Genotypic and phenotypic characterization of food-associated *Lactobacillus plantarum* isolates for potential probiotic activities

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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 susceptibility and resistance is 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^{TM}, 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^{TM} 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®25922™) and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC®25923™). 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 zone diameter was calculated using sliding callipers for each disc and any zone of 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™ 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 ± 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), *gyr*B (626 bp), *pur*K1 (525 bp), *gdh* (441 bp), *mut*S (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 (*pur*K1) and 74 (*gyr*B) 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^{TM} 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^{TM} 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 ± 0.01 to within a range from 4.30 ± 0.02 (21B) to 3.74 ± 0.01 (WCFS1) (see Figure 3). The media acidification continued to reach an average value of 3.55 ± 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, *rec*A) 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*, *gyr*B, *pur*K1, *gdh* and *mut*S) 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^{TM} 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 Gram-positive 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.

References

Achtman M, Wain J, Weill FX *et al.* Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathog* 2012;**8(6)**: e1002776.

Benincasa C, Muccilli S, Amenta M *et al.* Phenolic trend and hygienic quality of green table olives fermented with *Lactobacillus plantarum* starter culture doi. *Food Chem* 2015;**186**,271-276, DOI: 10.1016/j.foodchem.2015.02.010.

Botta C, Langerholc T, Cencic A *et al.* In vitro selection and characterization of new probiotic candidates from table olive microbiota. *PLoS ONE* 2014;**9(4)**: e94457, DOI:10.1371/journal.pone.0094457.

Cao H, Zheng W, He S *et al.* Identification of up-regulated proteins potentially involved in the antagonism mechanism of *Bacillus amyloliquefaciens* G1. *Antonie van Leeuwenhoek* 2013;**103**:1395-1404.

Charteris WP, Kelly PM, Morelli L. *et al.* Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. *J. Food Prot* 1998; **61**:1636-1643.

Corsetti A, Prete R, Garcia-Gonzalez N. Lactic Acid Bacteria: *Lactobacillus* spp.: *Lactobacillus plantarum. Reference Module in Food Sciences*. *Elsevier*, 2018;1–9.

Cozzolino A, Vergalito F, Tremonte P. *et al.* Preliminary evaluation of the safety and probiotic potential of *Akkermansia muciniphila* DSM 22959 in comparison with *Lactobacillus rhamnosus* GG. *Microorganisms* 2020; **8**:189, DOI: 10.3390/microorganisms8020189.

Danielsen M, Wind A. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int J Food Microbiol* 2003;**82**:1-11.

de la Rivas B, Marcobal A Munoz R. Allelic diversity and population structure in *Oenococcus oeni* as determined from sequence analysis of housekeeping genes. *Appl Environ microbiol* 2004;**70**:7210-7219, DOI: 10.1128/AEM.70.12.7210–7219.2004.

de la Rivas B, Marcobal A, Munoz R. Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiol* 2006*;***152**:85–93, DOI:10.1099/mic.0.28482-0.

EFSA. Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance (Technical guidance). *The EFSA Journal* 2008;**732**: 1–15.

Elisha BG, Courvalin P. Analysis of genes encoding d-alanine: d-alanine ligase-related enzymes in *Leuconostoc mesenteroides* and *Lactobacillus* spp. *Gene* 1998;**152**:79–83.

Elkins CA, Mullis LB Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. *Appl Environ Microbiol* 2004;**70**:7200–7209.

FAO/WHO (2002) Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food London, Ontario, Canada, April 30 and May 1, 2002.

FAO/WHO. Probiotics in food. Health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition, Paper 85 Roma, 2006.

Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;**39**:783-791.

Garcia-Gonzalez N, Prete R, Battista N *et al.* Adhesion properties of food-associa*ted Lactobacillus plantarum* strains on human intestinal epithelial cells and modulation of IL-8 release. *Front Microbiol* 2018;**9**:2392, DOI: 10.3389/fmicb.2018.02392.

Gardner NJ, Savard T, Obermeier P *et al.* Selection and characterization of mixed starter cultures for lactic acid fermentation of carrot, cabbage, beet and onion vegetable mixtures. *Int J Food Microbiol* 2001;**64**(3):261-275, DOI:10.1016/S0168-1605(00)00461-X.

Gueimonde M, Borja Sánchez B, de los Reyes-Gavilán CG *et al.* Antibiotic resistance in probiotic bacteria *Front Microbiol* 2013;**4**:202, DOI:10.3389/fmicb.2013.00202.

Hamilton-Miller JMT, Shah S. Vancomycin susceptibility as an aid to the identification of lactobacilli. *Lett Appl Microbiol* 1998;**26**:153-154.

