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Phenotypic and Genome-based Approaches for Antibiotic Resistance Assessment in Lactic Acid Bacteria: a Focus on the Genera *Leuconostoc* and *Lactobacillus*

S.S.D. AGR/16

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Abstract

Antibiotics represent one of the largest therapeutic categories used in human and veterinary medicine for the treatment of infectious diseases caused by bacterial agents (Fair *et al*., 2014). Before the introduction of antibiotics, these diseases were the leading cause of morbidity and mortality in human populations (Aminov, 2016). However, overprescribing and misuse of antibiotics in medicine, aquaculture and agriculture has tremendously raised the emergence and spread of antibiotic resistant bacteria, which constitute a serious problem for the health of both humans and animals (Berendonk *et al*., 2015). In fact, these microorganisms can be present in food as: i) part of the natural microbial population of raw materials, ii) part of technological cultures intentional added during the food processing, or iii) a result of environmental contamination (Verraes et al., 2013). Thus, foodstuffs could represent vectors for the spread of antibiotic resistance (AR) along the food chain to the human gastrointestinal tract, where the effectiveness of therapies could be compromise (Founou *et al*., 2016). Lactic acid bacteria (LAB) have been extensively used as probiotics and starter cultures due to their long history of safe use and several strains have the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (Kechagia *et al*., 2013; Casado Muñoz *et al*., 2016), which include members of the genus *Leuconostoc* and *Lactobacillus* (Ricci *et al*., 2017). However, limited information on the antimicrobial susceptibility profiles of *Leuconostoc* spp. is available, as well as their possible involvement in the dissemination of AR determinants between bacteria. On the other hand, even though more than 35 *Lactobacillus* species meet the criteria of QPS proposed by EFSA, a considerable number of resistant lactobacilli has been reported.

In the present study, the antimicrobial susceptibility profiles and the genetic basis of the resistance were investigated for both the genera *Leuconostoc* and *Lactobacillus* through the application of standard methods, such as phenotypic testing, conjugation experiments and PCR assay, and whole-genome sequencing (WGS)-based approaches.

In the first part of the dissection, the contribution of these two approaches in the characterization of AR features for the genus *Leuconostoc* was investigated, revealing that genome-based analysis was more informative than conventional molecular techniques, providing data about any resistance gene or mutation present in a single microbial genome. Indeed, WGS-based analysis revealed the presence of genes coding for aminoglycoside resistance, such as *aad6*, *sat*4 and *aphA*-3, for streptogramin A resistance, as *vat*E, and for tetracycline, such as *tet*(S), in the genome sequence of the multidrug resistant *L. mesenteroides* subsp. *mesenteroides* LbE16 strain. In addition, this

approach highlighted for the first time the presence of a *erm*(B)-bearing *Tn*917 transposon in the genome of *L. mesenteroides* subsp. *dextranicum* LbE15, which showed high similarity (99%) to the nucleotide sequence of the corresponding transposon of *E. faecalis*, *B. subtilis*, *S. aureus*, and several species of *Streptococcus*. Antibiotic susceptibility testing and conjugation experiments allowed to confirm the resistance phenotype for those *Leuconostoc* strains and provided the first evidence of the erythromycin resistance transfer between *L. mesenteroides* and *E. faecalis*, supplying novel proof that AR LAB can act as a reservoir of acquired AR genes. Moreover, the gene *lsa*A was found for the first time in *L. pseudomesenteroides* LMG 11482^T and *L. fallax* LMG 13177^T through genome-based analysis, and its role in the clindamycin and quinupristin-dalfopristin resistance phenotype characterizing *L. pseudomesenteroides* strains was confirmed through the relative quantification of the gene expression. Therefore, standard methods should be combined with the WGS-based approaches to validate the hypothesis emerged from the *in silico* prediction of AR features. Moreover, manual annotation of the data obtained from homology based-methods against a reference AR database has crucial importance to minimize false positive and/or false negative outputs.

In the last part of the dissection, the combination of phenotypic susceptibility testing and genome-based analysis was performed for the whole genus *Lactobacillus*, which has a crucial economic and scientific impact in food productions and human health as probiotics and starter cultures. This analysis revealed a positive correlation between phenotype and genotype for the 67% of the cases examined, where the genes *aac*(3), *lsa* and *cml*(A) involved in the resistance towards aminoglycoside, clindamycin and chloramphenicol, respectively, were found for the first time in *Lactobacillus* strains. In addition, acquired determinants coding for tetracycline and erythromycin resistance were simultaneously detected in *L. amylophilus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T. Whereas, *L. ingluviei* DSM 15946^T harboured the *Tn*916-like transposon carrying the genes *tet*(M) and *tet*(L), highlighting the potential of these AR genes to be horizontally transferred to other microorganisms.

The results reported in this study may be utilized as a starting point for the generation of new and more focused scientific protocols and regulatory procedures based on WGS approaches for the safety assessment of *Leuconostoc* and *Lactobacillus* strains employed as starter cultures, food preservatives or probiotic by food and probiotic stakeholders. The implementation of AR genes available for LAB could result in a paradigm shift from phenotype-to genotype-based assessment of the resistance not only for pathogens, but also for food-borne and technological bacteria. Therefore, WGS-based approaches could be used as a tool for the surveillance of the emergence and spread of AR determinants in bacteria, providing: (i) an important initial contribution to the

identification of genes potentially associated with resistance; and (ii) relevant information about the possibility of AR genes to be spread along the food chain.

Preface

The ever-increasing magnitude of antibiotic resistance (AR) is a global public health challenge. Indeed, the overprescribing of antibiotics has tremendously raised the emergence and spread of antibiotic resistant bacteria in the food chain (Verraes *et al*., 2013). Food may act as a vector for the transfer of AR bacteria and resistance genes to humans.

Whole-genome sequencing (WGS) is becoming an important tool in surveillance the emergence and spread of AR (Schürch and van Schaik, 2016). Indeed, WGS offers the unprecedented advantage of providing genetic information at the whole genome level, thus making it ideal for uncovering all possible genetic determinants of antimicrobial resistance in a single microbial genome (Chan, 2016).

This project aims at investigating the contribution of standard methods and WGS-based analysis in the research area of antimicrobial resistance in the specific case of lactic acid bacteria. To achieve this purpose, antibiotic susceptibility testing, PCR assay, conjugation experiments, WGS sequencing and genome-based analysis were applied to *Leuconostoc* strains isolated from cheese and to the type strains of the genus *Leuconostoc*. While, the antibiotic susceptibility profile of the whole genus *Lactobacillus* was performed under the phenotypic and genomic point of view thanks to the availability of genomes of almost all species of this genus.

Structure of the thesis

This thesis is structured as follows: after the introduction, chapters were divided in two parts, the first regards the characterization of the AR profiles for the genus *Leuconostoc* through the application of standard approaches (antibiotic susceptibility testing, PCR assay and conjugation experiments) combined with WGS-based analysis. While, the second part focus on the improvement of the current knowledges about the AR in the whole genus *Lactobacillus* from the phenotypic and genomic point of view.

Chapter 1 consists in a general overview of the basic concepts about AR, from the role of the antibiotics in medicine to the emergence of resistant bacteria, including the main issue linked to the presence of those bacteria in the food chain, which could act as vector to the human

gastrointestinal tract. Finally, the available technologies for the surveillance of the emergence and spread of resistance are elucidated.

Part 1 contains three chapters: the first one includes the characterization of the antibiotic susceptibility profiles of dairy *Leuconostoc* and the transfer of resistance genes *in vitro* and in a food matrix (Chapter 2). Chapter 3 regards the use of WGS for the identification of AR genes in three resistant *Leuconostoc mesenteroides* strains isolated from cheese, while Chapter 4 concerns the application of genome-based analysis for the assessment of AR in the genus *Leuconostoc*.

Part 2 is about the characterization of the antibiotic susceptibility profiles and the utilization of genome-based analysis to reveal the resistance genetic basis of the whole genus *Lactobacillus*, which is probably the most widely used as probiotics and starter cultures in a variety of foods, also due to their long history of safe use.

Finally, Chapter 6 highlights the improvement of the current knowledges about the AR in lactic acid bacteria, including the genus *Leuconostoc* and *Lactobacillus*, due to the increasing availability of genome sequences and the advantages provided by the use of genome-based analysis for the identification of the AR determinants and the possibility of those genes to be spread along the food chain.

Publications

Several parts of this project have been already published:

- Results in Chapter 2 have been already published and presented as a poster:
    - **Flórez AB[*], Campedelli I[*], Delgado S, Alegría A, Salvetti E, Felis GE, Mayo B, Torriani S.** 2016. Antibiotic susceptibility profiles of dairy *Leuconostoc*, analysis of the genetic basis of atypical resistances and transfer of genes in vitro and in a food matrix. PloS One, 11: 1. (*: contributed equally to this work)
    - **Flórez AB, Campedelli I, Delgado S, Alegría Á, Salvetti E, Felis GE, Torriani S, Mayo B.** Whole genome analysis as a tool for the safety assessment of antibiotic resistance in food-processing bacteria. EFSA's 2nd Scientific Conference "Shaping the Future of Food Safety, Together", Milan; October 14th–16th, 2015

- Part of the results in Chapter 3 have been already published on the Genome Announcements Journal:
    - **Campedelli I*, Flórez AB*, Salvetti E, Delgado S, Orrù L, Cattivelli L, Alegría Á, Felis GE, Torriani S, Mayo B.** 2015. Draft Genome Sequence of Three Antibiotic-Resistant *Leuconostoc mesenteroides* Strains of Dairy Origin. Genome Announc, 3: e01018-15 (*: contributed equally to this work)

- Part of the results in Chapter 5 have been presented as a poster:
    - **Campedelli I, Salvetti E, Clarke S, Mathur H, Ross RP, Hill C, Rea MC, Torriani S, O'Toole PW.** Phenotypic and genome-based assessment of antibiotic resistance in the *Lactobacillus delbrueckii* group. LAB12 symposium, Egmond Aan Zee, Netherland; August 27th–31st, 2017

All other results are in preparation for publication.

Chapter 1

Basic concepts on Antibiotic Resistance

1.1 Antibiotics and Antibiotic Resistance (AR)

Antibiotics are natural, synthetic, or semi-synthetic compounds, which either kill or inhibit the growth of microorganisms, specially bacteria, at defined concentrations and they are used to treat or prevent infections in humans and animals (Founou *et al*., 2016). The five major mechanisms of antibiotic action are: i) inhibition of cell wall synthesis, ii) inhibition of protein synthesis via the bacterial ribosome, iii) inhibition of DNA or RNA synthesis, iv) inhibition of the folic acid pathway of nucleic acid synthesis, and v) disruption of cell membrane integrity (Adu-Oppong *et al*., 2017). Based on action mechanisms, the first class includes β-lactams, whereas aminoglycosides, chloramphenicol, macrolides, streptogramins and tetracycline belong to the second class. Quinolones and rifampicin are classified as members of the third class, while sulphonamides and trimethoprim inhibit the energy metabolisms of microbial cells representing members of the fourth class. Whereas, glycopeptides belong to the fifth class (van Hoek *et al*., 2011).

In the 1940s, the introduction of antibiotics revolutionized medicine, saving the lives of millions of people with pneumonia, sepsis, meningitis, severe wound infections and urinary tract infections (Aminov, 2017). Moreover, many modern medical practices, such as organ transplantation, chemotherapy for cancer and orthopaedic surgery, would be high-risk procedures without the availability of antibiotics (WHO, 2011).

However, the increasingly large-scale production and consumption of antibiotics has had widespread effects on the microbial biosphere (von Wintersdorff *et al*., 2016).

In fact, the extensive use of these compounds in clinical and agricultural practice creates selective pressure on bacterial systems targeted by antibiotics, leading the evolution and increase of antibiotic resistance (AR) (Adu-Oppong *et al*., 2017; Andersson and Hughes, 2014), and compromising the activity of nearly all antibiotics that have been developed (Lee Ventola, 2015).

It is estimated that in the United States more than two million people every year are affected by antibiotic-resistant infections, resulting in at least 23,000 dying (Hampton, 2013). In particular, methicillin resistant *Staphylococcus aureus* was estimated to be responsible for 60-80% of nosocomial infections leading to 19,000 deaths per year in the United States (Fair and Tor, 2014).

In Europe each year, the number of infections and deaths caused by the most frequent multidrug-resistant bacteria (*S. aureus*, *Escherichia coli*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) was estimated at ~ 400,000 and 25,000, respectively (Davies, 2013).

In this sense, nowadays, the ever-increasing magnitude of AR is a global public health challenge (Howard *et al*., 2014), limiting treatment options for bacterial infections and thereby reducing clinical efficacy while increasing treatment costs and mortality (von Wintersdorff *et al*., 2016).

Generally, the AR is the capability of bacteria to survive to the exposure of a defined antibiotic concentration (Acar and Röstel, 2001). The resistance can be mediated by four main mechanisms: i) drug efflux; ii) reducing the permeability of the cell wall or membrane; iii) target over expression, modification, or protection; and iv) enzymatic inactivation of the drug (Adu-Oppong *et al*., 2017). In detail, bacterial efflux pumps actively transport antibiotics out of the cell, keeping the intracellular concentration low and preventing the drug from reaching inhibitory concentrations (Blair *et al*., 2015). This mechanism includes efflux pumps that are either specific to single antimicrobials or classes, such as the tetracycline efflux transporters (Chopra and Roberts, 2001) or nonspecific, multidrug-resistance efflux pumps (Sun *et al*., 2014).

Whereas, reducing the permeability of the cell wall or cell membrane and limiting antibiotic entry into the bacterial cell is achieved by the reduction of porin expression or by the replacement of porins with more-selective channels (Blair *et al*., 2015). The hydrophilic molecules, such as β-lactams, tetracycline and some fluoroquinolones, are particularly affected by changes in outer membrane permeability since they often use water-filled diffusion channels, as porins, to cross this barrier (Pagès *et al*., 2008).

The third resistance mechanism class represents a common strategy for bacteria to develop AR, where modification and protection of the target site results in decreased affinity for the antibiotic molecules (Munita and Ariasm 2016). One of the best-studied examples of target protection mechanisms is the tetracycline resistant determinants *tet*(M) and *tet*(O). They interact with the ribosome and dislodge the antibiotic from the binding site (Roberts and Schwarz, 2009). While, the most common target modification mechanism is represented by the erythromycin ribosome methylase (Erm) enzymes (Blair *et al*., 2015), which methylate the 16S rRNA sequence altering

the drug-binding site and preventing the binding of the macrolides, lincosamides and streptogramins (Roberts, 2008).

Finally, the fourth resistance strategy includes drug inactivation enzymes, which add specific chemical group to the antibiotic or destroy the molecule itself, rendering the antibiotic unable to interact with its target. This is one of the most successful bacterial strategies to survive to the presence of antibiotics. Many types of modifying enzymes have been described, which mainly catalyse i) acetylation (aminoglycosides, chloramphenicol, streptogramins), ii) phosphorylation (aminoglycosides, chloramphenicol), and iii) adenylation (aminoglycosides, lincosamides) (Munita and Arias, 2016).

1.2 AR dissemination and risks in the food chain

The use of antibiotics has always been associated with the development of resistance. Indeed, the presence of antibiotic in a specific environment leads to the elimination of susceptible bacterial cells, selecting those unusual strains that are able to survive to the antibiotic exposure through a Darwinian selection process. Those resistant variants multiply, becoming the predominant bacterial population, and transmit their genetic resistance traits to offspring or to other microorganisms (Holmes *et al*., 2016)

Therefore, bacteria can be intrinsically resistant to certain antibiotics or can acquired this trait from other microorganisms or from the environment. The intrinsic (innate) resistance to antibiotics is related to the general physiology or anatomy of a microorganism, representing an inherent trait in certain bacterial species and it is not affected by use or misuse of antibiotics (Capita and Alonso-Calleja, 2013). However, the vast majority of AR bacteria have been emerged as a result of genetic changes, acquired through mutation or by the uptake of genetic material by horizontal transfer from other bacterial strains (van Hoek *et al*., 2011). Spontaneous mutations of a locus on the microbial chromosome usually lead to changes in an antimicrobial target and are transmissible vertically. Whereas, horizontal gene transfer (HGT) allow the exchange of extrachromosomal genetic material between bacteria, mostly through the transference of mobile genetic elements, such as plasmids and transposons (Soucy *et al*., 2015).

The transmission of genetic material from one organism to another by HGT can greatly contribute to the dispersal of AR, because it can occur between closely or distantly related species and in diverse environments (Huddleston, 2014; Wang *et al*., 2006). Moreover, it can also alter the safety status of strains belonging to those microbial species Generally Recognized

as Safe (GRAS) or those with a Qualified Presumption of Safety (QPS), as defined respectively by the Food and Drug Administration (FDA) and the European Food and Safety Authority (EFSA), through the acquisition of virulence or AR traits (Rossi e*t al*., 2014).

HGT was defined as "the nongenealogical transmission of genetic material from one organism to another" and has a primary role in bacterial evolution since it causes genome rearrangements by the integration and/or deletion of genetic regions (Rossi *et al*., 2014). Effectively, there are no barriers among the ecological compartments in the microbial world and the microbiota of different compartments may easily exchange the genetic pool through mobile elements (Aminov, 2011).

The three major independent gene transfer mechanisms associated with HGT in prokaryotes are transformation, transduction and conjugation (Syvanen, 2012).

Transformation is the uptake of exogenous DNA from the environment and has been reported in both Gram-positive and Gram-negative bacteria. Bacteria capable of taking up DNA are called "competent". In this process, naked DNA is acquired by the recipient bacteria and either incorporated into the host genome by homologous recombination or transposition (van Hoek *et al*., 2011).

Transduction is a process in which the phage particles are packaged with bacterial DNA instead of phage, thus a bacteriophage acts as vector and inserts DNA into recipient cell. There are two type of transduction: i) generalized, in which any segment of bacterial DNA can be incorporated during the cell lysis; and ii) specialized, in which the DNA adjacent to the phage insertion site is packaged into the phage head as a result of prophage imprecise excision from the host genome (Soucy *et al*., 2015).

Whereas, conjugation requires physical contact between a donor and a recipient mediated by a conjugation pilus, through which genetic material is transferred. This transfer mechanism is considered particularly effective at spreading of AR genes among bacteria (Mathur and Sing, 2005).

Therefore, either direct and indirect hazards are associated to the horizontal resistance gene transfer. The direct hazard is the presence of foodborne resistance bacteria in foodstuffs, which can be transmitted to people through ingestion or contact, where they can cause infectious illness. The indirect hazard to human health is represented by the HGT of mobile genetic elements from non-pathogenic to pathogenic bacteria (Capita and Alonso-Calleja, 2013).

Food products of animal origins are often contaminated with bacteria, and thus likely constitute the main route of transmitting resistant bacteria and resistance genes from food animals to people (WHO, 2011). Therefore, AR bacteria and/or genetic resistance determinants present in soil,

water, human or animal faecal material may contaminated raw material used in the food productions, making final product potential reservoir of AR. In particular, animal products may contain AR bacteria as a result of faecal contamination during slaughter. Plant products may be contaminated with resistant bacteria during production due to the use of irrigation water contaminated with human and/or animal faeces or by sewage discharges. Foodstuffs may also be contaminated after food processing by the environment, thus it is referred as post-contamination. Finally, foods can be contaminated with AR bacteria and/or resistance determinants originating from other foodstuffs during handling by consumer, this is called cross-contamination (Verraes *et al*., 2013).

Thus, farmers, abattoir workers and food handlers as well as consumers are the larger number of people directly at risk of acquiring AR bacteria through the food chain (Founou *et al*., 2016).

Moreover, the application of processing technologies or preservation treatments in food industry to obtain safe and stable products, such as cooling, acidification, freezing, UV radiation treatment and modified atmosphere packaging, creates stress conditions for the microbial populations present in the raw materials, enhancing the development of virulence and phenotypic AR traits (Verraes *et al*., 2013).

Recently, fermented foods have been considered as potential vectors of AR genes, due to the presence of several stresses in this environment, such as low pH, high salt concentration and antimicrobial compounds, and of the high number of living bacteria, which can induce the exchange of such genes and promote HGT events (Casado Muñoz *et al*., 2014; Bautista-Gallego *et al*., 2013). Gene exchange may enhance survival of lactic acid bacteria (LAB), which naturally occur in fermented foods and beverages, and pathogens, thus representing an important risk for spread of AR genes in the gastrointestinal tract (van Reenen and Dicks, 2011).

Consequently, infections normally responding to antibiotic treatment may become difficult and sometimes impossible to cure, causing treatment failures and increasing of morbidity, mortality and society costs (WHO, 2011; Levy and Marshall, 2004).

The emergence of AR microorganisms along the food chain is thus a major global health issue. Therefore, food safety aspects should be addressed in order to reduce the AR risks for human and animal health.

In this context, the absence of acquired resistance factors in a candidate probiotic or starter culture for food and feed productions should be determined prior to approval for QPS status by EFSA (EFSA, 2012).

LAB have been extensively used as probiotics and starter cultures due to their long history of safe use and several strains having the QPS status (Kechagia *et al*., 2013; Casado Muñoz *et al*.,

2016), which include members of the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* (Ricci *et al*., 2017). However, since they are present in human gastrointestinal tract (GIT), and are also intentionally added to the diet, concerns have been raised about the AR in these beneficial bacteria (Casado Muñoz *et al*., 2016). In fact, microorganisms of which large number are present in foodstuffs or in human GIT are more likely to transfer AR genes than microorganisms of which only small numbers are present (Verraes *et al*., 2013).

1.3 AR in lactic acid bacteria (LAB)

LAB are integral components of fermented foods, where they carry out the fermentation processes leading to the final processed products. Moreover, members of the LAB group are the most widely used as probiotics due to their health-promoting features (Venema and Meijerink, 2015; Devirgiliis *et al*., 2013). In addition, several LAB species are also highly represented within the resident microbiota of GIT in healthy humans (Gerritsen *et al*., 2011). However, the role of LAB as reservoir of AR determinants with transmission potential to pathogens species is increasingly reported (Sharma *et al*., 2014; Devirgiliis *et al*., 2013; Toomey *et al*., 2009a; Mathur and Singh, 2005), thus representing a potential risk for human health.

In particular, *Lactobacillus* species are abundant in both food and GIT microbiota and several strains are widely employed as probiotic supplements. Therefore, this genus may have an important role in the genetic exchanges between the transient (foodborne) and the resident colonizers of human and animal GIT (Devirgiliis *et al*., 2011). The *Leuconostoc* genus represents another member of the LAB group, which is important for food technology especially for dairy industry. Indeed, some species of this genus with acid-producing lactococci compose the mesophilic starter cultures used in the production of butter, cream, soft and semi-hard cheeses (Edam and Gouda) and blue-veined cheeses, such as Roquefort (Hemme and Foucaud-Scheunemann, 2004).

