	purK1	gdh	mutS				
WCFS1	Human saliva	CL3	1	1	1	1	1	1	1				
ATCC14917 <sup>TM</sup>	Pickled cabbage	CL3	2	2	2	1	2	2	2				
IMC510	Human gut	CL3	3	3	1	1	3	3	3				
IMC513	Human gut	CL1	4	2	3	1	2	2	4				
O5	Table olives	CL1	5	4	3	1	2	4	5				
013	Table olives	CL3	6	3	1	1	3	5	6				
N14	Table olives	CLI	7	5	3	1	2	6	7				
C9O4	Table olives	CLI	8	6	3	1	2	7	8				
C9S2	Table olives	CL2	9	2	3	1	2	2	9				
21B	Sourdough	CL1	10	7	3	1	2	8	10				
CF1	Sourdough	CL3	11	3	4	1	3	9	11				
LAB1	Raw-milk cheeses	CL1	12	2	5	1	2	10	12				
LAB30	Raw-milk cheeses	CL6	13	1	6	2	3	11	13				
LAB32	Raw-milk cheeses	CL2	14	2	3	1	2	12	14				
LAB40	Raw-milk cheeses	CL5	15	2	7	3	3	13	15				
LAB49	Raw-milk cheeses	CL4	16	2	7	3	1	14	16				
LAB62	Raw-milk cheeses	CL4	17	2	8	1	3	15	17				
LT21	Raw-milk cheeses	CL6	18	3	6	2	3	16	18				
LT52	Raw-milk cheeses	CL5	19	2	9	4	3	17	19				
LT53	Raw-milk cheeses	CL4	20	2	10	1	4	13	20				
LT99	Raw-milk cheeses	CL6	21	1	6	2	5	18	18				
LT100	Raw-milk cheeses	CL4	22	2	7	1	3	19	21				

Table 2. Genetic properties of *Lb. plantarum* strains and isolates characterized in the study.

GroupNaGroup 1PenicillinInhibitors of cell wall synthesisVancomycinGroup 2TetracyclineInhibitors of protein synthesisChloramphenGroup 2TetracyclineInhibitors of protein synthesisChloramphenGroup 3Ciprofloxacinother: StreptograminsQuinopristin/Group 3CiprofloxacinInhibitors of nucleic acid synthesisRifampicin	AT 149 me M I S M	rcc v 17 <sup>T</sup> 15 R S	VCSF1 S R S	IMC 510 MS R S	IMC 513 R R	O5 MS R	O13 MS R	N14 MS	C9O4 MS	<b>C9S2</b> R	21B	CF1	LAB1	LAB 30	LAB 32	LAB 40	LAB 49	LAB 62	LT 21	LT 52	LT 53	LT 99	LT 100
GroupNaGroup 1PenicillinInhibitors of cell wall synthesisVancomycinSynthesisAmpicillinGroup 2TetracyclineInhibitors of protein synthesisChloramphenInhibitors of protein synthesisErytromycinGroup 3Clindamycinother: StreptograminsQuinopristin/Group 3CiprofloxacinInhibitors of nucleic acid synthesisRifampicin	149 me I S N nicol	917 <sup>T</sup> 1S R S 1S	S R S	510 MS R S	513 R R	O5 MS R	O13 MS R	N14 MS	C9O4 MS	C9S2 R	21B	CF1	LAB1	30	32	40	49	62	21	52	53	99	100
GroupNaGroup 1PenicillinInhibitors of cell wall synthesisVancomycinGroup 2TetracyclineInhibitors of protein synthesisChloramphenGroup 2ErytromycinChloramphen StreptograminsChloramphenother: StreptograminsQuinopristin/Group 3CiprofloxacinInhibitors of nucleic acid synthesisRifampicin	ume M	1S R S 1S	S R S	MS R S	R R	MS R	MS R	MS	MS	R	D												
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Group 2TetracyclineInhibitors of protein synthesisChloramphen ErytromycinErytromycin Clindamycin GentamycinKanamycin Streptograminsother: StreptograminsGroup 3Ciprofloxacin Rifampicin synthesis	M nicol S	<b>1S</b>			3	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Inhibitors of protein synthesisChloramphen StreptomycinOther: StreptograminsErytromycin Clindamycin Gentamycin StreptomycinOther: StreptograminsQuinopristin/ Cliprofloxacin Rifampicin Novobiocin	nicol		MS	MS	MS	S	MS	MS	MS	MS	MS	MS	S	S	MS	S	MS	MS	MS	S	MS	MS	S
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Clindamycin Gentamycin Kanamycin Streptomycin Group 3 Inhibitors of nucleic acid synthesis Novobiocin	5	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	s vnloa
Gentamycin Kanamycin Streptomycin other: Streptogramins Group 3 Inhibitors of nucleic acid synthesis Novobiocin	5	s	S	MS	S	S	MS	S	S	S	S	MS	MS	MS	S	MS	MS	MS	S	S	MS	S	MS ded
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Streptomycin other: Streptogramins Group 3 Inhibitors of nucleic acid synthesis Novobiocin	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R ://
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Novobiocin	Ν	<b>1S</b>	MS	MS	MS	S	MS	S	MS	MS	MS	MS	MS	R	S	S	S	S	S	S	S	MS	ance-arti
	Ν	1S	S	MS	MS	S	MS	S	MS	MS	S	MS	S	S	S	S	S	S	S	S	MS	MS	MS abs
*Susceptibility and resistance	is expressed as R	2: resista	ant; MS	: mode	erately	susce	ptible	; or S:	suscept	ible.	2												act/doi/10.1093/femsle/fnaa076/5835222 by University College Cork user