Holliman RE, Bone GP. Vancomycin resistance of clinical isolates of lactobacilli. *J Infect* 1988;**16**:279-283.

Hummel AS, Hertel C, Holzapfel, WH *et al.* Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Appl Environ Microbiol* 2007;**73**:730-739.

Kim KS, Morrison JO, Bayer AS. Deficient autolytic enzyme activity in antibiotic-tolerant lactobacilli. *Infect Immun* 1982;**36**:582-585.

Kleerebezem M, Boekhorst J, van Kranenburg, R *et al.* Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc Natl Acad Sci USA* 2003;**100**:1990-1995.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;**33**:1870-1874.

Luxananil P, Promchai R, Wanasen S *et al.* Monitoring *Lactobacillus plantarum* BCC 9546 starter culture during fermentation of Nham, a traditional Thai pork sausage. *Int J Food Microbiol* 2009;**129**(3):312-315, DOI: 10.1016/j.ijfoodmicro.2008.12.011.

Maiden MC, Bygraves JA, Feil E e*t al.* Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA.* 1998;**95**(6):3140-5.

Marthur S, Singh R. Antibiotic resistance in food lactic acid bacteria - a review. *Int J Food Microbiol* 2005;**105**:281–295.

Miyashita M, Yukphan P, Chaipitakchonlatarn W *et al. Lactobacillus plajomi* sp. nov. and *Lactobacillus modestisalitolerans* sp. nov., isolated from traditional fermented foods. *Int J Syst Evol Microbiol* 2015;**65**:2485-2490.

Molenaar D, Bringel F, Schuren FH *et al.* Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *J Bacteriol* 2005;**187**(17):6119-6127.

Morelli L. In vitro assessment of probiotic bacteria: from survival to functionality. *Int. Dairy J.* 2007;**17**: 1278-1283.

Nicas TI, Cole CT, Preston DA *et al.* Activity of glycopeptides against vancomycin resistant gram-positive bacteria. *Antimicrob Agents Chemother* 1989;**33**:1477-1481.

Oh PL, Benson AK, Peterson DA *et al.* Diversification of the gut symbiont *Lactobacillus reuteri* as a result of host-driven evolution. *ISME J.* 2010;**4**(3):377–87.

Petrovic T, Dimitrijevic S, Radulovic Z *et al.* Comparative analysis of the potential probiotic abilities of lactobacilli of human origin and from fermented vegetables. *Arch Biol Sci* 2012;**64**:1473–1480.

Pisano MB, Patrignani F, Cosentino S *et al.* Diversity and functional properties of *Lactobacillus plantarum*-group strains isolated from Italian cheese products. *Dairy Sci Technol* 2011;**91**(1):65–76.

Prete R, Tofalo R, Federici E *et al.* Food-Associated *Lactobacillus plantarum* and yeasts inhibit the genotoxic effect of 4-nitroquinoline-1-oxide. *Front Microbiol* 2017;8:2349, DOI:10.3389/fmicb.2017.02349.

Prete R, Long SL, Gallardo AL *et al.* Beneficial bile acid metabolism from *Lactobacillus plantarum* of food origin. *Sci Rep* 2020;10:1165, DOI: 10.1038/s41598-020-58069-5.

Ragon M, Wirth T, Hollandt F *et al.* A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathog* 2008;**4**(9):e1000146.

Rubio R, Aymerich T, Bover-Cid S *et al.* Probiotic strains *Lactobacillus plantarum* 299 V and *Lactobacillus rhamnosus* GG as starter cultures for fermented sausages. *Lwt-Food Sci Technol* 2013;**54**(1): 51-6.

Ruoff KL, Kuritzkes DR, Wolson JS *et al.* Vancomycin-resistant Gram-positive bacteria isolated from human sources. *J Clin Microbiol* 1988;**26**:2064-2068.

Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;**4**:406-425.

Siezen RJ, van Hylckama Vlieg JET. Genomic diversity and versatility of *Lactobacillus plantarum*, a natural metabolic engineer. *Microb Cell Fact* 2011;10:S1-S3.

Simpson WJ, Hammond JRM, Miller RB. Avoparcin and vancomycin - Useful antibiotics for the isolation of brewery lactic acid bacteria. *J Appl Bacteriol* 1988;**64**:299-309.

Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA.* 2004;101:11030-11035.

Teuber M, Meile L, Schwarz F. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie Van Leeuwenhoek* 1999;76:115–137.

Tremonte P, Pannella G, Succi M *et al.* Antimicrobial activity of *Lactobacillus plantarum* strains isolated from different environments: A preliminary study. *Int Food Res J* 2017;**24**: 852-859.