Members of both *Lactobacillus* and *Leuconostoc* genera are considered non-pathogenic species (Ricci *et al*., 2017; Sanders *et al*., 2010), although the emergence of AR *Lactobacillus* strains in food has been recently reviewed by Abriouel *et al*. (2015) and some studies have described resistant strains of the *Leuconostoc* genus isolated from Italian and Spanish cheeses (Alegría *et al*., 2013; Morandi *et al*., 2013). Therefore, they may represent reservoirs of AR genes horizontally transmissible to pathogens through the food chain.

Tetracycline resistance determinants are the most frequently described AR in foodborne LAB (Devirgiliis *et al*., 2011; Thaker *et al*., 2010), due to its extensive use as growth promoter in the '60s and '70s (Wegener, 2003). The resistance to this antibiotic in *Lactobacillus* species was found to be commonly mediated by the genes *tet*(M) and *tet*(S) (Abriouel *et al*., 2015), which encode for ribosomal protection proteins (Thaker *et al*., 2010).

Similarly, Morandi *et al*. (2013) found the gene *tet*(S) in *Leuconostoc* spp as the most commonly tetracycline resistance gene followed by *tet*(L) and *tet*(M). Moreover, the latter two genes were found in combination in two *Leuconostoc citreum* strains. In addition, *tet*(S) determinant was associated to the tetracycline resistance exhibited by *Leuconostoc* spp. isolated from raw milk, raw pork meat and Irish beef abattoir (Toomey *et al*., 2010; Wang *et al*., 2006; Gevers *et al*., 2003a). The genes *tet* are also frequently transferred among bacteria, due to the association with transposable elements (Thaker *et al*., 2010). The best characterized mobile genetic element carrying tetracycline resistance determinants is represented by *Tn*916, which is a 18 kb conjugative transposon harbouring the gene *tet*(M) and it display broad host range toward both Gram-positive and Gram-negative bacteria (Clewell *et al*., 1995). The presence of the gene *tet*(M) within the transposon *Tn*916 has been reported for a foodborne strain of *Lactobacillus paracasei* (Devirgiliis *et al*., 2009).

This association with transposable elements, such as conjugative transposons, have been frequently reported also for erythromycin resistance genes, which are also among the most widespread AR determinants in foodborne LAB (Simeoni *et al*., 2008; Ammor *et al*., 2007). The resistance gene *erm*(B) was detected in several *Lactobacillus* species isolated from fermented foods, in which it was located both on plasmids or chromosome (Nawaz *et al*., 2011). In addition, *erm*(B) and other methylase genes, such as *erm*(C), *erm*(G) and *erm*(T), were associated to lincosamide resistance in lactobacilli isolated from fermented meat (Abriouel *et al*., 2015).

Both *Lactobacillus* and *Leuconostoc* species are intrinsically resistant to vancomycin due to the presence of terminal D-Alanine-D-lactate residue in the peptidoglycan instead of D-Alanine-D-Alanine dipeptide, which prevents vancomycin binding (Goldestain *et al*., 2015; Ogier *et al*., 2008). Moreover, most *Lactobacillus* species are intrinsically resistant to aminoglycosides (gentamicin, kanamycin, streptomycin and neomycin), ciprofloxacin and inhibitors of folic acid synthesis, such as sulphonamides and trimethoprim. However, they are generally susceptible to chloramphenicol, ampicillin, penicillin, clindamycin, linezolide and quinupristin-dalfopristin (Abriouel *et al*., 2015).

With regard to *Leuconostoc* strains, they are generally susceptible to macrolides and tetracycline and resistant to quinolones and glycopetides (Hemme and Foucaud-Scheunemann, 2004).

Mobile genetic elements, such as plasmids, insertion sequences, transposons and introns, are widespread in LAB, enhancing their ability to exchange genetic information between strains of the same species, different species, or different genera (Morelli *et al*., 2004). In particular, several mobile elements have been found in lactobacilli, including IS*L2* in *Lactobacillus helveticus*, IS*L3* in *Lactobacillus delbrueckii*, IS*1223* in *Lactobacillus johnsonii*, IS*1163* and IS*1520* in *Lactobacillus casei*, and IS*Lp11* in *Lactobacillus plantarum* (Nicoloff and Bringel, 2003).

Insertion elements was observed to be horizontally transferred among LAB during cheese manufacturing, most likely through conjugation (van Reenen *et al*., 2011). In fact, conjugative transposons are widespread in LAB and have been found to confer resistance to tetracycline, erythromycin, chloramphenicol and kanamycin (Mathur and Singh, 2005). These genetic elements play a crucial role in the spread of AR genes among bacteria of different species.

1.4 Methods for AR surveillance

The development of AR among bacteria is a serious concern. For this reason, in 2012 EFSA provided a method to identify resistance to antimicrobials of human and veterinary importance in bacterial strains intended for use as food and feed additives, including *Lactobacillus* and *Leuconostoc* species, based on the determination of antibiotic susceptibility profiles and analysis of the genetic basis of the resistance.

The susceptibility to a relevant range of antibiotics should be established through internationally recognized and standardised methods, which allow to determine the Minimum Inhibitory Concentration (MIC) of the antibiotics.

Dilution methods are the most widely used ones for the determination of MIC values, since they offer the possibility to estimate the concentration of the tested antibiotic in the agar (agar dilution) or broth medium (macrodilution or microdilution), providing the quantitative measure of the *in vitro* antimicrobial activity against bacteria (Balouiri *et al*., 2016).

For agar dilution, solutions with defined numbers of bacterial cells are spotted directly onto the nutrient agar plates that have incorporated different antibiotic concentrations. After incubation, the presence of bacterial colonies on the plates indicates growth of the organism.

Broth dilution uses liquid growth medium containing two-fold dilutions of the antibiotic in tubes with a minimum volume of 2 mL, which is termed macrodilution, or with smaller volumes using 96-well microtiter plate, called microdilution. Then, each tube or well is inoculated with a defined number of bacterial cells (1-5 × 10^5 cfu/mL). After incubation, the presence of turbidity or a sediment indicates growth of the organism (Wiegand *et al*., 2008).

The principal disadvantages of the macrodilution method were the tedious, manual undertaking, risk of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test (Balouiri *et al*., 2016). The practice of manually preparing serial dilutions of the antibiotics results in a precision of this method equal to plus or minus 1 two-fold concentration (Jorgensen and Ferraro, 1998).

The miniaturization and mechanization of the test by use microtiter plates enhance the reproducibility of the method, lead to have pre-prepared panels and save reagents and space, which represent the major advantages of the microdilution technique.

Microtiter plates are typically prepared using dispensing instruments, which aliquot precise volumes of pre-weighed and -diluted antibiotics in broth into each of the 96 wells contained in the plates, allowing to test approximately 8 antibiotics in a range of 10 two-fold dilutions per plate (Jorgensen and Ferraro, 2009). However, microtiter-based systems are commercially available, such as vetMIC [National Veterinary Institute, Uppsala, Sweden (http://www.sva.se/en/service-and-products/vetmic)], which contain dried antimicrobials in serial two-fold dilutions that can be stored for 2 years at room temperature (Huys *et al*., 2010).

In both agar and broth dilution approaches, the MIC is defined as the lowest concentration of the antimicrobial agent that prevents visible growth of a microorganism under defined conditions and it is usually expressed in μg/mL or mg/L (Wiegand *et al*., 2008).

To distinguish sensitive and resistant microorganisms to a particular antibiotic, the European Committee for Antimicrobial Susceptibility Testing (EUCAST) established epidemiological cut-off values (ECOFFs), which are defined as the MIC value that corresponds to the upper limit of the wild-type population of a particular species. ECOFF are determined from the MIC distribution analysis of a large number of isolates. The common ECOFF for resistance to a specific antibiotic corresponds to the MIC value that splits the population with acquired resistance mechanisms (non-wild-type) from the wild-type population that has no resistance (Martínez *et al*., 2015).

Therefore, a strain is defined as resistant when it has a MIC value higher than the ECOFF for a specific antibiotic (EFSA, 2012).

The detection of MIC values above the ECOFF requires further investigation to determine the structural nature and the genetic basis of the resistance, in order to verify the absence of acquired and transferable resistance determinants in a candidate strain for technical application (EFSA, 2012).

Nucleic acid-based detection systems offer rapid and sensitive methods to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms (Fluit *et al*., 2001). Standard polymerase chain reaction (PCR) with amplicon sizing by gel electrophoresis are especially useful for identifying genes, which encode antimicrobial resistance. These assays are highly specific, especially if there are no other nucleic sequences harboured by the organism which share significant homology with the target genetic material of the primers employed (Cockerill, 1999). However, the direct detection of resistance genes by PCR techniques has limited utility, because only a few resistance genes are firmly associated with phenotypic resistance (Jorgensen and Ferraro, 2009).

The introduction of next-generation sequencing technologies has increased the possibilities of rapidly characterizing AR genes harboured by bacteria. In fact, whole-genome sequencing (WGS) allow to relatively easily detect any resistance gene or resistance mutation in a bacterial genome, becoming an important tool in surveillance, clinical diagnostics, and infection control of AR (Schürch and Schaik, 2017). Indeed, the application of WGS analysis to characterize the molecular mechanisms of the resistance is more informative than other molecular methods, such as PCR or microarrays (Ellington *et al*., 2017).

Since the first published bacterial genome sequence, from *Haemophilus influenzae* Rd, in 1995, the sequencing techniques have been remarkably advanced over the years and have greatly contributed to the increase in available nucleotide sequences. The genome of *H. influenzae* Rd was sequenced using classical Sanger sequencing, while the majority of available bacterial genomes today have been sequenced using technologies that reached the market after 2005 (Loman and Pallen, 2015).

WGS projects carried out before 2011 employed one of the triad of Illumina, SOLiD, or 454 sequencing technologies (van Dijk *et al*., 2014). However, bacterial genome sequencing is currently conducted almost exclusively on Illumina sequencers due to the discontinuation of Roche's 454 sequencing platform and the low rates of adoption of SOLiD and IonTorrent systems (Schürch and Schaik, 2017). The Illumina systems generates short reads that are insufficiently large to cover repeat elements in bacterial genomes, resulting in fragmented genome assembly. Sequencing technologies producing longer reads, such as PacBio (Pacific Biosciences) and Oxford Nanopore's MinION system, can overcome this limit and allow the

complete assembly of the bacterial genomes. However, a high error rate of PacBio and MinIon reads and the high cost per base compared with short-read sequencing techniques represent important disadvantages for their application in clinical diagnostics and routine AR surveillance (Schürch and Schaik, 2017).

Although more informative than conventional molecular techniques, WGS is not a simple task, especially when the data have been generated through short-read technology. Detection of defined resistance genes can be achieved either by BLAST analysis of draft genomes towards a gene-based database or by mapping individual reads to the same type of database (Ellington *et al*., 2017).

Therefore, several databases that store nucleotide and protein sequence information for AR genes have been developed, such as Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009; http://ardb.cbcb.umd.edu/), Antibiotic Resistance Gene Annotation (ARG-ANNOT) (Gupta *et al*., 2014; http://en.mediterranee-infection.com/article.php?laref=283%26titre=arg-annot), Comprehensive Antibiotic Resistance Database (CARD) (McArthur *et al*., 2013; http://card.mcmaster.ca/), ResFams (Gibson *et al*., 2015, http://www.dantaslab.org/resfams/) and ResFinder (Zankari *et al*., 2012; http://cge.cbs.dtu.dk/service/ResFinder/).

ARDB is a manually curated specialist AR gene database which combines information from several existing resources and offering AR gene (sub)type and ontology information (Liu and Pop, 2009). The site is functional and user-friendly, however the last update was in July 2009 representing the major concern for this database (Xavier *et al*., 2016).

ResFinder version 3.0, updated in September 2017, is a database that provides exhaustive information on acquired AR genes from sequenced or partially sequenced bacterial isolates (Zankari *et al*., 2012). The current version of ResFinder allows to set the identity and length coverage thresholds, which are respectively: i) the minimum percentage of nucleotides that are identical between the best matching resistance gene in the database and the corresponding sequence in the genome, and ii) the minimum number of nucleotides of the query sequence that must overlap the resistance gene in the database. However, the information currently contained in the database is specific for acquired genes and therefore does not include AR mechanisms mediated by chromosomal mutations representing its main limitation in the assessment of microbial AR features. In addition, the database accepts only nucleotide (and not protein) sequence queries for comparison (Xavier *et al*., 2016).

In contrast, ARG-ANNOT is a curated AR gene database, which predicts resistance function on the basis of chromosomal point mutations using a local blast program in Bio-Edit software

(Gupta *et al*., 2014). However, the last update of this database was in July 2015 and no web interface is available for the sequence analysis.

ResFams implements an alternative method in order to identify known and novel resistance genes with high precision and accuracy through profile HMMs, which are built on resistance proteins compiled from CARD, LacED and Jacoby and Bush's collection of curated β-lactamases (Gibson *et al*., 2015).

CARD is an actively curated database of molecular sequence reference data for prediction of AR genotype from genomic data and it is focused on comprehensive biocuration of the molecular sequences underlying AR, including intrinsic resistance, dedicated resistance genes, and acquisition of resistance via mutation of antimicrobial targets and associated elements (Jia *et al*., 2017). Since its introduction in 2013, regular updates have been announced, with the most recent one in October 2017.

These databases have dramatically advanced the field of genomic analysis of AR, allowing sequence-based identification of known resistance determinants (Adu-Oppong *et al*., 2017).

However, the potential 'added value' of WGS should be understood to determine the food-safety and clinical implications of AR and also the validity of data generated by these novel technologies should be challenged against phenotypic methods to distinguish susceptible from resistant isolates (Ellington *et al*., 2017).

Accurate prediction of resistance phenotypes through WGS analysis has been recently reported in different studies on pathogenic bacteria (McDermott *et al*., 2016; Tyson *et al*., 2015; Zhao *et al*., 2016; Liu *et al*., 2015; Zankari *et al*., 2012). In particular, Zankari *et al*. (2012) performed WGS and antimicrobial susceptibility tests on 200 isolates originating from Danish pigs, which cover four bacterial species, such as *Salmonella* Typhimurium, *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium*. The combination of the data obtained from the two approaches revealed high concordance (99.74%) between predicted resistance genes and the phenotype observed. Thus, the authors highlighted that antimicrobial resistance testing based on WGS is an alternative to conventional phenotypic methods (Zankari *et al*., 2012).

Similarly, strong correlation between resistance phenotypes and genotypes (99.2%) using *in vitro* susceptibility testing and WGS was determined for 114 *Campylobacter* strains, belonging to the *C. coli* and *C. jejuni* species (Zhao *et al*., 2016). In addition, the WGS ability to accurately predict resistance phenotypes in *E.coli* was reported by Tyson *et al*. (2015). In this study, thirty resistance genes and a number of resistance mutations were identified among the 76 multidrug resistance *E. coli* isolates, which highly correlated with the identified phenotypes (Tyson *et al*., 2015).

Moreover, WGS could be particularly effective for identifying and characterizing AR traits in slow-growing bacteria, such as *Mycobacterium tuberculosis*, for which phenotypic susceptibility testing is an expensive, technically challenging and time-consuming approach (Ellington *et al*., 2017).

Therefore, the increasing use of next generation WGS and whole community sequencing (metagenomics) is revolutionizing the surveillance of AR resulting in a paradigm shift from phenotype to genotype-based diagnostics of resistance (McArthur and Wright, 2015).

In fact, WGS has the potential to be a powerful tool for AR surveillance for clinical and food safety applications. However, no studies are available on the employing of WGS-based methods in the characterization of AR in LAB, probably due to the limited information about the genetic basis of the resistance for those bacteria.

PART I

Chapter 2

Antibiotic susceptibility profiles of dairy *Leuconostoc* and transfer of resistance genes *in vitro* and in a food matrix

2.1 Introduction

Leuconostoc is a genus of heterofermentative lactic acid bacteria (LAB), which produces gas (carbon dioxide, CO_2), and aroma compounds, thus playing an important role in the fermentation of several products including foods (Björkroth and Holzapfel, 2006). Green vegetation and roots are considered the natural niches of *Leuconostoc*, from which they can easily propagate to the raw materials (vegetables, fruits, cereals, meat and milk) utilized in the production of fermented foods (Hemme and Foucaud-Scheunemann, 2004). Therefore, they are frequently found as part of the natural LAB community involved in the manufacture and ripening of several fermented foods and beverages, such as kimchi, olives, meat, cacao beans, wine, pulque, and dairy products (Riveros-Mckay *et al*., 2014; Björkroth and Holzapfel, 2006; Hemme and Foucaud-Scheunemann, 2004).

In dairy technology, *Leuconostoc* strains are beneficial for numerous technological aspects linked to their capacity to produce organic acids, CO_2, dextrans and, especially, aromatic compounds, such as diacetyl, acetaldehyde and acetoin (Alegría *et al*., 2013; Hemme and Foucaud-Scheunemann, 2004). The level of diacetyl required to produce the desired aroma is low, between 1.5 and 5 ppm, due to its low threshold of perception. The diacetyl can be further transformed into acetoin and 2,3-butanediol, which do not give any aroma. This unfavourable transformation may be reduced when fermented dairy products are cooled after the aroma production. In addition, the incorporation of oxygen in fermented milk favours diacetyl production (Hemme and Foucaud-Scheunemann, 2004).

The production of CO_2 by *Leuconostoc* strains through the heterofermentation of glucose is crucial to create the desired openness in pressed ripened Dutch cheeses, such as Edam, Gouda

and other brined salted cheese varieties. In particular, these openings allow the *Penicillium roqueforti* colonization in Roquefort cheese (Hemme and Foucaud-Scheunemann, 2004).

For these characteristics, well-characterized strains are intentionally added as starter or adjunct cultures in many production processes to control the fermentations and contribute to the organoleptic and rheological properties of the final product (Nieto-Arribas *et al*., 2010; McSweeney and Suosa, 2000). *Leuconostoc* spp. have been commonly found as part of the microbial population of the mesophilic mixed starter cultures used in the production of Dutch-type cheeses together with *Lactococcus* spp. (Frantzen *et al*., 2017). In particular, *Leuconostoc pseudomesenteroides* and *Leuconostoc mesenteroides* subsp. *cremoris* strains have been isolated from two Danish mesophilic cheese starters and they contribute to the diacetyl and eye formation in Gouda cheese through their heterofermentative metabolism and ability to degrade citrate (Pedersen *et al*., 2014; Pedersen *et al*., 2013). However, the presence of *Leuconostoc* strains in milk and consequently in dairy products is even due to contamination during milking and/or manufacturing, enhanced by their peculiarity of surviving for a long time on the surface of the materials used during production processes (Hemme and Foucaud-Scheunemann, 2004). Therefore, *Leuconostoc* species contribute to the non-starter LAB population of dairy environments (Alegría *et al*., 2013), playing a crucial role in the maintaining flavour and peculiarity of tradition dairy products (Nieto-Arribas *et al*., 2010). Indeed, *L. mesenteroides* and *L. lactis* strains have been isolated from traditional dairy products produced in the western Tianshan Mountains of China and in Mongolia, respectively (Dan *et al*., 2014; Zuo *et al*., 2014). Strains belong to the species *L. lactis*, *L. pseudomesenteroides* and *L. mesenteroides* have been found in non-seasoned cheeses produced in the Vlašić mountain region in central Bosnia (Terzic-Vidojevic *et al*., 2014). Moreover, *L. mesenteroides* subsp. *mesenteroides* strains have been found in Algerian camel milk samples, which have exhibited also inhibitory activity against *Listeria* (Benmechernene *et al*., 2013). Genotypic and technological properties, antibiotic susceptibility and antimicrobial activity of 35 *Leuconostoc* strains isolated from different Italian raw milk cheeses were investigated by Morandi *et al*. (2013). The authors provided new evidence concerning the resistance of *Leuconostoc* to antimicrobial agents, and report one *L. pseudomesenteroides* strains which harbored the gene *tet*M and the transposon *Tn*916. Therefore, *Leuconostoc* strains may represent vectors for the spread of AR genes along the food chain, and their uncontrolled dissemination in the environment may represent a risk for human health. Particularly, the transmission of AR determinants carried on mobile genetic elements or on plasmids to other microorganisms by horizontal gene transfer (HGT) can greatly contribute to the dispersal of AR, because it can occur between closely or distantly related species and in

diverse environments (Huddleston, 2014; Rossi *et al*., 2014; Aminov, 2011; Wang *et al*., 2006). Three major independent gene transfer mechanisms - namely conjugation, transduction, and transformation - are associated with HGT (Soucy *et al*., 2015). Among these mechanisms, conjugation is considered particularly effective at spreading of AR genes among bacteria; though it has been mostly studied under laboratory conditions (Rossi *et al*., 2014). Therefore, commensal bacteria can act as reservoirs of resistance genes and likely play a key role in the dissemination of AR genes in microbial ecosystems, including foodstuffs (Djordjevic *et al*., 2013; Rolain, 2013; Verraes *et al*., 2013). Thus, addressing the possibility of food-borne commensal bacteria, including LAB, being a potential source for the transfer of antimicrobial resistance genes is one issue of great importance in the field of public health. Very limited information on the antimicrobial susceptibility profiles of *Leuconostoc* spp. is available, as well as their possible involvement in the dispersal of antimicrobial resistance determinants between bacteria. However, their long history of safe consumption in traditional fermented foods has led to the conclusion that *Leuconostoc* are Generally Regarded As Safe (GRAS) microorganisms. In this sense, the European Food Safety Authority (EFSA) (EFSA, 2012) considers *Leuconostoc* to be suitable for the qualified presumption of safety (QPS) approach to their safety assessment, which requires that technological strains intended to be introduced into the food chain should lack acquired or transferable resistance determinants to antimicrobials of clinical and veterinary importance to prevent lateral spread of these (van Reenen *et al*., 2011). Thus, deeper investigations are greatly needed to examine the safety of food-borne *Leuconostoc* strains.

In this context, the main aims of this study were: i) to determine the antibiotic resistance/susceptibility patterns of 32 LAB strains of the *Leuconostoc* genus originating from traditional Italian and Spanish cheeses; ii) to identify the genetic basis of potentially atypical resistances encountered; and iii) to investigate the horizontal exchange capability of specific AR from selected *Leuconostoc* strains to *Enterococcus faecalis* and *Listeria innocua*; the transferability was studied both under *in vitro* conditions and in a food matrix.