Wouters D, Grosu-Tudor S, Zamfir M *et al.* Applicability of *Lactobacillus plantarum* IMDO 788 as a starter culture to control vegetable fermentations. *J Sci Food Agric* 2013;**93**(13): 3352-61.

Xu H, Liu W, Zhang W *et al.* Use of multilocus sequence typing to infer genetic diversity and population structure of *Lactobacillus plantarum* isolates from different sources. *BMC Microbiol* 2015;**15**:241, DOI: 10.1186/s12866-015-0584-4.

Zago M, Fornasari ME, Carminati D *et al.* Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiol* 2011;**28**(5):1033– 1040.

Zago M, Lanza B, Rossetti L *et al.* Selection of *Lactobacillus plantarum* strains to use as starters in fermented table olives: Oleuropeinase activity and phage sensitivity. *Food Microbiol* 2013;**34**(1):81–87.

Zhang H, Liu L, Hao Y et al. Isolation and partial characterization of a bacteriocin produced by *Lactobacillus plantarum* BM1 isolated from a traditionally fermented Chinese meat product. *Microbiol Immunol* 2013;**57**(11):746–755.

Zhou JS, Pillidge CJ, Gopal PK *et al.* Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int J Food Microbiol* 2005;**98**:211–217.

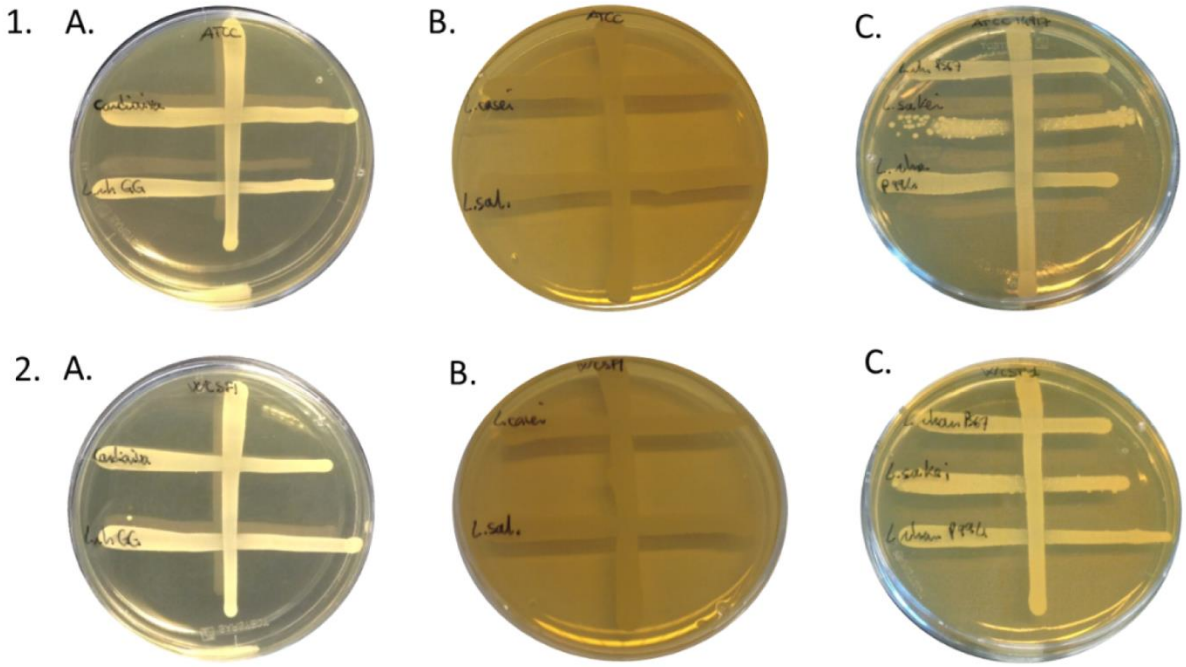

Figure 1. Interaction assessment of *Lb. plantarum.* Here, shown ATCC14917^{TM} (1) and *Lb. plantarum* WCFS1 (2) are applied as donor strains (centre line in each agar plate) against **A.** *Lb. reuteri* CARDIOVIVA^{TM}, *Lb. rhamnosus* GG. **B.** *Lb. casei* NCDO161, *Lb. salivarius* UCC118. **C.** *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.