2.2 Materials and Methods

Bacterial strains and growth conditions. The 32 *Leuconostoc* strains analysed in this study were selected from the collections of the Department of Biotechnology of Verona University and that of Microbiology and Biochesmistry of IPLA-CSIC; they have previously been identified to the species level as *Leuconostoc mesenteroides* (*n* = 18), *Leuconostoc citreum* (11), *Leuconostoc*

lactis (2), and *Leuconostoc carnosum* (1). Strains originated mainly from the chain production of traditional Italian cheeses (Monte Veronese, Caciotta, and Taleggio) and traditional Spanish (Cabrales, Casín, and Gamonedo) cheeses (Table 2.1).

Table 2.1. Source of isolation of the *Leuconostoc* strains of this study.

Source of isolation (details of the cheese)	Producer	LAB species	Strain	Details of isolation
Monte Veronese cheese (PDO[a] cheese from raw cows' milk, northern Italy)	A	*L. citreum*	ZF15-4	15 day-old cheese
			LE36	60 day-old cheese
			LE46	120 day-old cheese
		L. carnosum	ZLM1	Cheese milk
		L. mesenteroides	Zcaf2	Curd
			ZF30-4	30 day-old cheese
			LE50	120 day-old cheese
	B		RLM4	Cheese milk
			Rcaf2	Curd
			RF15-2	15 day-old cheese
			LE30	30 day-old cheese
			RF60-1	60 day-old cheese
Taleggio cheese (PDO cheese from raw or pasteurized cows' milk, northern Italy)	C		LbE15, LbE16	Ripened cheese
	D		LCT26a, LbT16	Ripened cheese
Caciotta cheese (pasteurized cows' milk cheese, northeast Italy)	D		LCT23, LCT25	Ripened cheese
Cabrales cheese (raw-milk, blue-veined, traditional PDO cheese, Northern Spain)	A	*L. mesenteroides*	3AC2	Curd
			3AC16	15-day old cheese
	B	*L. citreum*	4AC4	Curd
			4AC15	15-day old cheese
		L. lactis	4AB2	Curd
Casín cheese (raw-milk, acid-coagulated, traditional PDO cheese, Northern Spain)	A	*L. mesenteroides*	CA2, CA5	Curd
		L. citreum	CA3	Curd
			CA6, CA7	7-day old cheese
		L. lactis	CA33	30-day old cheese
Gamonedo cheese (raw-milk, blue-veined, smoky, traditional PDO cheese, Northern Spain)	A	*L. citreum*	GA3, GA5	Curd
			GA22	30-day old cheese

[a]PDO, protected designation of origin status.

The type strains *L. citreum* LMG 9849^T, *L. mesenteroides* subsp. *cremoris* LMG 6909^T, *L. mesenteroides* subsp. *dextranicum* NCFB 529^T, *L. mesenteroides* subsp. *mesenteroides* NCFB 523^T were obtained from the BCCM/LMG Bacteria Collection, Ghent, Belgium and NCFB, National Collection of Food Bacteria (now NCIMB). Unless otherwise stated, strains were grown at 30°C in de Man Rogosa and Sharpe (MRS) broth (Fluka, Milan, Italy).

Enterococcus faecalis OG1RF and *Listeria innocua* LMG 11387^T were used as recipients in mating experiments; these were performed as reported previously (Rizzotti *et al*., 2009a). The recipients and the strains used as reference for PCR detection of AR genes (see below) were cultivated at 30 or 37°C in Brain Heart Infusion (BHI) medium (Fluka).

Bacteria were kept in liquid cultures with 20% (w/vol) glycerol at -80°C for long term storage.

Determination of phenotypic resistance. The minimum inhibitory concentration (MIC) of several antibiotics were determined according to Alegría *et al*. (2013), using VetMIC (National Veterinary Institute of Sweden, Uppsala, Sweden) plates for LAB, containing serial 2-fold dilutions of 16 antibiotics (ampicillin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, linezolid, neomycin, penicillin, rifampicin, streptomycin, tetracycline, trimethoprim, vancomycin, and virginiamycin). As the concentration range of erythromycin, clindamycin, and virginiamycin in the VetMIC plates was not sufficient to measure the actual MIC to some strains, these were analysed by microdilution in Elisa plates with 2-fold dilutions of the antibiotics (obtained from Sigma-Adrich, St. Louis, Mo., USA). In addition, a mixed formulation of Iso-Sensitest medium (Oxoid, Basingstoke, United Kingdom) (90%) and MRS (10%), known as LSM (Klare *et al*., 2005), was used for testing tetracycline resistance phenotype of some strains.

Briefly, individual LAB colonies grown on Mueller–Hinton agar plates (Oxoid, Basingstoke, Hampshire, UK) were suspended in 2 mL sterile saline solution (Oxoid) to obtain a density corresponding to McFarland standard 1 (approx. 3×10^8 cfu/mL). This suspension was diluted 1:1,000 in Mueller–Hinton broth (final concentration 3×10^5 cfu/mL) and then 100 μL of this inoculum was added to each well of the VetMIC plate. Following a 48-h incubation at 30°C, MICs were visually read as the concentration at which inhibition of growth occurred.

In accordance with EFSA (EFSA, 2012), a bacterial strain should be considered phenotypically resistant when it is not inhibited at a concentration of a specific antimicrobial equal or higher than the established microbiological breakpoint or epidemiological cut-off (ECOFF) value.

However, one or two Log_2 dilution deviations of the MICs from the cut-offs have been reported to be within the normal inter- and intra-laboratory variation in AR analyses (Huys *et al*., 2010).

DNA extraction, PCR detection of AR genes and sequencing of amplicons. Total genomic DNA was extracted and purified from 2-mL overnight cultures using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, USA), following the manufacturer's instructions. Isolation of plasmid DNA was performed following the method of O'Sullivan and Klaenhammer (1993) with minor modifications. Instead of the original solutions, the denaturation and neutralization steps were done by using the solutions of the commercial Plasmid Mini Kit (Qiagen, Hilden, Germany). Plasmid profiles were analysed by electrophoresis on 0.7% agarose gels in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA), stained with ethidium bromide (0.5 mg mL^{-1}), and visualized and photographed under UV light with a G. Box equipment (Syngene, Cambrigde, UK).

The presence of genes associated with resistance to erythromycin [*erm*(A), *erm*(B), *erm*(C), *msr*A], tetracycline [*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tet*(W)], and chloramphenicol (*cat*), was determined in the resistant strains by PCR amplification using the primers and conditions reported by Hummel *et al*. (2007a) and Rizzotti *et al*. (2009b; 2005) (Table 2.2).

For sequencing, the PCR products were purified with the Wizard SV Gel and PCR Clean-Up system according to the manufacturer's instructions (Promega Corporation) and sent to GATC Biotech (Costance, Germany). Sequence similarity searches were performed using the BLAST network service (http://blast.ncbi.nlm.nih.gov/).

Table 2.2. Primers and positive control strains used for the detection of antibiotic resistant genes. The PCR amplification was performed using conditions reported by Hummel *et al*. (2007a) for the gene *cat*, and Rizzotti *et al*. (2009b; 2005) for the genes *erm*(A)-*erm*(C) and *tet*(K)-*tet*(W), respectively.

Target gene	**Primer name**	**Sequence (5'-3')**	**Amplicon size (bp)**	**Positive control strain**
erm(A)	ermA-I	TCT AAA AAG CAT GTA AAA GAA	645	*S. pyogenes* 190
	ermA-II	CTT CGA TAG TTT ATT AAT ATT AGT		
erm(B)	ermB-I	GAA AAG GTA CTC AAC CAA ATA	639	*S. pyogenes* C61
	ermB-II	AGT AAC GGT ACT TAA ATT GTT TAC		
erm(C)	ermC-I	TCA AAA CAT AAT ATA GAT AAA	642	*S. epidermidis* DST-ST12
	ermC-II	GCT AAT ATT GTT TAA ATC GTC AAT		
*mrs*A	mrsA-I	GCA AAT GGT GTA GGT AAG ACA ACT	399	*E. faecium* FAIR-E 349
	mrsA-II	ATC ATG TGA TGT AAA CAA AAT		
tet(K)	tetK-1	TCG ATA GGA ACA GCA GTA	169	*S. epidermidis* DST-SE20
	tetK-2	CAG CAG ATC CTA CTC CTT		
tet(L)	tetL-1	ATA AAT TGT TTC GGG TCG GTA AT	1,077	*E. faecalis* DST-ET10
	tetL-2	AAC CAG CCA ACT AAT GAC AAT GAT		
tet(M)	tetM-1	GTG GAC AAA GGT ACA ACG AG	406	*S. epidermidis* DST-ST11
	tetM-2	CGG TAA AGT TCG TCA CAC AC		
tet(O)	tetO-1	AAC TTA GGC ATT CTG GCT CAC	515	*E. faecalis* Jtet
	tetO-2	TCC CAC TGC TCC ATA TCG TCA		
tet(S)	tetS-1	CAT AGA CAA GCC GTT GAC C	669	*E. gallinarum* DST-ET14
	tetS-2	ATG TTT TTG GAA CGC CAG AG		
tet(W)	tetW-1	GAG AGC CTG CTA TAT GCC AGC	168	*B. animalis* subsp. *lactis* DST-Bl
	tetW-2	GGG CGT ATC CAC AAT GTT AAC		
cat	Entcatfw	ATG ACT TTT AAT ATT ATT RAW TT	540	*E. faecalis* FAIR-E 279
	Entcatrev	TCA TYT ACM YTA TSA ATT ATA T		

Identification of the antibiotic resistant strains. Identification of strains at species and subspecies level was carried out using molecular biology based methods and selected phenotypic tests.

Amplification and sequencing of the 16S rRNA gene and three protein-coding genes, i.e. the genes encoding the α-subunit of ATP synthase (*atpA*) and phenylalanyl-tRNA synthase α-subunit (*pheS*), were carried out according to the indications of Rizzotti *et al*. (2005) and De Bruyne *et al*. (2007), respectively. Primer sequences and PCR conditions used in each case were those described by the corresponding reference (Table 2.3).

Table 2.3. Primers and conditions used for the identification of dairy *Leuconostoc*.

Target gene	Primer name	Sequence (5'-3')	Amplicon size (bp)	Reference for primers and PCR conditions
16S rRNA	Lac16S-f Lac16S-r	AAT GAG AGT TTG ATC CTG GCT GAG GTG ATC CAG CCG CAG GTT	1,535	Rizzotti *et al*., 2005
atpA	atpA-20-F atpA-26-R	TAY RTY GGK GAY GGD ATY GC TTC ATB GCY TTR ATY TGN GC	1,011	De Bruyne *et al*., 2007
pheS	pheS-21-F pheS-23-R	CAY CCN GCH CGY GAY ATG C GGR TGR ACC ATV CCN GCH CC	411	De Bruyne *et al*., 2007

The *atpA* and *pheS* sequences of the isolates and type strains of species within the *Leuconostoc* genus (Table 2.4) were used for phylogenetic analyses using MEGA version 6 software (Tamura *et al*., 2013).

Leuconostoc mesenteroides strains were tested for their ability to ferment some carbohydrates using the protocol reported by Björkroth and Holzapfel (2006). Briefly, strains were grown in Basal MRS-medium (pH 6.5) supplemented with a selected carbohydrate to give a concentration of 1%. After incubation at 30°C for 7 days, acid production was indicated by a change from purple to yellow in the colour of the bromocresol purple indicator dye.

Table 2.4. GenBank accession numbers for nucleotide sequences of the genes *atpA* and *pheS* used for the phylogenetic analyses. The *atpA* and *pheS* sequences of *L. mesenteroides* LbE15, LbE16, and LbT16 were obtained from the sequencing of the PCR-amplicons obtained in this study.

Species	Strain	Accession number	
		atpA	***pheS***
L. carnosum	LMG 23898^T	AM711275	AM711282
L. citreum	LMG 9849^T	AM711202	AM711152
L. fallax	LMG 13177^T	AM711284	AM711193
L. gelidum	LMG 18297^T	AM711204	AM711160
L. holzapfelii	LMG 23990^T	AM711273	AM711209
L. inhae	LMG 22919^T	AM711190	AM711167
L. kimchii	LMG 23787^T	AM711220	AM711195
L. lactis	LMG 8894^T	AM711253	AM711267
L. mesenteroides subsp. *cremoris*	LMG 6909^T	AM711203	AM711159
L. mesenteroides subsp. *dextranicum*	LMG 6908^T	AM711185	AM711155
L. mesenteroides subsp. *mesenteroides*	LMG 6893^T	AM711176	AM711145
L. mesenteroides subsp. *suionicum*	LMG 11499^T	AM711177	AM711198
L. mesenteroides	Zcaf2	KT692960	KT692961
L. pseudomesenteroides	LMG 11482^T	AM711175	AM711197

DNA hybridization. This analysis was performed at the Dairy Research Institute of Asturias (IPLA) of the Spanish National Research Council (CSIC). Briefly, total and plasmid DNA from erythromycin and tetracycline resistant strains was independently digested with *Pst*I, *Pst*I and *Eco*RI, and *Hin*dIII or *Pst*I, and *Nsi*I restriction enzymes (Takara, St Germain en Laye, France). After electrophoresis, the DNA was blotted onto Hybond-N nylon membranes (GE Healthcare, Buckinghamshire, UK) using a standard protocol (Sambrook and Russell, 2001). An internal segment of the erythromycin resistance [*erm*(B)] and tetracycline resistance [*tet*(S)] genes, both amplified by PCR, were used as probes after labelling with Digoxigenin (Roche, Basel, Switzerland). Labelling, hybridization under high-stringency conditions, and detection was performed using the non-radioactive DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche) following the manufacturer's recommendations. AR genes were detected by chemoluminescence using an ImageQuant 350 Digital Imaging System (GE Healthcare, Pittsburgh, USA).

Filter mating. Selected strains were included in filter mating experiments with *L. innocua* LMG 11387^T and *E. faecalis* OG1RF. The two recipient strains were plasmid-free and susceptible to chloramphenicol, erythromycin, and tetracycline (Rizzotti *et al.*, 2009a). The latter strain was further resistant to rifampicin (50 μg/mL) and fusidic acid (25 μg/mL).

Mating experiments were conducted on 0.45 μm nitrocellulose filters (25 mm diameter) (Millipore, Milan, Italy). After overnight incubation, donor and recipient cultures were mixed at a ratio of 1:10, to obtain 1×10^7 and 1×10^8 cfu/mL, respectively. Aliquots of the mating mixtures were filtered, and then 2 mL of sterile peptone physiological solution (PPS; 0.85 g/L NaCl, 1 g/L peptone) was passed through the filter to trap the cells more tightly into the membrane. Filters were incubated over the surface of BHI agar plates without any selective agents for 24 h at 37°C. Afterwards, the filters were washed with 2 mL of PPS and the suspended bacteria were analysed by plate counting. Appropriate culture conditions were applied for separate counting of donor, recipient and tranconjugant cells. Briefly, MRS supplemented with 16 μg/mL tetracycline, or 8 μg/mL chloramphenicol or 4 μg/mL erythromycin was used for counting the different *Leuconostoc* donors; BHI with 50 μg/mL rifampicin plus 25 μg/mL fusidic acid was used for counting the recipient strain *E. faecalis* OG1RF; and Listeria Selective Agar (LSA, Oxoid) base was used for enumerating the recipient strain *L. innocua* LMG 11387^T. Transconjugants of *E. faecalis* and *L. innocua* were selected on BHI agar supplemented with rifampicin and tetracycline or chloramphenicol or erythromycin, at the same concentrations reported above, or on LSA supplemented with one of the antibiotics, respectively.

Transfer frequency was expressed as the number of transconjugants per recipient.

Food mating. Only donor and recipient strains giving transconjugants in filter mating experiments were used.

All the following procedures were performed using sterile tools under a sterile cabinet. To perform mating trails, Monte Veronese cheese slices (8 cm^3 – 40 mm × 20 mm × 10 mm) were placed in Petri dishes and the surface was inoculated with a mixed culture (0.5 mL) of donor and recipient strains. Inoculum was prepared from overnight cultures that were centrifuged at 8000 × for 5 min. The pellets were washed twice with PPS, suspended in PPS and mixed to obtain 1×10^7 and 1×10^8 cfu/mL of donor and recipient strains, respectively. After incubation at 37°C for 24 h, the cheese slices were washed with 1 mL sterile PPS, and counts of donors, recipients and transconjugants were determined using the culture conditions reported above. Four replicates for experiment were conducted.

Characterization of transconjugants. Presumptive transconjugants were isolated from selective agar plates and grown in BHI broth with appropriate antibiotics. To distinguish them from donor mutants, they were typed with primer Hpy1 (5'-CCGCAGCCAA-3') using the Random Amplification of Polymorphic DNA (RAPD)-PCR technique as reported by Akopyanz *et al*. (1992). Then, transconjugants were checked for the presence the AR gene by specific PCR.

Finally, the effect of such transfer on the phenotype was examined by determining the MIC of the specific antibiotic as described above.

2.3. Results and Discussion

2.3.1 Determination of phenotypic resistance. The MIC values of several antibiotics encompassing nearly all important pharmacological classes was determined by broth microdilution in VetMIC plates for 32 LAB strains belonging to the genus *Leuconostoc* isolated from Italian and Spanish traditional cheeses. The MICs obtained for the 16 different antibiotics and the relative ECOFF values are summarized in Table 2.5. To distinguish resistant from susceptible strains, the MICs were compared to the epidemiological cut-off (ECOFF) values reported by Casado Muñoz *et al*. (2014), Flórez *et al*. (2005), Danielsen and Wind (2003) and defined according to the European Commission SCAN (2007) and EFSA (2012) for the genera *Lactobacillus* and *Leuconostoc*. When not defined, the breakpoint values suggested by the National Committee for Clinical Laboratory Standards (2011) and Geenen *et al*. (2010) for staphylococci were considered.

As expected, all analyzed strains were insensitive to high concentrations of vancomycin (MIC ≥ 128 μg/mL), since this is a common trait for species belonging to the genus *Leuconostoc* (Ogier *et al*., 2008). Such intrinsic characteristic is linked to the presence of D-Ala-D-Lactate in their peptidoglycan rather than a D-Ala-D-Ala dipeptide (Hemme and Foucaud-Scheunemann, 2004). Moreover, they all were resistant to trimethoprim (MICs ≥ 8 μg/mL) for the absence of the folic acid synthesis pathway (Katla *et al*., 2001). However, a broad MIC distribution (from 8 to 128 μg/mL) of this antimicrobial was observed.

In contrast, all strains were susceptible to the beta-lactams ampicillin and penicillin G, to gentamycin and linezolid (MICs lower than the microbiological ECOFFs). Some studies have previously shown that *Leuconostoc* strains isolated from dairy and meat products are susceptible to many of these antibiotics and in particular to the beta-lactams (Morandi *et al*., 2013; Ammor *et al*., 2007). A broad MIC distribution characterized the remaining antibiotics, wherein we can find one or more resistant strains, belonging to different species.

Concerning aminoglycosides, most of the strains (23 out of the 32) exhibited resistance to kanamycin (MICs ≥ 16 μg/mL). The MIC distribution of kanamycin was broad, ranging from 2 to 128 μg/mL with one strain (*L. mesenteroides* LbE16) being resistant to more than 128 μg/mL. Kanamycin resistance was found in *L. mesenteroides* (9 strains), *L. citreum* (11), *L. lactis* (2) and

L. carnosum (1). This observation corroborates data reported in previous studies, in which the profiles of kanamycin resistance in *Leuconostoc* spp. vary largely among strains (Alegría *et al*., 2013; Adimpong *et al*., 2012).

Table 2.5. Distribution of MICs of 16 antibiotics for LAB strains belonging to the genus *Leuconostoc* originated from Italian and Spanish cheese milk and dairy products.

Antibiotic	No. of isolates with the following MICs (µg/mL)											
	<1	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**ECOFF* (µg/mL)**
Gentamycin	7	15	10									16
Kanamycin			1		8	**6**	**13**	**2**	**1**		**1**	16
Streptomycin			2	2	3	22	2		**1**			64
Neomycin	7	4	16	3	1		1					8
Tetracycline			19	11			**1**		**1**[a]			8
Erythromycin	29	**2**									**1**[a]	1
Clindamycin	31										**1**[a]	1
Chloramphenicol				**12**	**18**	**1**	**1**					4
Ampicillin	27	5										2
Penicillin G	32											1
Vancomycin										**32**[a]		≥ 32
Virginiamycin	23	8							**1**			4
Linezolid		1	26	5								≥ 8
Trimethoprim					**1**	**7**	**3**	**1**	**20**[a]			8
Rifampicin	9	7	13	**2**	**1**							≥ 4
Ciprofloxacin				15	15	1		**1**				>32

*Epidemiological cut off (ECOFF) values for strains were based on those provided by Casado Muñoz *et al*. (2014), Flórez *et al*. (2005), Danielsen and Wind (2003) and defined according to EFSA (2012), and the European Commission, SCAN (2007) for the genera *Lactobacillus* and *Leuconostoc*. When not defined, the breakpoint values suggested by the CLSI (2011) and Geenen *et al*. (2010) for staphylococci were considered. Resistant strains with a MIC value higher than the ECOFF reported in the table are indicated in bold.

[a] MICs exceed the highest antimicrobial concentration tested; MICs of the antibiotics should be read as > the actual figure.

MICs of the streptomycin were between 2 and 128 µg/mL, with only one strain (LbE16) being resistant to 128 µg/mL. Although this, the data obtained here suggest that the cut-off of

streptomycin and kanamycin for *Leuconostoc* should be updated, for which evaluating MICs in a larger number of strains is encouraged.

MICs of neomycin were lower than the breakpoint (8 μg/mL) for all strains, except *L. mesenteroides* LbE16 and CA5 (32 and 8 μg/mL, respectively).

The lack of cytochrome-mediated transport is thought to be responsible for the resistance of anaerobic and facultative bacteria to aminoglycosides (Bryan and Kwan, 1981). However, the presence of strains isolated from the same environment showing low and high MICs to aminoglycosides is largely unexplained and needs to be addressed further. High MICs may also anticipate the presence of dedicated (acquired) resistance genes (Rojo-Bezares *et al*., 2006; Ammor *et al*., 2008). Low rates of resistance to aminoglycosides have also been observed by Rodríguez-Alonso *et al*. (2009) and Morandi *et al*. (2013) for *Leuconostoc* strains isolated from artisan Galician and Italian raw milk cheeses, respectively.

All strains displayed resistance to chloramphenicol (MICs ≥ 4 μg/mL) with MICs between 4 and 32 μg/mL. Previous reports have indicated that most *Leuconostoc* species are susceptible to this broad spectrum antibiotic, since the proposed microbiological breakpoint was higher, i.e. 16-32 μg/mL (Flórez *et al*., 2005). The possibility of an intrinsic resistance of *Leuconostoc* species to chloramphenicol exists, which would reduce the horizontal transferability of this resistance to other bacterial species. However, this possibility cannot exclude the presence of dedicated genes providing resistance to this antibiotic, especially in the two strains displaying a high level MIC to chloramphenicol.

Concerning ciprofloxacin, a second-generation quinolone that inhibit bacterial nucleic acid synthesis, only a strain of *L. citreum* (CA7) was considered resistant, displaying a MIC value higher than 32 μg/mL. On the contrary, Morandi *et al*. (2013) found that 83% of the 35 examined strains belonging to different *Leuconostoc* species showed phenotypic resistance to such antimicrobial. This discrepancy could be due to the different susceptibility method used for the AR surveys: disc diffusion in agar (Morandi *et al*. 2013) versus microdilution (this work).

MICs of rifampicin were between <1 and 8 μg/mL, with three strains (*L. citreum* CA3 and CA6, and *L. lactis* CA33) which could be considered resistant (MICs ≥ 4 μg/mL). Rifampicin is a broad-spectrum antibiotic that inhibits the function of RNA polymerase in eubacteria (Alifano *et al*., 2015). Mutations in the gene *rpoB* encoding the RNA polymerase β-chain have been previously reported to confer resistance to rifampicin in two LAB strains, namely *L. mesenteroides* ATCC 8293 and *O. oeni* PSU-1 (Marcobal *et al*., 2008). Whether this is the case in our strains has yet to be demonstrated.

As regards the antimicrobials belonging to the macrolide-lincosamide-streptogramin (MLS) family, all strains showed a MIC ≤ 1 μg/mL for virginiamycin, except LbE16 (MIC 128 μg/mL). This streptogramin was used for decades as an animal growth promoter; however it was banned in the European Union (EU) in 1999, because of its structural relatedness to some therapeutic antimicrobial drugs used for humans. Resistance of LAB to streptogramins, including virginiamycin, is considered less common among many other protein synthesis inhibitors (Vannuffel and Cocito, 1996).

Most strains displayed erythromycin MICs below or equal to the EFSA's cut-off (1 μg/mL) except for *L. mesenteroides* LbE15 which proved to be resistant to high level of this macrolide antibiotic (MIC >256 μg/mL). All examined strains showed clindamycin MICs lower than the cut-off (1 μg/mL), except again for *L. mesenteroides* LbE15 that was resistant to such antibiotic (MIC >256 μg/mL). As erythromycin, clindamycin belongs to the MLS phenotype, and a considerable cross-resistance with erythromycin occurs due to the overlapping ribosomal binding sites of these two antibiotics (Devirgiliis *et al*., 2013). A phenotypic erythromycin resistant strain of *L. mesenteroides/L. pseudomesenteroides* isolated from the Spanish traditional blue-veined Cabrales cheese has been already reported (Flórez *et al*., 2005), however the nature of such resistance was not investigated further. Phenotypic clindamycin resistance in Gram-positive bacteria, such as in staphylococci and enterococci, has been reported to be either constitutive or inducible. Identification of strains carrying the latter resistance type may fail by using a microdilution method (Sasirekha *et al*., 2014).

Finally, tetracycline MICs ranged between 2 and > 64 μg/mL and two strains (LbE16 and LbT16) grow at ≥ 32 μg/mL of tetracycline. Atypical resistance levels to tetracycline have been reported in several studies for LAB strains isolated from dairy and meat foodstuffs (Verraes *et al*., 2013; Ammor *et al*., 2007). However, only two strains of *Leuconostoc* spp. with tetracycline resistance were found in independent studies where strains from beef abattoirs (Toomey *et al*., 2010) and raw pork meat (Gevers *et al*., 2003c) were analyzed. To the best of our knowledge, this is the first report in which *Leuconostoc* with phenotypic resistance to tetracycline were detected from traditional dairy products.

It is noteworthy that several strains of *Leuconostoc* with resistance to three or more antimicrobial classes (multi-drug resistant; MDR) were identified in this work. All MDR *Leuconostoc* showed resistance to at least four antimicrobials (intrinsic and non-intrinsic), and one strain proved to be resistant to nine of them. Particularly, MDR was observed in *L. citreum* LE46 and in four *L. mesenteroides* strains, as shown in Table 2.6.

Table 2.6. Antibiotic resistant *Leuconostoc* strains characterized in this study.

Species	Strain	Source of isolation [a]	Fermentation of				Phenotypic antibiotic resistance (MIC µg/mL)[b]	Resistance gene(s)[c]
			Arabinose	Fructose	Sucrose	Trehalose		
L. citreum	LE46	Monte Veronese cheese	nd	nd	nd	nd	CM (32), KM (128), TM (>64)	-
L. mesenteroides subsp. *mesenteroides*	Zcaf2	Curd of Monte Veronese cheese	+	+	+	+	CM (16), KM (16), TM (32)	-
L. mesenteroides subsp. *mesenteroides*	LbE16	Taleggio cheese	+	+	+	+	CM (8), EM (1), KM (512), NM (32), SM (128), TC (>64), TM (32), VI (>8)	*tet*(S)
L. mesenteroides subsp. *cremoris*	LbT16	Taleggio cheese	-	-	-	-	CM (4), TC (32), TM (8)	-
L. mesenteroides subsp. *dextranicum*	LbE15	Taleggio cheese	-	+	+	+	CL (>16), CM (8), EM (>8), KM (32), TM (16)	*erm*(B)

[a] For more details see Table 2.1.
[b] All strains were insensitive to vancomycin (>128 µg/mL).
[c] The genes were detected by PCR
CL: clindamycin; CM: chloramphenicol; EM: erythromycin; KM: kanamycin; NM: neomycin; SM: streptomycin; TC: tetracycline; TM: trimethoprim, VI: virginiamycin.
n.d.: not detected.

In detail, *L. citreum* LE46 and *L. mesenteroides* Zcaf2 showed the higher chloramphenicol MIC value (32 and 16 μg/mL, respectively). *L. mesenteroides* LbT16 was resistant/insensitive to tetracycline, in addition to chloramphenicol, trimethoprim and vancomycin. The strains LbE15 and LbE16 showed simultaneous resistance/insensitivity to vancomycin, chloramphenicol, erythromycin, kanamycin and trimethoprim, and the first strain was further resistant to clindamycin, and the second to neomycin, streptogramin, tetracycline and virginiamicin. These findings confirmed the data of Rodríguez-Alonso *et al*. (2009) and Morandi *et al*. (2013) that have reported the presence of *Leuconostoc* strains resistant to multiple antibiotics in artisanal raw milk cheeses.

2.3.2 Identification of the MDR leuconostocs. In order to accurately characterize the *Leuconostoc* strains (LbE15, LbE16, LbT16, LE46, Zcaf2) showing atypical AR a series of further experiments were performed. Firstly, molecular identification at the species level was carried out using amplification and sequence analysis of 16S rRNA gene, to confirm the previous analysis on the identity of the strains. The genus *Leuconostoc* was revised in the last year, after the present study, with the reclassification of *Leuconostoc mesenteroides* subsp. *suionicum* (Gu et al., 2012), as *Leuconostoc suionicum* species, and the description of *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* as novel subspecies (Jeon *et al*., 2017). Since, the 16S rRNA gene sequence data do not allow the discrimination of the four described subspecies of *L. mesenteroides* (*mesenteroides*, *cremoris*, *dextranicum*, and *suionicum*), therefore additional analysis were carried out using more divergent protein-coding genes, i.e. *atpA*, and *pheS* (Rahkila *et al*., 2014).

Comparative 16S rRNA gene sequence analysis confirmed that the strain LE46 belonged to the species *L. citreum* (99.6% sequence identity) and the other four strains to *L. mesenteroides* (99.9%). This was further confirmed by sequence analysis of *phe*S (accession number: KT692962). The Neighbour-joining tree of the concatenated *atpA* and *pheS* partial gene sequences revealed low relatedness (92–96%, respectively) between our *L. mesenteroides* strains and the same sequences from *L. mesenteroides* subsp. *suionicum* LMG 11499^{T}, which has been recently reclassified as *L. suionicum* (Jeon *et al*., 2017). For Neighbour-joining tree see Figure 2.1. Significantly higher values, in the range of 99.0–99.8%, were found with the other three *L. mesenteroides* subspecies, due to their close phylogenetic relationships. As DNA analysis did not give a conclusive identification, strains were classified at the subspecies level by conventional phenotypic approach based on their different capacity to ferment L-arabinose, fructose, sucrose, and threalose. The carbohydrate fermentation profiles varied among the strains, as shown in

Table 2.6. The strain LbT16 was easily identified as *L. mesenteroides* subsp. *cremoris* since members of this subspecies utilize a limited number of carbohydrates (Björkroth and Holzapfel 2006). The ability to ferment or not the pentose arabinose allowed the differentiation of the other strains: Zcaf2 and LbE16 were ascribed to *L. mesenteroides* subsp. *mesenteroides*, while LbE15, that did not utilize arabinose, was included in *L. mesenteroides* subsp. *dextranicum*.

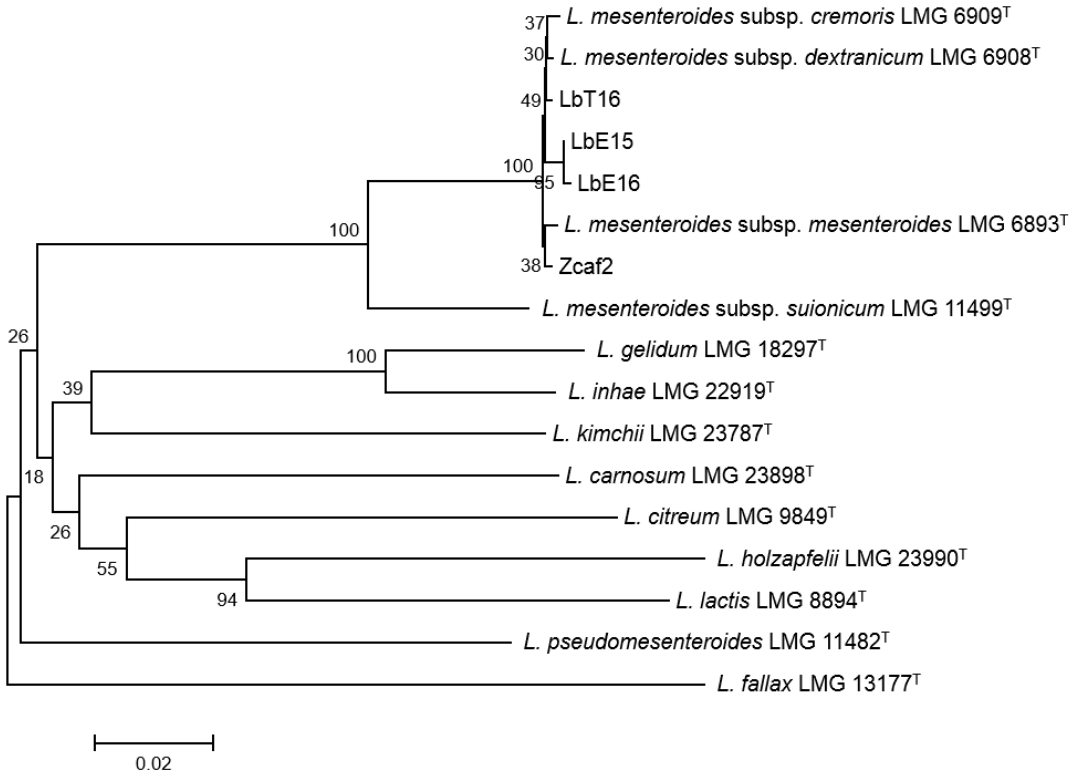

Figure 2.1. Phylogenetic tree obtained from the concatenated *atp*A and *phe*S gene sequences of the four *Leuconostoc* strains showing atypical AR profiles and 12 *Leuconostoc* type strains with *L. fallax* LMG 13177^T as an outgroup. The tree was reconstructed using maximum composite likelihood method. Bootstrap values (1,000 replicates) are shown as a percentage at the branching points. The scale bar represents the number of nucleotide substitutions per site.

2.3.3 Molecular detection of resistance genes. To detect genetic determinants responsible for the resistance phenotypes observed in the strains LbE15, LbE16, LbT16, LE46, and Zcaf2, the presence of well-known structural genes associated with resistance to antibiotics which inhibit protein synthesis, such as tetracycline [*tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), and *tet*(W)], erythromycin [*erm*(A), *erm*(B), *erm*(C), and *msr*A], and chloramphenicol (*cat*), was investigated by PCR amplification. All positive controls produced an amplicon of the expected size (data not shown). The results are summarized in Table 2.6.

The *erm*(B) gene was found only in the strain LbE15, to which it should confer its erythromycin-resistance. This gene has been shown to provide MLS resistance, coding for a methylase enzyme that modifies the 23S rRNA macrolide binding sites (van Hoek *et al*., 2011). This gene found in *L. mesenteroides* LbE15 displayed 99% similarity with that carried by *Streptococcus pneumoniae* MDRSPN001, *Staphylococcus aureus* HZW450, *Streptococcus agalactiae* CUGBS591, *Listeria monocytogenes* LM78, *Enterococcus faecium* e194 and *Enterococcus faecalis* V583, suggesting that this erythromycin resistance determinants could represent an acquired character for LbE15 and thus it could be transfer to other microorganisms.

Neither *erm*(A) and *erm*(C) genes, coding for rRNA methylases (Roberts, 2008), or the efflux gene *msr*A, coding for an ATP-binding transporter (Roberts, 2008), were not detected in the strains examined. Among LAB, *erm*(B) is the best studied and the most widely spread gene conferring erythromycin resistance (Devirgiliis *et al*., 2013; Thumu and Halami, 2012a; Nawaz *et al*., 2011; Ammor *et al*., 2007). In addition, *erm*(B) has been identified as one of the most common resistance genes in Spanish and Italian commercial cheeses (Flórez *et al*., 2014). However, to our knowledge, this gene has never been described in *Leuconostoc* species.

Analysis of the tetracycline-resistant leuconostocs showed that, among the screened genes, only *tet*(S), coding for a ribosomal protection protein (Thaker *et al*., 2010), was present in the strain LbE16 and its nucleotide sequence was identical to that carries by *L. monocytogenes* LM78, *E. faecalis* C386, and *S. dysgalactiae* subsp. *equisimilis* NTUH_1743. This finding suggests that tetracycline resistance represent an acquired character for *L. msesentoroides* LbE16 and it could act as vector for the AR dissemination in the food chain.

In contrast, the absence of all the tested resistance determinants [*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(L) and *tet*(K)] in *L. mesenteroides* LbT16 may suggest a new mechanism of resistance which can be due either to acquired genes or to a mutation of indigenous genes (EFSA, 2012). Indeed, the possible presence of a false positive phenotype linked to specific growth requirements of LbT16 can be excluded, since its resistance was confirmed in different media added with tetracycline, i.e. MRS, LSM, and Mueller–Hinton broth.

The tetracycline resistance genes are largely spread among LAB and more than one gene has been reported to be present in some strains (Ammor *et al*., 2007). Few data are available on the abundance of the genes *tet* in food-borne *Leuconostoc* strains. Two previous investigations carried out on a limited number of strains have reported the presence of the gene *tet*(S) in AR strains belonging to the species *L. mesenteroides* (Toomey *et al*., 2010) and *L. citreum* (Gevers *et al*., 2003c) isolated from meat processing lines. In addition, Morandi *et al*. (2013) found *tet* determinants in tetracycline-susceptible *Leuconostoc* strains isolated from dairy products,

unveiling *tet*(M) as the most frequent gene, followed by *tet*(L) and *tet*(S). Furthermore, these authors found *tet*(L) and *tet*(M) together in two *L. citreum* strains, and the genes *tet*(M) and *int* (the transposon integrase gene of the Tn916/Tn1545 family) in a strain of *L. pseudomesenteroides*.

As regards chloramphenicol resistance, the gene *cat* could not be amplified from the genomic DNA of the five resistant strains. Therefore, the genetic basis of chloramphenicol resistance could not be determined and further research will be needed to elucidate the underlying resistance mechanism. The gene *cat* encodes a chloramphenicol acetyl transferase, and was selected because it is the commonest chloramphenicol resistance gene in LAB (Hummel *et al*., 2007a).

2.3.4 Location of *erm*(B) and *tet*(S) in the resistant *L. mesenteroides* strains. As many other LAB, *Leuconostoc* species harbour one or several plasmids of various sizes (Jung *et al*., 2012a; Makarova *et al*., 2006) without known functions, except for replication (cryptic). Plasmid profiling revealed at least three plasmids in each *L. mesenteroides* LbE15 (Figure 2.2, Panel A2 line 1) and LbE16 (Figure 2.3, Panel A line 4).

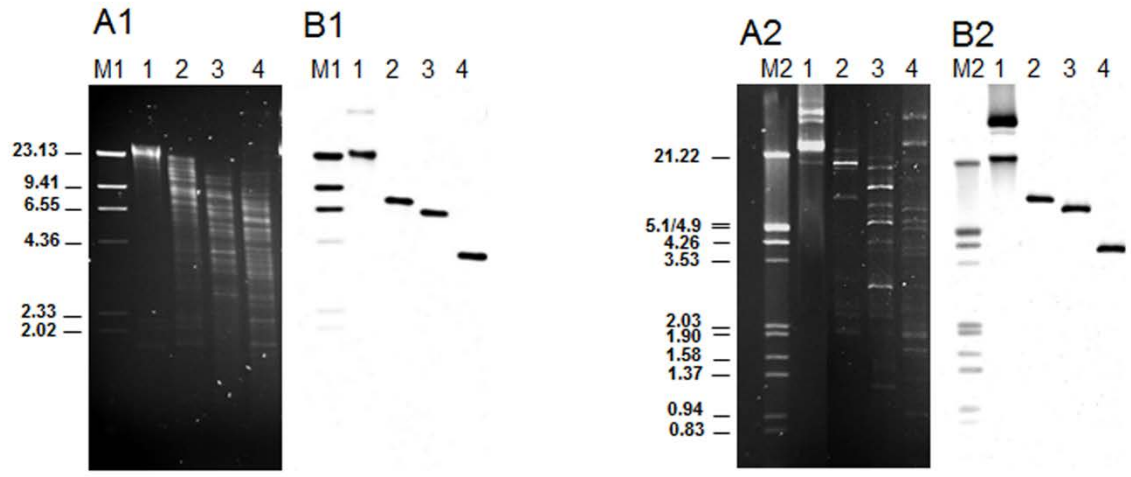

Figure 2.2. Gel electrophoresis (A) and Southern blot analysis (B) of total genomic (A1) and plasmid DNA (A2) from *L. mesenteroides* subsp. *dextranicum* LbE15. Lines order in the two gels: 1, undigested DNA; 2, DNA digested with *Pst*I; 3, DNA digested with *Pst*I and *Eco*RI; 4, DNA digested with *Hind*III. As a probe, an internal segment of *erm*(B) obtained by specific PCR and labelled with digoxigenin was used. M, molecular weight markers: M1, digoxigenin-labelled, *Hind*III-digested lambda DNA; M2, digoxigenin-labelled, *Eco*RI and *Hind*III-digested lambda DNA. The size of the fragments of the molecular weight markers (in kbp) is indicated on the left.

Hybridization experiments using as a probe internal segments of *erm*(B) and *tet*(S), respectively, were used to identify the genetic location of these genes in the strains *L. mesenteroides* LbE15 and LbE16. Chemiolumiscence signals were obtained at the same positions in both the total and plasmid DNA samples from the strain *L. mesenteroides* LbE15 (Figure 2.2, Panel B1 and B2). Identical hybridization pattern of undigested total and plasmid DNA in *L. mesenteroides* LbE15, and also in total and plasmid digested DNA, which pointed out towards the erythromycin resistance gene linked to the largest plasmid of the strain (Figure 2.2). The plasmid codification of *erm*(B) leads us to suppose that *L. mesenteroides* LbE15 might have gained the erythromycin resistance by HGT event, supporting the hypothesis that this resistance is an acquired feature of LbE15.

On the contrary, the presence of hybridization signals in total DNA (undigested-digested), but not in plasmid DNA proved that tetracycline resistance was encoded on the bacterial chromosome of the strain *L. mesenteroides* LbE16 (Figure 2.3). Location of this gene in the *L. mesenteroides* genome has yet to be reported.

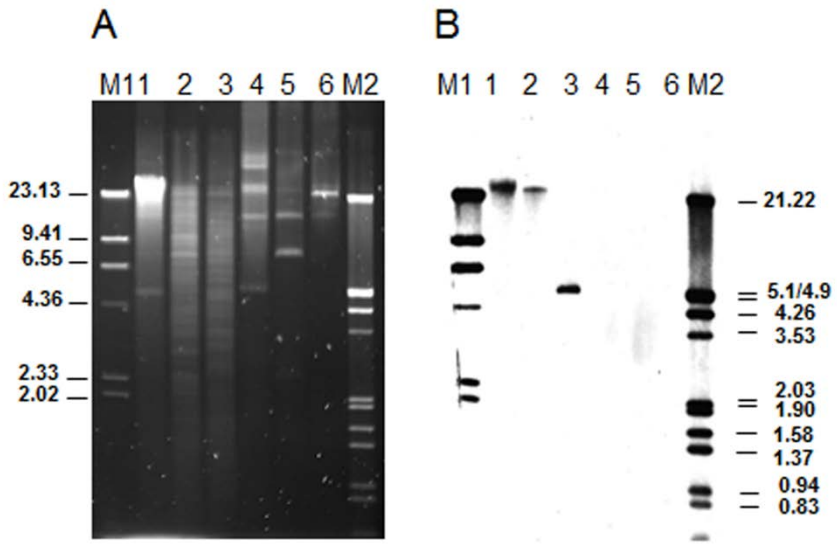

Figure 2.3. Gel electrophoresis (A) and Southern blot analysis (B) of total genomic and plasmid DNA from *L. mesenteroides* subsp. *mesenteroides* LbE16. Order: 1, undigested total DNA; 2 and 3, total DNA digested with *Pst*I, and *Nsi*I, respectively; 4 undigested plasmid DNA; 5 and 6, plasmid DNA digested with *Pst*I, and *Nsi*I, respectively. As a probe, an internal segment of *tet*(S) obtained by specific PCR and labelled with digoxigenin was used. M, molecular weight markers: M1, digoxigenin-labelled, *Hind*III-digested lambda DNA; M2, digoxigenin-labelled, *Eco*RI and *Hind*III-digested lambda DNA. The size of the fragments of the molecular weight markers (in kbp) is indicated on the left and right side of the picture.