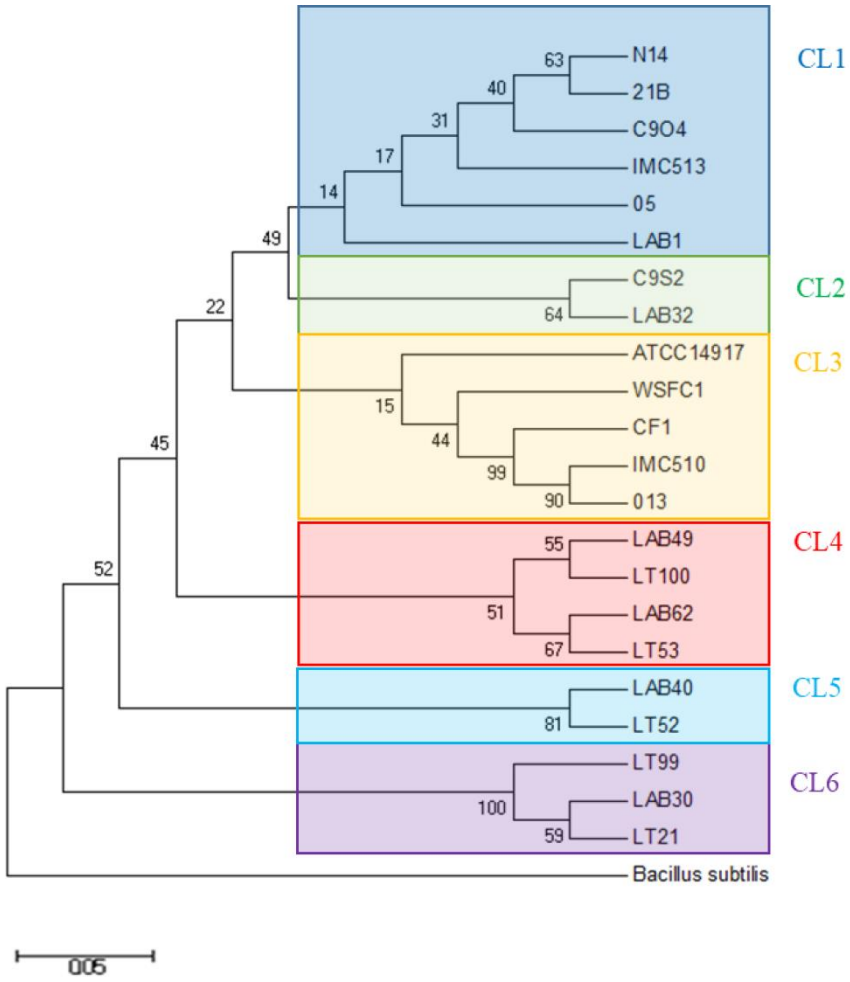

Figure 2. Genetic relationship of the *Lb. plantarum* isolates characterized in this study. Neighbor-joining phylogenetic tree infers evolutionary distance using the Neighbor-Joining method, computed based on the concatenated nucleotide sequence of six MLST housekeeping loci. Bar scale indicates phylogenetic 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.