2.3.5 Filter mating experiments. Conjugation and mobilization of various mobile genetic elements are believed to play key roles in the dissemination of AR in bacteria (Schwarz *et al*., 2017). Therefore, filter mating trials were conducted *in vitro* using *L. mesenteroides* LbE15 and LbE16, which harbored acquired resistance determinants, as donors and *E. faecalis* OG1RF and *L. innocua* LMG 11387^{T} as recipients in order to investigate the transferability of AR. Both these recipient strains have been shown to be susceptible to erythromycin (MIC 1 µg/mL) and tetracycline (MIC 1 µg/mL) and plasmid free (Rizzotti *et al*., 2009a). Further, both recipients have already been used successfully in previous mating studies involving enterococci (Rizzotti *et al*., 2009a; Cocconcelli *et al*., 2003).

Transfer of AR genes to *L. innocua* LMG 11387^{T} was never achieved. However, transconjugants were obtained in the conjugation between *L. mesenteroides* subsp. *dextranicum* LbE15 and the recipient *E. faecalis* strain. Transfer was low, but detectable, with an average frequency of 3.2×10^{-8} transconjugants per recipient. All presumptive transconjugants, grown onto plates containing the selective antibiotics (erythromycin plus rifampicin), were isolated from each mating experiment and subjected to RAPD-PCR fingerprinting using the primer *Hpy*1 to exclude the presence of mutant donors. They displayed the same RAPD-PCR profile as the recipient strain *E. faecalis* OG1RF, thus confirming that they were true transconjugants and not reverted mutants (data not shown). The transconjugants displayed increased average MIC values of > 64 (erythromycin) in comparison to the original recipient MIC of 1 µg/mL. Thereafter, transfer of the genes *erm*(B) to transconjugants was verified by specific PCR, since they were selected during the experiments by their resistance phenotype. Results revealed that this genetic determinant could be PCR amplified from the transconjugants, whereas amplification was negative when DNA from *E. faecalis* OG1RF was used as a template (data not shown). Further, sequence analysis of a *erm*(B) gene fragment (549 pb) from donor and selected transconjugants showed, as expected, 100% identity. These findings demonstrated that transfer of the *erm*(B) gene and its associated phenotype between *L. mesenteroides* and enterococci can occur in laboratory conditions.

Previous studies have reported the *in vitro* transfer of *erm*(B) from different LAB species, such as *Lactobacillus fermentum*, *Lactococcus lactis*, *Lactobacillus reuteri* and *Lactobacillus salivarius*, to enterococci and lactococci (Nawaz *et al*., 2011; Toomey *et al*., 2009b; Lampkowska *et al*., 2008; Ouoba *et al*., 2008). However, until now, no successful conjugal transfer has been described for *Leuconostoc* strains. Indeed, to our knowledge, the only previous study of Toomey *et al*. (2010) did not obtain transconjugants when attempting to transfer tetracycline resistance from *L. mesenteroides* strains harbouring *tet*(S).

2.3.6 Food mating experiments. Since the laboratory transfer assays do not mimic the *in vivo* conditions, mating trials were also conducted in food using the same donor and recipient strains as above. The mating experiment was done onto the surface of Monte Veronese cheese. Twenty presumptive transconjugants were obtained, which were verified as before. An estimated conjugation frequency of around 2.2×10^{-7} per recipient was calculated, which was considerably higher (up to ~16,000-fold) than that seen under standard filter mating conditions.

The present study demonstrates that HGT events can be realized in a food matrix, and that *Leuconostoc* strains could represent potential vectors of AR genes in dairy products. The possibility of transfer of AR from commensal food-borne bacteria has been studied extensively in laboratory conditions, but only a limited number of researches have been conducted in real food matrices (Rossi *et al*., 2014). Furthermore, almost all these investigations have considered meat-based foods as environmental niches for HGT among bacteria, especially enterococci (Gazzola *et al*., 2012; Rizzotti *et al*., 2009a; Cocconcelli *et al*., 2003). In this context, the results of the present study appear of relevance, as the transmission of AR gene between *Leuconostoc* and *E. faecalis* was shown for the first time in filter mating experiments, under ideal conditions, and in a complex ecosystem, like that of the cheese. In addition, it was observed that the frequency of the transfer events found in Monte Veronese cheese was higher than those found in laboratory media; these data are in accordance with Davies and Davies (2010) who suggested that frequencies of conjugative transmission in nature are probably some orders of magnitude higher than those under laboratory conditions.

2.4. Conclusions

Resistance to different antibiotics was detected among strains of the genus *Leuconostoc* isolated from traditional Italian and Spanish cheeses. Some resistances, such as those to vancomycin, chloramphenicol and trimethoprim are - or can be - indicative of intrinsic nature, suggesting the need of future evaluation of MICs in a larger number of *Leuconostoc* strains. However, resistances of a reasonable acquired origin were also found. As such, a correlation between atypical erythromycin and tetracycline resistance and the presence of *erm*(B) and *tet*(S) genes, respectively, was encountered. The high similarity of these AR determinants shared with the sequence of erythromycin and tetracycline genes of *L. monocytogenes*, *Enterococcus* spp. and *Staphylococcus* spp. strains confirmed that these genetic elements represent acquired resistance character and they could be horizontal transfer to other microorganisms in the food chain.

Indeed, the data presented in this study provide the first evidence of the erythromycin resistance transfer by conjugation between *L. mesenteroides* and *E. faecalis* both *in vitro* and in cheese, supplying novel proof that AR LAB can act as a reservoir of acquired AR genes. Moreover, the high frequency of the transfer events in Monte Veronese cheese comparted with those identified for laboratory media highlights the importance to continue research on the quantification of HGT of AR genes from food-borne bacteria to pathogens and human through food. In addition, the study of mobile genetic elements in commensal bacteria is crucial to better understand the epidemiology of AR genes to implement the surveillance of AR in food.

The recent improvements in sequencing technologies and the increasing availability of genome sequences can provide unprecedented insights into the makeup and genetic organization of AR genes. Therefore, the application of genome sequence analysis represents an important starting point to improve the current knowledge on the molecular basis of AR in the LAB species, including those belong to the genus *Leuconostoc*.

Chapter 3

Identification of antibiotic resistance genes in three resistant *Leuconostoc mesenteroides* strains of dairy origin using whole-genome sequencing

3.1 Introduction

Leuconostoc mesenteroides is a lactic acid bacteria (LAB) species, which was revised in the last year with the reclassification of *Leuconostoc mesenteroides* subsp. *suionicum* (Gu *et al*., 2012), as *Leuconostoc suionicum* species (Jeon *et al*., 2017), and the description of *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* as novel subspecies (Jeon *et al*., 2017). Therefore, the *L. mesenteroides* species is now divided into the subspecies *mesenteroides*, *dextranicum*, *cremoris* and *jonggajibkimchii*. It comprises Gram-positive, catalase-negative, facultatively anaerobic, non-spore-forming, and spherical heterofermentative, with coccus shapes and relatively low G + C content bacteria (Ogier *et al*., 2008; Hemme and Foucaud-Scheunemann, 2004).

L. mesenteroides strains are commonly found in association with food substrates, both of plant and animal origin (Silva *et al*., 2015; Nionelli *et al*., 2014; Björkroth and Holzapfel, 2006). Moreover, members of this species are reported to be mainly responsible for the fermentation of various vegetables, such as kimchi (a Korean fermented vegetable food) and sauerkraut (pickled cabbage), and dairy products, such as cheese (Chun *et al*., 2017; Di Cagno *et al*., 2013, Jung *et al*., 2012b; Breidt, 2004; Cibik *et al*., 2000).

In the dairy industry, *L. mesenteroides* strains are naturally present as contaminants in many traditional cheese varieties or they are deliberately added as adjunct cultures (Pedersen *et al*., 2014; Alegría *et al*., 2013; Ali *et al*., 2013). Indeed, their capacity to produce aromatic compounds, such as acetaldehyde, acetoin and diacetyl, in addition to lactic and acetic acid, carbon dioxide and dextrans, contribute to the development of desirable sensory traits of dairy products (Kothari and Goyal, 2015; Nieto-Arribas *et al*., 2010; Hemme and Foucaud-Scheunemann, 2004; McSweeney and Suosa, 2000).

Recently, *L. mesenteroides* strains have been proposed as biopreservative cultures for food products and as potential probiotics (Giles-Gómez *et al*., 2016; de Paula *et al*., 2015; de Paula *et al*., 2014). In particular, *L. mesenteroides* subsp. *mesenteroides* SJRP55 isolated from water buffalo mozzarella cheese showed both probiotic and biopreservative features. In fact, it was characterized by good adhesion properties, β–galactosidase activity, dextran production and viability in fermented milk during storage and sensitive to most of the tested antibiotics, which make this strain a potential candidate for the industrial application as a probiotic strain (de Paula *et al*., 2015). In addition, *L. mesenteroides* SJRP55 produces bacteriocins, which are able to inhibit the growth of *Listeria* spp. strains, representing a promising biopreservative culture in fermented milk (de Paula *et al*., 2014).

Although *L. mesenteroides* strains are generally considered to be non-infectious agents in humans and to be suitable for the qualified presumption of safety (QPS) status from the European Food Safety Authority (EFSA) (EFSA, 2012), they were associated with certain human diseases such as brain abscess, endocarditis, nosocomial outbreaks, and central nervous system tuberculosis (Barletta *et al*. 2017; Bou *et al*., 2008; Albanese *et al*., 2006).

In addition, our previous study reported the presence of multidrug resistant *L. mesenteroides* strains in traditional Italian cheese. In particular, these strains displayed atypical resistance to erythromycin and clindamycin (LbE15), kanamycin, streptomycin, tetracycline and virginiamycin (LbE16), and tetracycline (LbT16) (Flórez *et al*., 2016). Preliminary analysis of the sequences revealed the presence of *erm*(B) in LbE15 and *tet*(S) in LbE16, coding for erythromycin (Roberts, 2008) and tetracycline (Thaker *et al*., 2010) resistance, respectively (Flórez *et al*., 2016). However, these genotypes could only partially explain the resistance phenotypes showed by these three *L. mesenteroides* strains.

With the development of high-throughput and low-cost sequencing technologies, genomic information-based approaches have been recently considered for the comprehensive understanding of antibiotic resistance (AR) traits of a microorganism (Gillings *et al*., 2017; Köser *et al*., 2014). Indeed, whole-genome sequencing (WGS) provides genetic information at the whole genome level, thus making available any resistance gene or mutation present in a single microbial genome (Chan, 2016).

In this context, the main aims of this study were: i) to determine the whole genome sequence of *L. mesenteroides* subsp. *dextranicum* LbE15, *L. mesenteroides* subsp. *mesenteroides* LbE16 and *L. mesenteroides* subsp. *cremoris* LbT16 through WGS approach; and ii) to characterize the genetic basis of the AR phenotypes previously identified for these three strains, through the analysis of the genome sequence.

3.2 Material and Methods

Bacterial strains and growth conditions. The three *L. mesenteroides* strains analysed in this study have been previously isolated from Taleggio Italian cheese and they have been identified as multidrug resistant bacteria (Flórez *et al*., 2016). These strains were grown at 30°C in de Man Rogosa and Sharpe (MRS) broth (Fluka, Milan, Italy) and were kept in liquid cultures with 20% (w/vol) glycerol at -80°C for long term storage.

DNA extraction and sequencing. Total genomic DNA was extracted and purified from 2-mL overnight cultures using the Wizard Genomic DNA purification kit (Promega Corporation, Madison, USA), following the manufacturer's instructions.
Whole-genome sequencing was performed using the Illumina HiSeq2000 platform at Beijing Institute of Genomics (BIG) (Beijing, China) with a paired-end library. The sequencing data have been deposited in DDBJ/EMBL/GenBank under the following accession numbers: LAYN01000000 (*L. mesenteroides* subsp. *dextranicum* LbE15), LAYU01000000 (*L. mesenteroides* subsp. *mesenteroides* LbE16) and LAYV01000000 (*L. mesenteroides* subsp. *cremoris* LbT16).

Quality control and genome assembly. For the raw sequencing data, the reads were cleaned by removing the Illumina's oligonucleotide adapter sequences and the duplicates using the CLC Bioinformatics Database software package (CLC bio, Aarhus, Denmark). Thus the reads quality was verified with FastQC software in order to confirm that the Q value of the reads was higher than 28. The clean reads were assembled using SPAdes Assembler version 3.5.0 (Bankevich *et al*., 2012). Finally the contigs obtained were reordered on the genome sequence of *L. mesenteroides* subsp. *mesenteroides* ATCC 8293^{T} (Accession number: CP000414) using MAUVE program (Rissman *et al*., 2009).

Genome annotation and identification of antibiotic resistance genes. The genomic sequences of the three *L. mesenteroides* strains were annotated by the National Center for Biotechnology (NCBI, http://www.ncbi.nlm.nih.gov/) Prokaryotic Genome Annotation Pipeline and using RAST server (Rapid Annotations using Subsystems Technology, http://RAST.nmpdr.org) (Aziz *et al*., 2008). This is a fully automated service for annotating bacterial and archaeal genomes, which identifies protein-encoding, rRNA and tRNA genes, assigns functions to the genes, predicts which subsystems are represented in the genomes and uses this information to reconstruct the metabolic network and pathways.

The annotated sequences were employed to query the Comprehensive Antibiotic Resistance Database (CARD, version 1.0.6, http://arpcard.mcmaster.ca) (McArthur *et al*., 2013) through the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) in order to identify all AR genes involved in the resistance phenotypes observed in our previous study (Flórez *et al*., 2016). A gene was annotated as putative AR determinant according to its best BLASTP hit in CARD with a threshold of amino acid sequence identity > 30% and query coverage > 70%. In addition, the amino acid sequences of all AR genes retrieved from CARD, resulting in a reference dataset of 2,163 amino acid sequences, were aligned against the annotated genome sequences of the collection and the best BLASTP hits were filtered as described above. In order to minimise putative false negative or false positive outputs, only the putative AR determinants obtained from both approaches were considered for subsequent analyses. In detail, each putative AR determinant was manually annotated querying the NCBI non–redundant (NR) protein database to verify its actual function in the resistome and to determine its involvement in acquired phenotypes.

Flanking regions of the AR genes. Sequences surrounding the AR genes from the strains *L. mesenteroides* LbE15, LbE16 and LbT16 were analysed by retrieving those contigs carrying resistance determinants from the whole genome sequencing data. The organization of these sequences were revealed though BLASTN and BLASTX alignments against the NCBI non-redundant database. Phylogenetic tree for the sequences identified through BLAST analysis were constructed using distance analysis as implemented in MEGA version 6 software (Tamura *et al*., 2013).

3.3 Results and Discussion

3.3.1 Assembly and annotation of the genome sequencing of the three *L. mesenteroides* strains. A total of 1,524,191, 1,682,147 and 1,416,327 paired-end reads (2 × 75-bp length on average) were obtained after quality control and trimming process, and they were assembled into 65, 86, and 66 contigs for strain LbE15, LbE16 and LbT16, respectively (genome coverage of about 200×). The length of the largest assembled contig was 259,998-bp, 285,382-bp and 382,195-bp for the genome of LbE15, LbE16 and LbT16, respectively. The three genomes contain 53 genes encoding RNAs, of which 3 for rRNAs and 50 for tRNAs (Table 3.1).

The genome of LbE15, LbE16 and LbT16 carries 1,939, 2,100 and 2,044 genes, respectively, of which 1,713, 1,949 and 1,900 represent coding sequences (Figure 3.1-3.3 A).

Table 3.1. Whole-genome information of the three *L. mesenteroides* strains LbT16, LbE16 and LbT15.

Feature	Statistics		
	LbT16	**LbE16**	**LbE15**
Genome size (bp)	1,906,463	2,036,196	2,008,120
No. of contigs	66	86	65
N_{50} (bp)	75,366	160,323	76,771
G+C content (%)	37	37	37
No. of genes	1,939	2,100	2,044
No. of coding sequences	1,713	1,949	1,900
No. of pseudogenes	172	97	90
No. of tRNAs	50	50	50
No. of rRNAs	3	3	3
Accession number	LAYV00000000	LAYU00000000	LAYN00000000

According to RAST annotation server, the annotated sequences were sorted into 24 subsystem categories of SEED database for the genome of *L. mesenteroides* subsp. *cremoris* LbT16, *L. mesenteroides* subsp. *mesenteroides* LbE16 and *L. mesenteroides* subsp. *dextranicum* LbE15 strain and the distribution of the genes for each category is reported in Figure 3.1-3.3 B.

A SEED subsystem is a collection of functional roles that together create a specific biological process or structural complex (Overbeek *et al*., 2005).

The most abundant category were represented by “Amino Acids and Derivatives”, “Protein Metabolism” and “Carbohydrates” for all three *L. mesenteroides* genomes. A recent report on the *L. mesenteroides* pan-genome features revealed that housekeeping processes, such as amino acid and protein metabolism, were more enriched in the core-genome than in the accessory/unique genome. In contrast, genes corresponding to carbohydrate transport and metabolism were more abundant in the accessory/unique-genome than in the core-genome suggesting that the fermentation features of *L. mesenteroides* species for carbohydrate compounds is variable among *L. mesenteroides* strains (Chun *et al*., 2017).

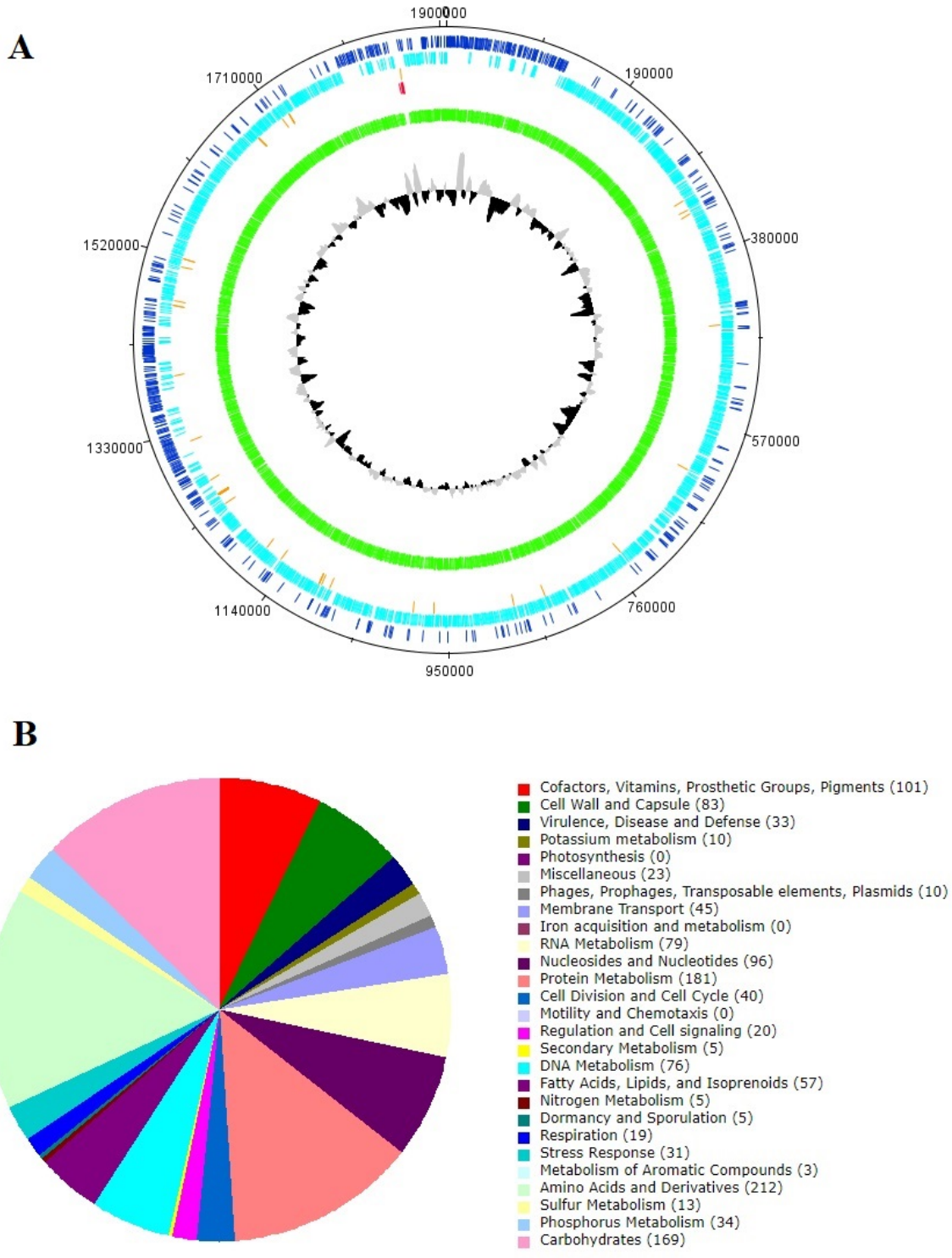

Figure 3.1. Circular representation (A) and functional annotation (B) of the genome sequence of *L. mesenteroides* subsp. *cremoris* LbT16. A) Plot of the genome sequence obtained with DNA Plotter (Carver *et al*., 2009): from the outer circle to the inner circle are circle 1, ORFs on the positive strand (blue); circle 2, ORFs on the negative strand (light blue); circle 3, tRNA (orange); circle 4, rRNA (red); circle 5, summary of ORFs (green); circle 6, GC content (light/dark grey). B) Subsystem category distribution of the genes annotated through RAST server (Aziz *et al*., 2008).

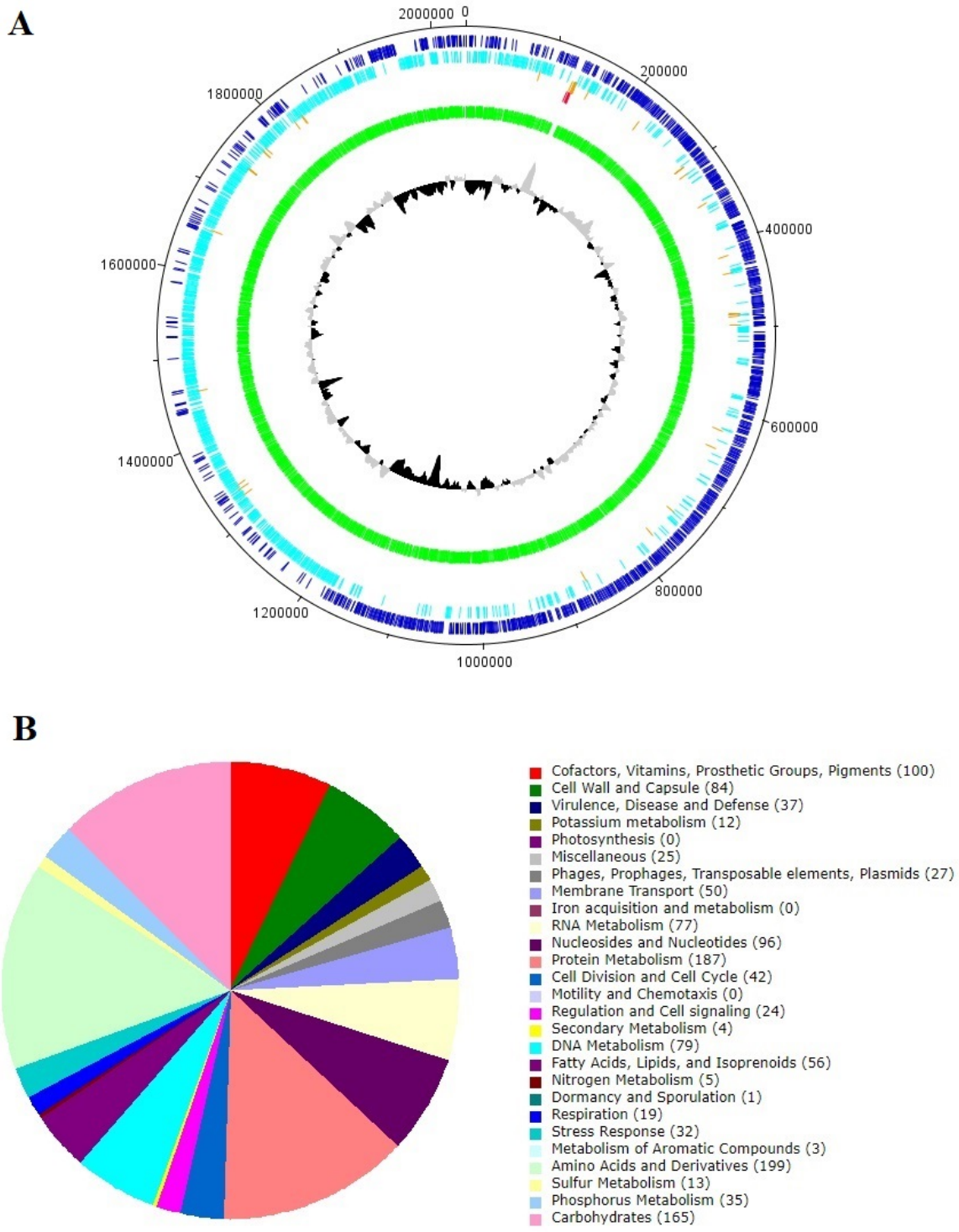

Figure 3.2. Circular representation (A) and functional annotation (B) of the genome sequence of *L. mesenteroides* subsp. *mesenteroides* LbE16. A) Plot of the genome sequence obtained with DNA Plotter (Carver *et al*., 2009): from the outer circle to the inner circle are circle 1, ORFs on the positive strand (blue); circle 2, ORFs on the negative strand (light blue); circle 3, tRNA (orange); circle 4, rRNA (red); circle 5, summary of ORFs (green); circle 6, GC content (light/dark grey). B) Subsystem category distribution of the genes annotated through RAST server (Aziz *et al*., 2008).

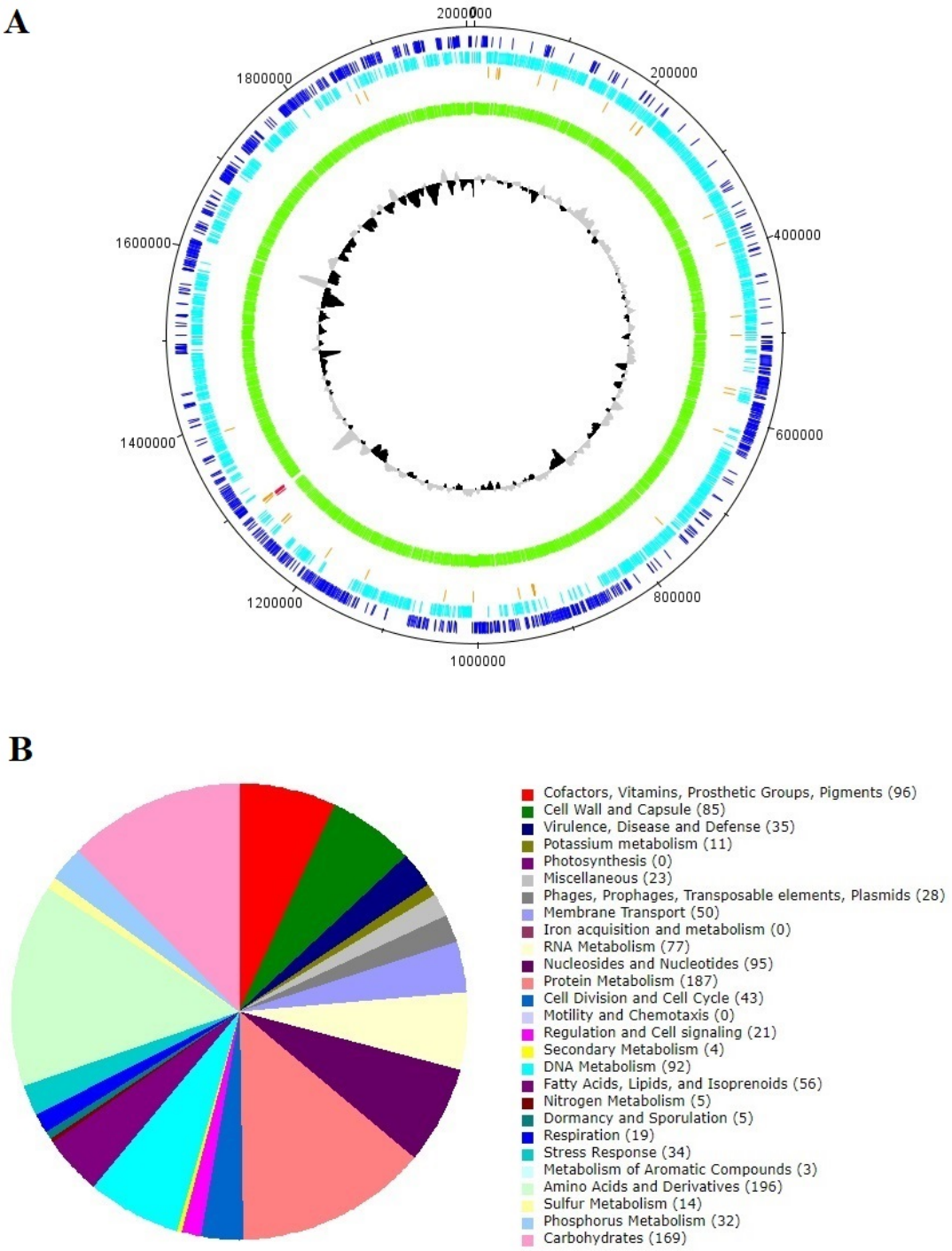

Figure 3.3. Circular representation (A) and functional annotation (B) of the genome sequence of *L. mesenteroides* subsp. *dextranicum* LbE15. A) Plot of the genome sequence obtained with DNA Plotter (Carver *et al*., 2009): from the outer circle to the inner circle are circle 1, ORFs on the positive strand (blue); circle 2, ORFs on the negative strand (light blue); circle 3, tRNA (orange); circle 4, rRNA (red); circle 5, summary of ORFs (green); circle 6, GC content (light/dark grey). B) Subsystem category distribution of the genes annotated through RAST server (Aziz *et al*., 2008).

Regarding gene involved in virulence, disease and defence, RAST server revealed a total of 33, 37, and 35 genes for LbT16, LbE16 and LbE15, respectively. This category includes genes coding for: adhesion; toxins and superantigens; bacteriocins, ribosomally synthesized antibacterial peptides; resistance to antibiotics and toxic compounds; invasion and intracellular resistance (Aziz *et al*., 2008).

In particular, a total of 21, 25 and 23 genes encoding for antibiotic and heavy metal resistance were found for LbT16, LbE16 and LbE15 strain, respectively. To better understand the genetic basis of the AR, the genome sequence of these *Leuconostoc* strains was further analysed through homology-based method against a set of AR reference sequences.

3.3.2 Identification of AR and their genetic organization. To reveal the relevant genes for AR carried by *L. mesenteroids* subsp. *cremoris* LbT16, *L. mesenteroides* subsp. *mesenteroides* LbE16 and *L. mesenteroides* subsp. *dextranicum* LbE15, sequence alignment of their genome sequences and the protein sequence of AR genes in CARD was performed. Based on the selection criteria, a total of seven gene sequences were identified among the three *L. mesenteroides* strains which showed high similarity to the sequence of AR genes in CARD (Table 3.2), except for *L. mesenteroides* subsp. *cremoris* LbT16 which lacked any known AR genes. Therefore, the resistance phenotypes revealed in our previous study (Flórez *et al*., 2016) for this strain may be due to unspecific mechanisms, such as activity of general efflux systems, or caused by a not-yet-reported gene.

The genome sequence analysis of *L. mesenteroides* subsp. *mesenteroides* LbE16 and *L. mesenteroides* subsp. *dextranicum* LbE15 confirmed the presence of tetracycline and erythromycin resistance genes, such as *tet*(S) and *erm*(B), which were already detected in these strains by PCR assay in our previous report (Flórez *et al*., 2016). In addition, this genome-based approach allowed the identification of five more AR gene sequences in the LbE16 genome encoding for aminoglycoside (*aad6*, *sat*4 and *aphA*-3), streptogramin A (*vat*E) and methylenomycin A (*mmr*) resistance (Table 3.2).

DNA sequences from the contigs in which the AR genes were identified and the open reading frames (*orf*s) flanking the AR genes were subjected to BLASTN, BLASTX and BLASTP analyses (http://blast.ncbi.nlm.nih.gov) to characterize the up- and down-stream regions of the AR genes. The flanking regions of AR genes found in strains LbE16 and LbE15 are schematically depicted in Figure 3.4.

Table 3.2. AR genes identified in the multidrug resistant *L. mesenteroids* subsp. *cremoris* LbT16, *L. mesenteroides* subsp. *mesenteroides* LbE16 and *L. mesenteroides* subsp. *dextranicum* LbE15 through the genome sequence analysis.

Strain	Resistance phenotype [a]	Gene ID	CARD genes	Resistance gene class
LbT16	Chloramphenicol Tetracycline Trimethoprim	-	-	-
LbE16	Chloramphenicol Erythromycin Kanamycin Neomycin Streptomycin Tetracycline Trimethoprim Virginiamycin	NODE_8_length_76144_cov_4.16338_ID_15_72291_74216	*tet*(S)	Ribosomal protection protein coding for tetracycline resistance
		NODE_69_length_1520_cov_2.01614_ID_139_1374_730	*vat*E (satG)	Acetyltransferase involved in streptogramin resistance
		NODE_28_length_13172_cov_1.39428_ID_55_4572_5207	*aad*6	Aminoglycoside nucleotidyltransferase
		NODE_28_length_13172_cov_1.39428_ID_55_5204_5746	*sat*4	Streptothricin acetyltransferase and streptothricin resistant determinant.
		NODE_28_length_13172_cov_1.39428_ID_55_5839_6633	*aphA*-3	Aminoglycoside phosphotransferase
		NODE_28_length_13172_cov_1.39428_ID_55_9096_7729	*mmr*	Methylenomycin A resistance protein
LbE15	Clindamycin Chloramphenicol Kanamycin Trimethoprim	NODE_19_length_33139_cov_4.9724_ID_39_27528_26791	*erm*(B)	rRNA methyltransferase encoding for macrolide-lincosamide-streptogramin resistance

[a] Resistance phenotypes of the three *L. mesenteroides* strains reported by Flórez *et al*. (2016)

In detail, three contigs harbouring AR genes were identified in the genome of *L. mesenteroides* LbE16 (Figure 3.4A). The tetracycline resistance gene *tet*(S) was identified in one of the contigs. Based on the size of the contig harbouring this AR determinant (171,788 bp), it is expected that the tetracycline resistance gene is located in the bacterial chromosome of LbE16. This observation is in accordance with the data reported by Flórez *et al*. (2016).

Further, a small contig contained two *orf*s, of which one showed extensive homology to virginiamycin resistance [*vat*(E) in Figure 3.4A] and a third contig harbouring a cluster of genes involved in AR were identified for the LbE16 strain. This gene cluster showed extensive homology to others AR determinants involved in resistance to aminoglycosides; namely, *aad6* encoding streptomycin resistance, *sat4* encoding resistance to streptothricin, *aphA-3* encoding kanamycin and neomycin resistance and *mmr* encoding methylenomycin A resistance (Figure 3.4A).

The *aad6–sat4–aphA-3* cluster of LbE16 revealed almost identical nucleotide sequences with a cluster which has been previously detected in staphylococci, campylobacter and enterococci (Qin *et al*., 2012). This cluster has also been detected in naturally occurring mobile genetic elements, such as plasmids and transposons; thus is supposed to be horizontally transferable between foodborne bacteria (Qin *et al*., 2012). Notably, upstream of the streptogramin/aminoglycosides resistance genes, an *orf* that could encode a plasmid-associated protein was identified (*rep*). Moreover, the nucleotide sequence around the *rep* gene shared a complete identity with those encoded by plasmids pKLC2 (Jung *et al*., 2012a), LkipL4726 (Oh *et al*., 2010) and pLCK1 (Kim *et al*., 2008), from *Leuconostoc carnosum*, *Leuconostoc kimchi* and *Leuconostoc citreum*, respectively, suggesting that these sequences may be located on a plasmid in the LbE16 strain.

Further studies are necessary to resolve the complete plasmid structure using hybrid assembly strategies, which typically employ Illumina pair-end library for contig assembly and the PacBio reads for subsequent scaffolding.

None of the *orf*s located in the other two contigs showed significant homology to plasmid sequences, suggesting they must be chromosomally encoded. However, sequences surrounding the antibiotic resistance genes showed homology with *orf*s encoding recombinase/transposase-like proteins (in yellow in Figure 3.4). In particular, the sequence upstream of *tet*(S) gene showed, at amino acidic level, 99% identity with transposase A of *Streptococcus dysgalactiae* subsp. *equisimilis* (Liu *et al*., 2008). As before, these elements may contribute to the horizontal transfer of these antibiotic resistances.

In contrast to LbE16, only one contig carrying AR genes was detected in the *L. mesenteroides* LbE15 genome. This contig harboured the erythromycin resistance gene *erm*(B) detected in this

strain (the *orf* in red in Figure 3.4B). Downstream of *erm*(B), two *orf*s encoding plasmid-replication proteins were identified; thereby supporting the association of erythromycin resistance with a plasmid, as previously highlighted by Flórez *et al*. (2016). Moreover, a Type III restriction-modification system was shown to be encoded upstream of the erythromycin resistance determinant. These genes shared the greatest homology (95%) to the corresponding region of the plasmid LkipL4704 from *L. kimchi* (Oh *et al*., 2010). Furthermore, two genes encoding mobile element proteins proved to be identical at nucleotide level to those of pLG1, a plasmid of *Enterococcus faecium* (Laverde *et al*., 2011). These two genes shared 99% identity with the *Tn*3 DDE-transposase of several species of staphylococci, streptococci and enterococci, and the resolvase of *Streptococcus pneumoniae*, respectively (Nesmelova and Hackett, 2010). Transposons are involved in the rapid adaptation of bacteria to changing environments, and their frequent location on plasmids may facilitate dissemination (Bellanger *et al*., 2014).

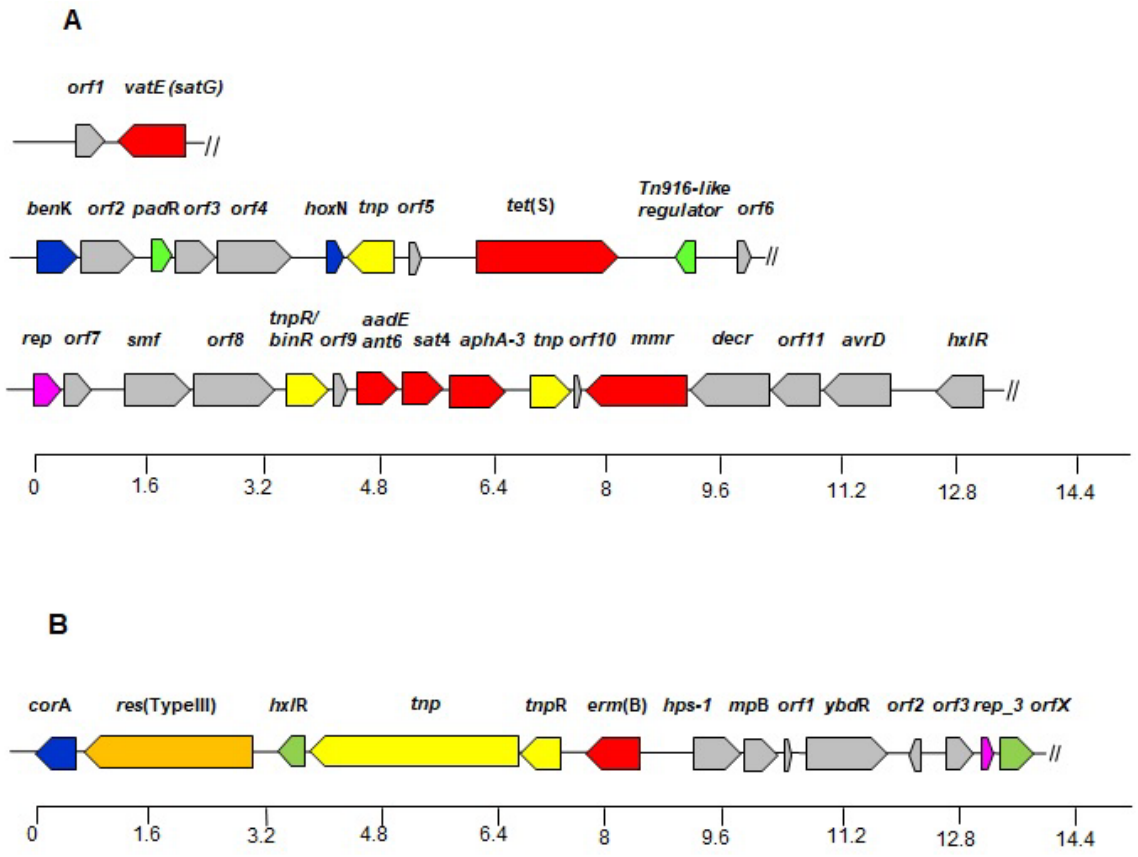

Figure 3.4. Diagram showing the genetic organization of DNA contigs around the antibiotic resistance genes identified in the genome of *L. mesenteroides* subsp. *mesenteroides* LbE16 (A) and *L. mesenteroides* subsp. *dextranicum* LbE15 (B) strains. Color code of genes and open reading frames (*orf*s): antibiotic resistance genes are in red; in yellow, genes encoding proteins involved in mobilization; in orange, genes of restriction-modification systems; in green, genes encoding regulatory proteins; in blue, genes involved in transport; in pink, genes encoding plasmid-associated replication proteins; in grey, genes belonging to other RAST subsystems (Aziz *et al*., 2008). The broken line symbol indicates the end of the contig.

A deeper gene sequence analysis revealed the high similarity of the region flanking the gene *erm*(B) with the nucleotide sequence of the *Tn*917-like transposon found in *E. faecalis* DS16 (Accession number: M11180.2) (Shaw and Clewell, 1985), *Bacillus subtilis* subsp. *subtilis* AG1839 (CP008698.1) (Smith *et al*., 2014), *Staphylococcus aureus* subsp. *aureus* SA268 (CP006630.1), *Streptococcus gordonii* KCOM 1506 (CP012648.1), *Streptococcus agalactiae* GBS6 (CP007572.1) (Srinivasan *et al*., 2014), *Streptococcus oralis* Uo5 (FR720602.1) (Reichmann *et al*., 2011), *Streptococcus pneumoniae* 11928 (FR671417.1) (Croucher *et al*., 2011) (Figure 3.5).

The strict similarity observed validates the hypothesis that LbE15 acquired the *erm*(B) from other microorganisms through horizontal gene transfer events

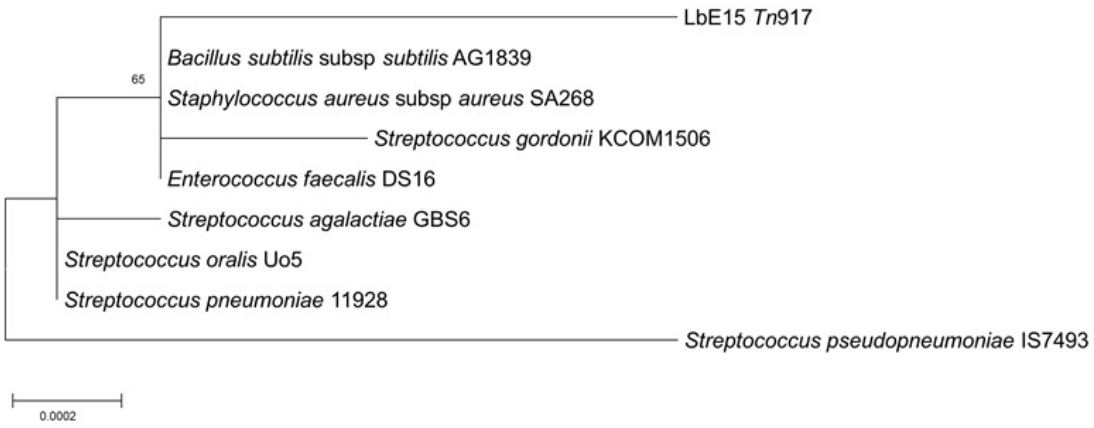

Figure 3.5. Phylogenetic tree based on the nucleotide sequence of the *Tn*917-like transposon. The tree was reconstructed by using Neighbor-joining method. Bootstrap values (1,000 replicates) are shown as a percentage at the branching points. The scale bar represents the number of nucleotide substitutions per site.

The *Tn*917 transposon confers on its host inducible resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics (Puopolo *et al*., 2007). It was originally identified on the 22-kb, nonconjugative, multiple resistance plasmid pAD2 of *Streptococus faecalis* DS16 (Tomich *et al*., 1979), reclassified as *E. faecalis* by Schleifer and Kilpper-Bälz (1984). A particular property of *Tn*917 is the ability to undergo enhanced transposition on exposure to low levels of erythromycin (Tomich *et al*., 1980, Puopolo *et al*., 2007).

The entire sequence of the *Tn*917 transposon identified in the genome of *L. mesenteroides* subsp. *dextranicum* LbE15 is shown in Figure 3.6. In detail, the transposon is 5,258 bp long and carried five different *orf*s, which were all on the same strand.