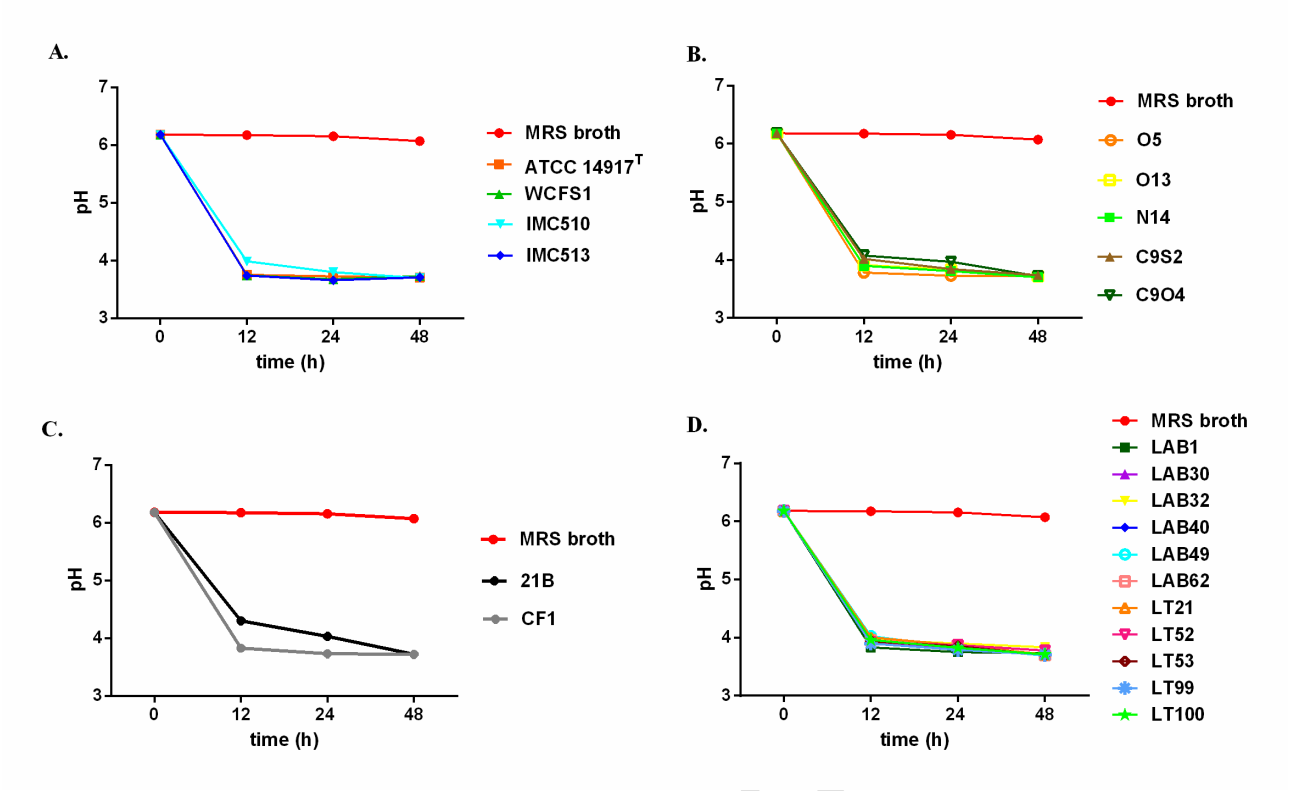

Figure 3. Acidification activity, over time, of *Lb. plantarum* isolates. **A.** Reference strains, **B.** Isolates from table olives, **C.** Isolates from sourdough, **D.** Isolates from raw-milk cheeses. Mean values from three biological replicates. All the isolates showed significant differences ($p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

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

Gene	Fragment size (bp)	Mean GC content (%)	No. of alleles	No. of polymorphic sites
pgm	656	42.0	7	16
ddl	702	41.9	10	8
*gyr*B	626	44.3	4	74
*pur*K1	525	46.5	5	5
gdh	441	48.3	19	38
*mut*S	594	46.7	21	29

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

Isolates/Strains	Origin	MLST cluster	ST	Allele no. at locus					
				pgm	*ddl*	*gyrB*	*purK1*	*gdh*	*mutS*
WCFS1	Human saliva	CL3	1	1	1	1	1	1	1
ATCC14917^{TM}	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
O13	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 3. Antimicrobial susceptibility and resistance of 22 *Lb. plantarum* strains and isolates.

Antimicrobial agents		Antimicrobial susceptibility of *L. plantarum* isolated strains[a]																					
		ATCC 14917[T]	WCSF1	IMC 510	IMC 513	O5	O13	N14	C9O4	C9S2	21B	CF1	LAB1	LAB 30	LAB 32	LAB 40	LAB 49	LAB 62	LT 21	LT 52	LT 53	LT 99	LT 100
Group	**Name**																						
Group 1	**Penicillin**	MS	S	MS	R	MS	MS	MS	MS	R	R	MS	R	MS	R	MS	MS	R	MS	R	R	MS	R
Inhibitors of cell wall synthesis	**Vancomycin**	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	Ampicillin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Group 2	**Tetracycline**	MS	MS	MS	MS	S	MS	MS	MS	MS	MS	MS	S	S	MS	S	MS	MS	MS	S	MS	MS	S
Inhibitors of protein synthesis	**Chloramphenicol**	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Erytromycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Clindamycin	S	S	MS	S	S	MS	S	S	S	S	MS	MS	MS	S	MS	MS	MS	S	S	MS	S	MS
	Gentamycin	R	R	R	R	MS	MS	S	R	R	R	R	R	R	R	R	R	R	S	R	R	MS	RS
	Kanamycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	Streptomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
other: Streptogramins	**Quinopristin/Dalfopristin**	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Group 3	**Ciprofloxacin**	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Inhibitors of nucleic acid synthesis	**Rifampicin**	MS	MS	MS	MS	S	MS	S	MS	MS	MS	MS	MS	R	S	S	S	S	S	S	S	MS	MS
	Novobiocin	MS	S	MS	MS	S	MS	S	MS	MS	S	MS	S	S	S	S	S	S	S	S	MS	MS	MS

*Susceptibility and resistance is expressed as R: resistant; MS: moderately susceptible; or S: susceptible.