In total agreement with the data reported by Shaw and Clewell (1985), the inverted repeats at the left and right terminal of the transposon are 38 bp long and they are identical (reported in Figure 3.6 as LR and RR, respectively). Moreover, a 38-bp internal repeat (IR) was found downstream from the *orf*3, which was nearly identical to the LR sequence and they were in the same orientation.

The *orf*s 1-3 were located by the left terminal repeat and they code for a leader peptide, an rRNA methyltransferase and a hypothetical protein, respectively.

Region characteristic of a promoter was located 55 bp upstream from the leader peptide start codon and has typical -10 (TATAAT) and -35 (TTGATA) sequence (Gilman and Love, 2016) (in blue in Figure 3.6). Similarly, the *orf*4 and *orf*5 are also preceded by a promoter region, which was found five bp upstream from the Shine-Dalgarno sequence of the *orf*4 and was characterized by the presence of -10 sequence TATAAA and the -35 hexamer TTAATG.

The *orf*4 and *orf*5 coded for a resolvase and a transposase enzyme, respectively, which are essential for the transposition of the *Tn*917 (Shaw and Clewell, 1985).

These findings corroborate the data reported by Flórez *et al.* (2016), confirming that erythromycin resistance is an acquired character of the LbE15 strain and the ability to undergo transposition from this strain to other microorganisms. Therefore, *L. mesenteroides* subsp. *dextranicum* LbE15 represents a reservoir of AR features and the set-up of *erm*(B) flanking regions increase the horizontal transferability and the spread of erythromycin resistance along the food chain.

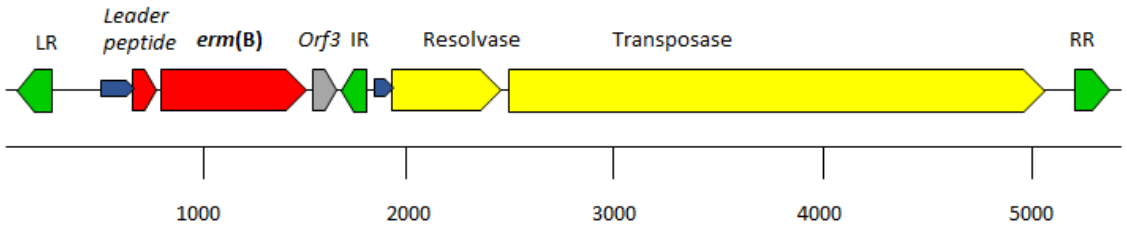

Figure 3.6. Diagram showing the genetic organization of *Tn*917 transposon identified in the genome of *L. mesenteroides* subsp. *dextranicum* LbE15. Color code of genes and open reading frames (orfs): antibiotic resistance genes are in red; in yellow, genes encoding proteins involved in mobilization; in blue, promoter regions; in green, repeat region; in grey, genes belonging to other RAST subsystems (Aziz *et al.*, 2008).

3.4 Conclusions

This study reported the draft genome sequence of three *L. mesenteroides* strains isolated from Italian soft cheese samples, namely *L. mesenteroides* subsp. *dextranicum* LbE15, *L. mesenteroides* subsp. *mesenteroides* LbE16 and *L. mesenteroides* subsp. *cremoris* LbT16.

The genome sequence analysis revealed the presence of seven different AR genes among the three *Leuconostoc* strain examined, five of which had not yet been identified. In fact, the *tet*(S) and *erm*(B) genes were already found in LbE16 and LbE15, respectively, in our previous study through the PCR assay.

However, the presence of genes coding for aminoglycoside resistance, such as *aad6*, *sat*4 and *aphA*-3, and for streptogramin A resistance, as *vat*E, in LbE16 was identified only through the analysis of its genome sequence.

Moreover, this analysis allowed to characterize the flanking region of the AR determinants, revealing the presence of a *erm*(B)-bearing *Tn*917 transposon in the genome of *L. mesenteroides* subsp. *dextranicum* LbE15. In particular, the sequence of this transposon was found to be almost identical to those previously reported for *E. faecalis*, *B. subtilis*, *S. aureus* and several species of *Streptococcus*, highlighting that *Tn*917 is highly conserved among different bacterial species.

Therefore, the whole-genome sequencing is more informative than conventional molecular techniques, providing data about any resistance gene or mutation present in the bacterium analysed. These peculiarities make WGS ideal as tool for the surveillance of emergence and spread of AR traits.

In conclusion, the complete genomes of the three *L. mesenteroides* strains reported here represent a fundamental starting point to improve the current knowledge regarding the molecular basis of AR in LAB and to evaluate its transference capability via horizontal gene transfer among food-borne bacteria.

Chapter 4

Genome-based assessment of antibiotic resistance in the genus *Leuconostoc*

4.1 Introduction

The genus *Leuconostoc* belongs phylogenetically to the phylum Firmicutes, class Bacilli, order Lactobacillales, and family *Leuconostoccaceae* (Lyhs *et al*., 2015). Nowadays, this genus comprises 14 species and 7 subspecies: *L. gelidum* subsp. *gelidum*, *L. gelidum* subsp. *gasicomitatum*, *L. gelidum* subsp. *aenigmaticum*, *L. inhae*, *L. carnosum*, *L. miyukkimchi*, *L. kimchii*, *L. rapi*, *L. palmae*, *L. lactis*, *L. citreum*, *L. hozapfelii*, *L. pseudomesenteroides*, *L. mesenteroides* subsp. *suionicum* (which has been recently reclassified as *L. suionicum*, Jeon *et al*., 2017), *L. mesenteroides* subsp. *mesenteroides*, *L. mesenteroides* subsp. *dextranicum*, *L. mesenteroides* subsp. *cremoris*, *L. mesenteroides* subsp. *jonggajibkimchii*, and *L. fallax*.

Members of the genus *Leuconostoc* are Gram-positive lactic bacteria, coccoid to ovoid-like morphology, which are usually arranged in pairs or small chains. They are not mobile, not spore forming, facultatively anaerobic, catalase-negative, non-proteolytic and unable to hydrolyse arginine due to the lack of arginine dehydrolase enzyme (Björkroth and Holzapfel, 2006). An interesting property of *Leuconostoc* spp. is the exclusive production of D-lactate from glucose, since most other lactic acid bacteria produce DL-lactate. Other end products of the glucose fermentation are represented by CO_2, ethanol and/or acetate (Ogier *et al*., 2008).

Leuconostoc spp. are environmental microorganisms generally found on green vegetation and roots. From this natural habitat they can easily propagate in various niches including plant materials, such as vegetables and silage, and fermentation food products from various row material (Hemme and Foucaund-Scheunemann, 2004). In particular, *Leuconostoc* spp. constitute the natural microbial population of fermented vegetable products, such as sauerkraut and kimchi, a typical Korean fermented cabbage dish from which the type strains of *L. kimchii* (IH25^T; Kim e*t al*., 2000) and *L. inhae* (IH003^T; Kim *et al*., 2003) have been isolated. Some *Leuconostoc* strains, such as *L. mesenteroides*, play an important role in the fermentation processes of plant

products, such as sauerkraut and cucumber (Beganović *et al*., 2011), and are also used as starter cultures in the fermentation of carrots and coffee beans (Dellaglio *et al*., 1995). Recently in Korea, *L. mesenteroides* strains have been isolated from various food products such as kimchi, soybeans, fish and molluscs (Kaur *et al*., 2017). In addition, strains belonging to *L. lactis* and *L. mesenteroides* subsp. *mesenteroides* species were even found in quinoa samples (Vera-Pingitore *et al*., 2016).

Leuconostoc spp. have been associated with wide variety of meat products, such as fresh and vacuum packaged meat, poultry, as well as processed and fermented meat products (Björkroth and Holzapfel, 2006). Indeed, the occurrence of leuconostocs including *L. carnosum*, *L. citreum*, *L. mesenteroides*, *L. gelidum* subsp. *gelidum*, and *L. gelidum* subsp. *gasicomitatum* in meat products has been reported in different studies (Rahkila *et al*., 2014; Oki *et al*., 2011; Shaw and Harding, 1989).

Despite the important role of *Leuconostoc* spp. in the production of fermented foods, these strains have been even related to negative aspects in human health, including the production of undesirable compounds in foods, such as biogenic amines (Moreno-Arribas *et al*., 2003) and the possibility of causing infections in subjects with compromised immune systems (Hemme and Foucaud-Scheunemann, 2004).

Frequently, leuconostocs associated with infections caused by multiple microorganisms were isolated from patients following vancomycin treatment. Indeed, the vancomycin resistance is an intrinsic feature of the species of the genus *Leuconostoc* and is due to the presence of pentadepsipeptide with D-Lactate at the C-terminal in the peptidoglycan instead of a D-Alanine (Ogier *et al*., 2008). Although this resistance is a well-known character for *Leuconostoc*, the resistance toward other antibiotics has been characterized in few reports. Generally, *Leuconostoc* strains are susceptible to antibiotics inhibiting the protein synthesis, such as erythromycin, chloramphenicol, clindamycin and tetracycline (Flórez *et al*., 2005, Ammor *et al*., 2007). High susceptibility toward ampicillin and penicillin has been detected in strains belong to *L. citreum*, *L. mesenteroides*, *L. lactis* and *L. pseudomesenteroides* isolated from fermented foods (Casado-Muñoz *et al*., 2014; Morandi *et al*., 2013). Moreover, acquired resistance to tetracycline and erythromycin has been reported for *L. mesenteroides* strains (Flórez *et al*., 2016).

The application of molecular methods, such as PCR assay and microarray analysis, is being very helpful in the determination of the genetic basis of the resistance phenotype. However, this approach is limited to a narrow number of antibiotic resistance (AR) determinants.

Therefore, whole-genome sequencing (WGS) is becoming an important tool in surveillance the emergence and spread of AR (Schürch and van Schaik, 2017). Indeed, WGS offers the

unprecedented advantage of providing genetic information at the whole genome level, thus making it ideal for uncovering all possible genetic determinants of antimicrobial resistance in a single microbial genome (Chan, 2016). WGS and whole-community sequencing (i.e., metagenomics) could revolutionize food safety assessment, resulting in a paradigm shift from phenotype- to genotype-based assay of AR (Ellington *et al*., 2017). To date, the genome sequence of 11 type strains of the genus *Leuconostoc* are available, representing an important starting point for the safety assessment of this relevant technological group of lactic acid bacteria (LAB).

To improve the current knowledge about the AR in the genus *Leuconostoc* and to verify the effectiveness of the WGS as a tool for surveillance of AR, the main aims of this study were: i) to identify known genes and genetic mutation associated with AR through the use of the genomic approach, and ii) to verify the correlation between AR determinants found and the resistance phenotypes observed.

4.2 Materials and Methods

Bacterial strains and growth conditions. A collection of 11 type strains of the genus *Leuconostoc* was set up based on the available genomes in the databases (Table 4.1). They were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium) and from the Spanish Type Culture Collection (CECT, Valencia, Spain). *Leuconostoc* strains were grown in de Man-Rogosa-Sharpe (MRS, Fluka, Italy) medium at 27°C for 48 h and kept in liquid cultures with 20% (w/vol) glycerol at -80°C for long term storage.

Genome sequence analysis to retrieve AR genes. The genomic sequences of the 11 type strains of the genus *Leuconostoc* were downloaded from NCBI using the Accession Number reported in Table 4.1, and they were annotated using RAST server (Rapid Annotations using Subsystems Technology, http://RAST.nmpdr.org) (Aziz *et al*., 2008). The annotated sequences were employed to query the Comprehensive Antibiotic Resistance Database (CARD, version 1.0.6, http://arpcard.mcmaster.ca) (McArthur *et al*., 2013) through the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) in order to identify all AR genes that could be involved in resistance phenotypes. A gene was annotated as putative AR determinant according to its best BLASTP hit in CARD with a threshold of amino acid sequence identity > 30% and query coverage > 70%.

Table 4.1. Genome features of the 11 type strains of the *Leuconostoc* genus analysed in this study.

Species	Strain ID	Source	Genome AN	Contig	Size (pb)	G+C content (%)	CDS[a]	Reference
L. carnosum	CECT 4024^T	Chill-stored meat	BACM00000000.1	2.407	3.234.408	40.9	3.446	Nam *et al*., 2011a
L. fallax	LMG 13177^T	Sauerkraut	AEIZ00000000.1	30	1.638.971	37.5	1.895	Nam *et al*., 2011b
L. gelidum subsp. *gasicomitatum*	CECT 5767^T	Packaged meat	FN822744.1	1	1.954.080	37	1.913	Johansson *et al*., 2011
L. gelidum subsp. *gelidum*	CECT 4026^T	Packaged meat	AEMI00000000.1	43	1.957.281	36	1.930	Kim *et al*., 2011a
L. inhae	CECT 7026^T	Kimchi	AEMJ00000000.1	893	2.298.088	36	2.757	Kim *et al*., 2011b
L. lactis	LMG 8894^T	Milk	AEOR00000000.1	1.151	2.011.205	42.6	2.079	Kim *et al.* (Unpublished)
L. mesenteroides subsp. *cremoris*	LMG 6909^T	Hansen's starter powder	ACKV00000000.1	126	1.638.511	37.9	1.753	n.a.
L. mesenteroides subsp. *dextranicum*	LMG 6908^T	Isolated in 1912	CP012009.1, CP012010.1^P	1	1.818.633; 36.094^P	38	1.696	Park and Shin (Unpublished)
L. mesenteroides subsp. *mesenteroides*	LMG 6893^T	Fermenting olives	CP000414.1, CP000415.1^P	1	2.038.396; 37.367 ^{P}	37.66	1.960	Makarova *et al*., 2006
L. suionicum	CECT 8146^T	1972, Sweden	CP015247.1, CP015248.1^P	1	2.026.850; 21.983 ^{P}	37.59	1.921	Jeon *et al.* (Unpublished)
L. pseudomesenteroides	LMG 11482^T	Cane juice	AEOQ00000000.1	1.160	3.244.985	38.3	3.451	Kim *et al*., 2011c

[a]: Number of predicted coding sequences
^{P}: Accession number of the plasmid sequence.
n.a.: Reference not available

In addition, the amino acid sequence of all AR genes retrieved from CARD, resulting in a reference dataset of 2,163 amino acid sequences (including aminoglycosides, lincosamides, macrolides, streptogramins, tetracyclines, phenicols, β-lactams, glycopeptides, and folate pathway inhibitors, such as trimethoprim), was aligned against the annotated genome sequences of the strain collection and the best BALSTP hits were filtered as described above. In order to minimise putative false negative or false positive outputs, only the putative AR determinants obtained from both approaches were considered for subsequent analyses. In detail, each putative AR determinant was manually annotated querying the NCBI non-redundant (NR) protein database in order to verify its actual function in the resistance.

Antimicrobial susceptibility testing. The minimum inhibitory concentration (MIC) of several antibiotics were determined using microdilution broth methods according to Clinical and Laboratory Standard Institute (CLSI; www.clsi.org), the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and ISO standard. In particular, 96-well microtiter plates containing serial two-fold dilutions of 16 antibiotics (ampicillin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, linezolid, neomycin, penicillin, quinupristin-dalfopristin, rifampicin, streptomycin, tetracycline, trimethoprim, and vancomycin) were prepared following the instruction reported by Wiegand *et al*. (2008). Briefly, antibiotic stocks at different concentrations were prepared: 5,120 μg/mL for gentamicin, streptomycin and neomycin; 2,560 μg/mL for vancomycin and ciprofloxacin; 1,280 μg/mL for trimethoprim, rifampicin, tetracycline and chloramphenicol; 320 μg/mL for penicillin, ampicillin, clindamycin and linezolid; and 160 μg/mL for quinupristin-dalfopristin and erythromycin. Starting from each initial stock, 10 dilutions were produced in order to obtain all the antibiotic solutions necessary for the preparation of the microtiter plates as shown in Figure S4.1. Finally, 50 μL of each antibiotic solutions in the 10 different concentrations were distributed in the microtiter plates.
MICs were evaluated in LAB susceptibility test medium (LSM) (Klare *et al*., 2005), a mixed formulation containing Iso-Sensitest broth (90%) and MRS broth (10%) as described in ISO 10932 IDF 223 document and recommended by EFSA (2012). Briefly, individual *Leuconostoc* colonies were grown overnight at 27°C in MRS broth, thus the suspension's turbidity was adjusted to an OD_{600} equal to 0.2, corresponding to a concentration of about 1×10^8 cfu/mL. This suspension was diluted 1:100 in LSM broth, and then 50 μL of this inoculum was added to each well of the microtiter plates prepared as described above (final concentration 5×10^5 cfu/mL). This test was performed in triplicate for each strain of the collection. Plates were incubated under aerobic conditions at 27°C for 48 h. MICs were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited. Epidemiological cut-off (ECOFF)

values were retrieved from EFSA (2012). Breakpoints for antibiotic not covered by EFSA were adopted from Ammor *et al*. (2007), and Danielsen and Wind (2003).

Phenotype-genotype correlation. A total of 176 phenotypic data points were generated from the 11 type strains of the genus *Leuconostoc* by antimicrobial susceptibility testing. Each interpretation of resistant or susceptible phenotype to a given antimicrobial agent was compared with the presence or absence of a known corresponding resistance gene(s) and/or structural gene mutations identifying through the genome sequence analysis. The overall correlation between phenotype and genotype was classified as positive when genomic data agreed with phenotypic testing, thus resistance and susceptible phenotypes correlated respectively with presence and absence of one or more AR genes. Otherwise, the correlation was considered negative.

Flanking regions of the AR genes. The genetic make-up of upstream and downstream sequences flanking clindamycin resistance genes were characterized performing a BLASTN and BLASTX alignment of the contigs carrying the AR genes against the NCBI NR database. This analysis was carried out for the strains *L. fallax* LMG 13177^{T} and *L. pseudomesenteroides* LMG 11482^{T}.

RNA isolation and real-time PCR. The relative quantification of the gene expression was performed for the gene *lsa*A of *L. pseudomesenteroides* LMG 11482^{T}. Cell cultures of this strain were grown at 27°C in MRS broth under three different conditions: in free-antibiotic medium, in the presence of clindamycin (4 μg/mL) and quinupristin-dalfopristin (1 μg/mL). The cells were collected in two different growth stages corresponding to OD_{600} equal to 0.2 and 0.8. For total RNA extraction, cells were washed with 1 mL of 10 mM Tris (pH 8) prepared in sterile diethyl pyrocarbonate (DEPC)-treated water. The pellet was treated with 500 μl of lysozyme 10 mg/mL and was incubated at 37°C for 1 h. After centrifugation (4°C, 8,000 rpm, 4 min) and elimination of the supernatant, the pellet was treated with 1 mL of Trizol solution and was incubated for 5 min at room temperature. Subsequently, 200 μL of chloroform were added and vigorously mixed. After centrifugation (4°C, 10,000 rpm, 15 min), the supernatant was treated with 500 μL of isopropyl alcohol and left for 10 min at room temperature. Total RNA was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C, washed with 1 mL of ethanol 75%, and dissolved in 35 μL of sterile water (RNAse- and DNAse-free). The purification and transcription of the RNA was performed using respectively Turbo DNA-free (Life Technologies, USA) and ImProm-II^{TM} Reverse Transcriptase (Promega) kit, following the manufacturer's instructions.
All real-time PCR reactions were performed in a FastStart Essential DNA Green Master added with the primers reported in Table 4.2 using a Light Cycler Nano (Roche, Switzerland). The

amplification program included an initial incubation at 94°C for 6 min followed by 45 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and finally 95°C for 10 s. At the end of the PCR, a dissociation curve was generated to verify the presence of unspecific products or primer dimers. Two independent biological replicates were performed for each growth condition and data were obtained from three technical replicates per sample.

The analysis of gene expression was performed using the $2^{-\Delta\Delta ct}$ method (Schmittgen and Livak, 2008) with 16S rRNA as endogenous control.

Table 4.2. Primers used for the relative quantification of the gene *lsa*A.

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
16S rRNA	16S-F	AGCGTTATCCGGATTTATTG	157	Yan *et al.*, 2016
	16S-R	CTACGCATTCCACCGCTACA		
*lsa*A	*lsa*A-F	CCCCAGACAATTCAAGACTC	137	This study
	*lsa*A-R	CTCGAAAATTTGCGCCAGAG		

4.3 Results and Discussion

4.3.1 Identification of putative AR genes. The genomic sequences of the 11 type strains of the genus *Leuconostoc* were retrieved from NCBI database, four of which were complete and closed, while the remaining genomes were subdivided into contigs. These seven genomes were annotated using RAST server, which identifies protein-encoding sequences and assigns functions to the genes (Aziz *et al*., 2008). The annotated genes of each genomes were aligned against the protein sequences of AR genes in CARD in order to retrieve all putative AR genes carried by the 11 *Leuconostoc* type strains. Based on the selection criteria, a total of 192 gene sequences were identified among the type strains analysed, which probably encode for resistance to tetracycline (1), erythromycin (7), clindamycin (42), penicillins (37), vancomycin (85), streptogramins (7), and trimethoprim (13) (Table 4.3). However, no genes were found linked to aminoglycosides and chloramphenicol resistance.

In particular, the genome sequence analysis revealed the presence of the gene *dfr* and penicillin binding protein (PBP) in all the type strains analysed, which are involved in trimethoprim and β-lactams resistance, respectively. However, the presence of these determinants is not necessary linked to the resistance, in fact only mutated DHFR enzymes and PBPs are able to avoid the antibiotic effect on microbial growth (Stanhope *et al*., 2008; Rosander *et al*., 2008).

Table 4.3. Putative AR resistance genes identified through the genome sequence analysis, which are involved in the resistance toward aminoglycosides (Am), tetracycline (TC), erythromycin (EM), clindamycin (CL), chloramphenicol (CM), penicillins (Pe), vancomcyin (VA), strepogramins (St), and trimethoprim (TM).

Strain	**Putative AR genes ***								
	Am	**TC**	**EM**	**CL**	**CM**	**Pe**	**VA**	**St**	**TM**
L. carnosum CECT 4024^T	-	-	-	*lmr*B	-	PBP2x (2)	*van*C, *van*HB, *van*HD (3), *van*HF, *van*HO, *van*N	-	*dfr*D
L. fallax LMG 13177^T	-	-	-	*lmr*B (2), *lmr*C, *lmr*D, *lsa*A	-	PBP1a (2), PBP2b, PBP2x	*van*E, *van*HA (2), *van*HB (2), *van*HO, *van*WB	*lsa*A, *vga*E	*dfr*A3
L. gelidum subsp. *gasicomitatum* CECT 5767^T	-	-	*mac*B, *erm*D	*lmr*B (3), *lmr*C, *lmr*D	-	PBP1a (2), PBP2b, PBP2x	*van*B, *van*HB, *van*HD (3), *van*HO (2), *van*YB	*vat*E	*dfr*G
L. gelidum subsp. *gelidum* CECT 4026^T	-	-	*mac*B, *erm*D	*lmr*B (3), *lmr*C, *lmr*D	-	PBP1a (2), PBP2b, PBP2x	*van*HB, *van*HD (3), *van*HO (2), *van*L, *van*YB, *van*WB	*vat*E	*dfr*A3
L. inhae CECT 7026^T	-	-	-	*lmr*B (3), *lmr*C, *lmr*D	-	PBP1a (2), PBP2b	*van*B, *van*HB, *van*HD (2), *van*HO (2), *van*YB	*vat*B	*dfr*E, *dfr*G
L. lactis LMG 8894^T	-	-	-	*lmr*C	-	PBP1a	*van*HD, *van*HO	-	*dfr*E, *dfr*G
L. mesenteroides subsp. *cremoris* LMG 6909^T	-	-	*mef*A/B	*lmr*B, *lmr*C	-	PBP1a (2), PBP2b, PBP2x	*van*HA, *van*HB (2), *van*HO (3), *van*L	-	*dfr*A26
L. mesenteroides subsp. *dextranicum* LMG 6908^T	-	-	-	*lmr*B (2), *lmr*C, *lmr*D	-	PBP1a (2), PBP2b, PBP2x	*van*HA, *van*HB, *van*HD, *van*HF, *van*HO (3), *van*L	-	*dfr*A26
L. mesenteroides subsp. *mesenteroides* LMG 6893^T	-	-	-	*lmr*B (2), *lmr*C, *lmr*D	-	PBP1a (2), PBP2b, PBP2x	*van*HA, *van*HB, *van*HD (2), *van*HF, *van*HO (3), *van*L, *van*YG1	-	*dfr*A26
L. suionicum CECT 8146^T	-	-	-	*lmr*B (2), *lmr*C, *lmr*D	-	PBP1a, PBP2b, PBP2x	*van*HA, *van*HB, *van*HD (3), *van*HF, *van*HO (3), *van*L	*vga*E	*dfr*A26
L. pseudomesenteroides LMG 11482^T	-	*otr*C	*car*A (2)	*lmr*B (3), *lmr*C, *lmr*D, *lsa*A	-	PBP1a (2), PBP2b, PBP2x	*van*HB (2), *van*HD, *van*HO (5), *van*N	*lsa*A	*dfr*C

*: the number of sequences identified per each AR genes were reported in bracket.

In details, DHFR is a dihydrofolate reductase enzyme that plays a crucial role in the DNA synthesis and represents the target for trimethoprim (Sköld, 2001). Whereas, PBPs catalyse the polymerization of the glycan strand (transglycosylation) and the cross-linking between glycan chains (transpeptidation) (Sauvage *et al*., 2008). β-lactams mimic the D-Ala-D-Ala dipeptide in an elongated conformation, that produces an imbalance in cell wall metabolism resulting in the growth inhibition or lysis (Zapun *et al*., 2016).

Regarding vancomycin, it is well known that the resistance to this antibiotic is an intrinsic feature of the genus *Leuconostoc* (Ogier *et al*., 2008). Indeed, *Leuconostoc* spp. are characterized by the presence of D-Ala-D-Lactate in their peptidoglycan rather than D-Ala-D-Ala (Hemme and Foucaud-Scheunemann, 2004). However, the analysis of the genome sequences identified *van* genes in all strains, and their role has to be deeper investigated. Frequently, *van* genes are organized in operons and they are usually found in vancomycin resistant enterococci, representing an acquired character for those microorganisms (Hill *et al*., 2010). These operons encode enzymes for synthesis and elimination, respectively, of low- and high-affinity precursors for ligase enzyme, thus removing the vancomycin-binding target (Courvalin, 2006).

Concerning the Macrolide-Lincosamide-Streptogramin (MLS) group, different genes were found including: i) *mac*B, *erm*D, *mef*A/B, and *car*A linked to macrolide resistance; ii) *lmr*B, *lmr*C and *lmr*D associated to lincosamide resistance; iii) *vat*E, *vat*B, and *vga*E related to streptogramin resistance; and iv) *lsa*A linked to clindamycin and quinupristin-dalfopristin resistance. In detail, the gene *erm*D codes for an rRNA methylase, which adds one or two methyl groups to a single adenine in the 23S rRNA sequence, reducing the macrolide affinity for the 50S ribosomal subunit (Roberts, 2008). *Vat* genes code for acetyltransferase enzymes, which modify streptogramins resulting in the disruption of the structure and inactivation of these antibiotics (Roberts, 2008). Whereas, the genes *vga*E, *mef*A/B, and *lmr*B encode for transporters belong to the Major Facilitators Superfamily (MFS) that pump out of the cell MLS antibiotics reducing their inner cellular concentration (Roberts, 2002). Moreover, members of the ABC (ATP-binding cassette) transporter family are involved in the resistance to MLS, and in this study *mac*B, *car*A, and *lsa*A were found as genes encoding for this group of transporter.

Regarding tetracycline, the sequence of the gene *otr*C was only identified in the genome of *L. pseudomesenteroides* LMG 11482^{T}, which encodes for an ABC transporter involved in the resistance to this antibiotic (Yu *et al*., 2012).

The genome analysis revealed even the presence of 64 gene sequences coding for drug efflux pumps in the 11 type strains examined (Figure 4.1). Efflux pumps belonging to the MFS and ABC superfamily were identified in all *Leuconostoc* strains, except for *L. lactis* LMG 8894^{T}.

Indeed, this strain carried three sequences associated to ABC transporters, but no genes coding for MFS transporters. Moreover, *L. fallax* LMG 13177^T and *L. mesenteroides* subsp. *cremoris* LMG 6909^T displayed the presence of gene sequence annotated as multidrug efflux pump. The ABC family utilizes ATP hydrolysis to drives the export of substrates, while MFS transporters use the proton motive force as energy source (Blanco *et al*. 2016). Moreover, these drug efflux pumps are characterized by a broad substrate specificity (Sun *et al*., 2014), which make difficult to correlate their presence to a specific AR phenotype. Efflux pumps are ancient, highly-conserved determinants, which have been selected long before the recent use of antibiotics for human and veterinary infection treatment (Blanco *et al*., 2016). These characteristics may explain the high spread of drug efflux pumps revealed in *Leuconostoc* strains.

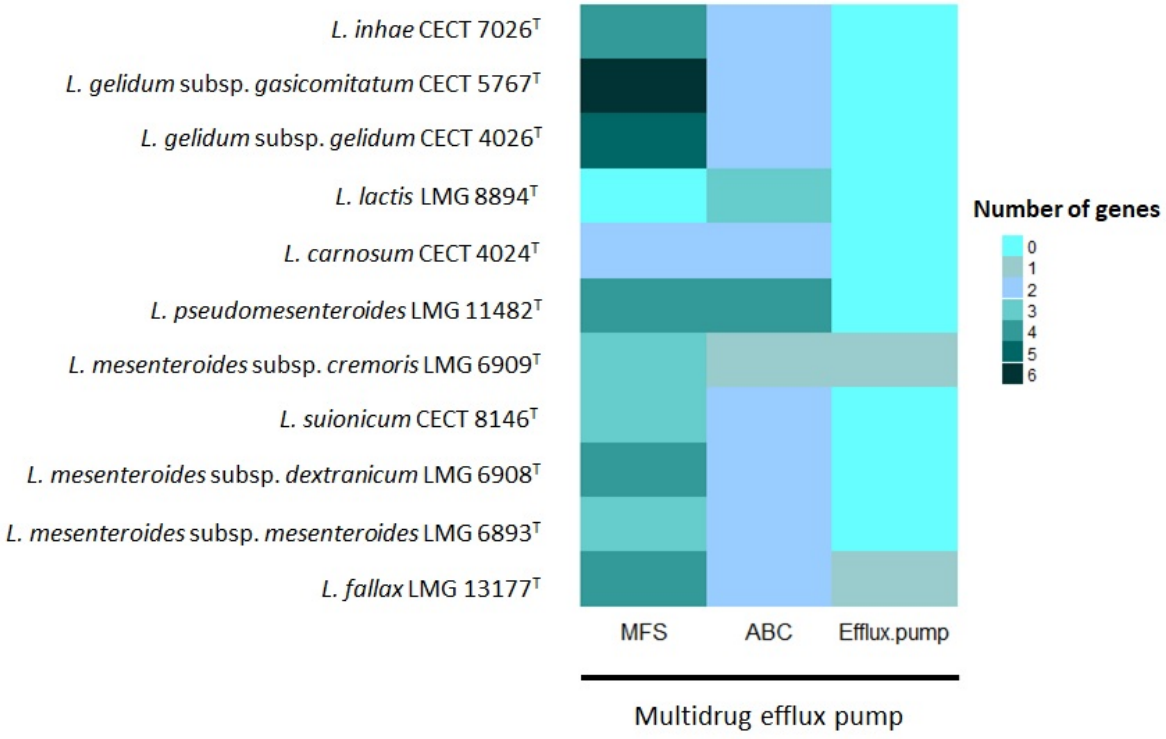

Figure 4.1. Distribution of gene sequences coding for drug efflux pumps identified in the 11 type strains of the genus *Leuconostoc*.

4.3.2 Determination of phenotypic resistance. In order to validate the genomic data, the MIC values for several antibiotics generally used in human and veterinary treatment were determined through broth microdilution plates for the 11 type strains of the genus *Leuconostoc*. The MIC values obtained for 16 antibiotics and the relative ECOFFs are reported in Table 4.4.

Table 4.4. MIC values of the *Leuconostoc* type strains for 16 antibiotics: gentamicin (GM); kanamycin (KM); streptomycin (SM); neomycin (NM); tetracycline (TC); erythromycin (EM); clindamycin (CL); chloramphenicol (CM); ampicillin (AM); penicillin (PC); vancomycin (VA); quinupristin-dalfopristin (QD); linezolid (LZ); trimethoprim (TM); ciprofloxacin (CI); rifampicin (RI) determined through the broth microdilution method. MICs higher than the ECOFF values defined by EFSA (2012), Ammor *et al*. (2007), and Danielsen and Wind (2003) are reported in bold.

Strain	**MIC (μg/mL)**															
	GM	**KM**	**SM**	**NM**	**TC**	**EM**	**CL**	**CM**	**AM**	**PC**	**VA**	**QD**	**LZ**	**TM**	**CI**	**RI**
L. carnosum CECT 4024^T	<0,5	16	4	>0,5	4	0,12	<0,03	4	**8**	1	**>128**	1	4	**16**	8	2
L. fallax LMG 13177^T	8	**256**	64	**16**	8	0,25	**4**	**16**	2	0,5	**>128**	**8**	4	**>64**	8	2
L. gelidum subsp. *gasicomitatum* CECT 5767^T	4	**64**	**128**	8	2	0,12	<0,03	4	**4**	0,25	**>128**	1	2	**>64**	4	1
L. gelidum subsp. *gelidum* CECT 4026^T	<0,5	4	8	<0,5	2	0,12	<0,03	4	1	0,06	**>128**	0,5	2	**16**	2	0,5
L. inhae CECT 7026^T	<0,5	<2	2	<0,5	1	0,06	<0,03	2	2	0,25	**>128**	1	1	2	4	1
L. lactis LMG 8894^T	8	**128**	32	**16**	2	0,25	<0,03	4	**8**	0,25	**>128**	1	1	**16**	4	0,5
L. mesenteroides subsp. *cremoris* LMG 6909^T	2	**32**	32	4	4	0,25	0,12	**8**	**4**	0,25	**>128**	1	4	**16**	2	1
L. mesenteroides subsp. *dextranicum* LMG 6908^T	4	**64**	**128**	**16**	1	0,25	0,12	4	**4**	0,25	**>128**	0,5	1	4	2	0,25
L. mesenteroides subsp. *mesenteroides* LMG 6893^T	4	**256**	**128**	**16**	4	0,25	0,12	**8**	**8**	1	**>128**	1	2	**64**	8	0,25
L. suionicum CECT 8146^T	16	**512**	**128**	**32**	8	0,25	0,12	**8**	**>16**	1	**>128**	1	2	**64**	2	0,25
L. pseudomesenteroides LMG 11482 ^{T}	8	**128**	**128**	**32**	8	0,5	**16**	**8**	**4**	0,5	**>128**	2	2	**>64**	4	0,5
ECOFF	16	16	64	8	8	1	1	4	2	1	nr	4	8	8	32	4

Nr: not required (EFSA, 2012)

Phenotypic resistance was interpreted based on the ECOFF values reported by EFSA (2012), Ammor *et al*. (2007), and Danielsen and Wind (2003), classifying a strain as resistant when the MIC value for a specific antibiotic was higher than the corresponding ECOFF.

As expected, all the 11 type strains analysed showed resistance to vancomycin (MIC > 128 µg/mL), a common trait for the species belonging to the genus *Leuconostoc* (Ogier *et al*., 2008), due to the D-Alanine-D-Lactate presence in the peptidoglycan (Hemme and Foucaud-Scheunemann, 2004). In contrast, all strains of the collection displayed susceptibility to tetracycline, erythromycin, penicillin, linezolid, ciprofloxacin, and rifampicin. In particular, low concentrations of erythromycin (< 1 µg/mL), penicillin (≤ 1 µg/mL) and rifampicin (≤ 1 µg/mL) were able to inhibit the growth of the *Leuconostoc* strains investigated. However, the MIC values of linezolid and ciprofloxacin covered only three two-fold dilution steps, ranging respectively from 1 to 4 µg/mL and from 2 to 8 µg/mL. These observations are in accordance to the high susceptibility to erythromycin as well as to tetracycline revealed for *Leuconostoc* spp. isolated from cheese (Morandi *et al*., 2013). Otherwise, the authors identified several strains belonging to *L. lactis*, *L. mesenteroides*, *L. pseudomesenteroides*, and *L. citreum* resistant to ciprofloxacin, and two *L. mesenteroides* strains showed even resistance to rifampicin (Morandi *et al*., 2013).

Moreover, most of the type strains showed resistance to trimethoprim (9 out of the 11 strains analysed) and ampicillin (8/11). Resistance to the latter antibiotic is not a common feature among the *Leuconostoc* species, indeed several strains isolated from foods have shown susceptibility to ampicillin (Morandi *et al*., 2013; Flórez *et al*., 2005). However, some *L. mesenteroides* and *L. lactis* strains have been recently reported as ampicillin resistant (Iullietto *et al*., 2016; Vera-Pingitore *et al*., 2016). Therefore, this resistance appears to be a variable trait within the genus *Leuconostoc*. Trimethoprim is a tetrahydrofolate reductase inhibitor that causes the block of the folate biosynthetic pathway inducing metabolic disruption (Cassir *et al*., 2014). The susceptibility to this antibiotic has been recently investigated for 13 *L. pseudomesenteroides* strains isolated from olive fermentation, revealing that only two strains were resistant to trimethoprim with MIC values > 64 µg/mL (Casado Muñoz *et al*., 2014)

As regards aminoglycosides, most strains showed resistance to kanamycin (8 out of 11 examined), displaying a broad MIC distribution, which ranged from 2 to 512 µg/mL. Moreover, 46 and 55% of the strains analysed showed resistance to streptomycin (MIC > 64 µg/mL) and neomycin (MIC > 8 µg/mL), respectively; whereas, all strains exhibited MIC values lower that 16 µg/mL for gentamycin, and thus were classified as susceptible toward this aminoglycoside. This is consistent with our data (Flórez *et al*., 2016), where gentamicin concentrations below and/or equal to 2 µg/mL were sufficient to inhibit the growth of the 32 strains of *Leuconostoc*

analysed. In addition, kanamycin and streptomycin resistance has been reported as a common trait characterizing some strains belonging to the genus *Leuconostoc* in previous studies (Vera-Pingitore *et al*., 2016; Morandi *et al*., 2013; Ammor *et al*., 2007).

Regarding chloramphenicol, which is a broad-spectrum antibiotic able to inhibit the protein synthesis, and generally the growth of *Leuconostoc* strains (Casado-Muñoz *et al*., 2014), five out of the 11 strains examined were characterized by MIC values higher than the ECOFF (4 µg/mL) and therefore they were classified as resistant. Moreover, only *L. fallax* LMG 13177^T and *L. pseudomesenteroides* LMG 11482^T showed resistance to clindamycin, and they were characterized by MIC values equal to 16 and 4 µg/mL, respectively. In addition, LMG 13177^T displayed resistance even toward quinupristin-dalfopristin.

Notably, *L. fallax* LMG 13177^T and *L. pseudomesenteroides* LMG 11482^T showed resistance to the highest number of antibiotics tested. Multidrug resistance phenotype has been previously reported for *L. pseudomesenteroides* strains, which included resistance to clindamycin, kanamycin, streptomycin, and trimethoprim (Casado Muñoz *et al*., 2014; Morandi *et al*., 2013).

4.3.3 Genotype-phenotype correlation. The AR data obtained from the genome sequence analysis and the phenotypic tests were compared in order to determine the correlation between genotype and phenotype. Overall, genotypic resistance correlated with the 66.5% of phenotypes obtained (Figure 4.2). In detail, genotype was in accordance with the phenotype for 117 of 176 phenotypic tests investigated, which included 86 cases representing susceptible phenotype toward a specific antibiotic linked to the absence of resistance determinants, and 31 cases for which the resistance phenotype correlated with the presence of one or more AR genes. Whereas, the inconsistency between phenotypic and genetic data was mostly represented by the presence of susceptible strains carrying AR determinants. This discrepancy mainly characterized clindamycin, penicillin, and quinupristin-dalfopristin, and it may be associated to the absence of gene expression. This could be due to the lack of a functional promoter or inducer (Gao *et al*., 2012), or of a proper post-transcriptional modification of the protein (Depardieu *et al*., 2007). High incongruity between phenotype and genotype was even observed for aminoglycosides and chloramphenicol. Although several *Leuconostoc* strains showed resistance to those antibiotics, no genes coding for these resistance phenotypes were identified. This may be due to novel resistance mechanisms (Gordon *et al*., 2014), or point mutations of the target, which results in a reduction of the affinity for the antibiotic (Blair *et al*., 2015). Otherwise, the cell membrane could create a barrier for the antibiotic due to its impermeability towards specific compounds, such as hydrophilic molecules, which results in the inhibition of the antibiotic activity (Danilchanka *et al*., 2008).

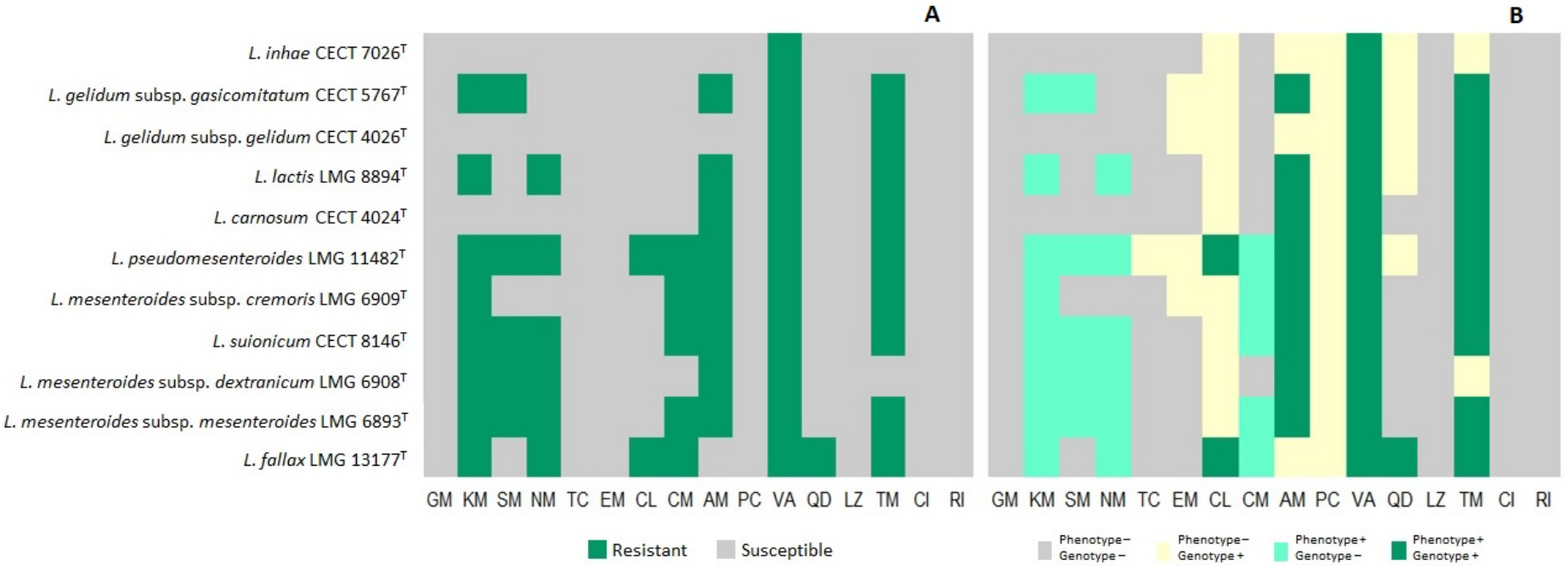

Figure 4.2. Antibiotic resistance profiles of the 11 type strains of the genus *Leuconostoc* and the relative correlation between genotype and phenotype. A) Resistant strains with MIC values higher than the relative ECOFFs are indicated in green, whereas sensitive strains are reported in grey. B) Positive correlations between genomic data and phenotypes observed are reported in green and grey, whereas negative correlation are indicated in yellow and blue. Antimicrobial abbreviations: GM, Gentamicin; KM, Kanamycin; SM, Streptomycin; NM, Neomycin; TC, Tetracycline; EM, Erythromycin, CL, Clindamycin; CM, Chloramphenicol; AM, Ampicillin; PC, Penicillin; VA, Vancomycin; QD, Quinupristin-dalfopristin; LZ, Linezolid; TM, Trimethoprim; CI, Ciprofloxacin; RI, Rifampicin.

4.3.4 Proof of the gene annotation to enhance the correlation. Each putative AR gene identified through the genomic approach was manually annotated in order to validate positive correlations and to clarify the observed discrepancies between genotype and phenotype. Moreover, analysis of the gene sequences involved in the resistance mechanism was performed aiming to the identification of genetic mutation associated with AR.

Regarding β-lactam, genes encoding for PBPs were detected in all genomes analyses, although the susceptibility toward penicillin was displayed by all the 11 *Leuconostoc* strains examined and few strains showed resistance to ampicillin. However, only the change of the PBPs sequence results in the expression of a β-lactam resistance phenotype, which includes both penicillin and ampicillin. In particular, the replacement of aspartic acid (D) at the position 399 of the PBP1a sequence with valine (V) results in the resistance phenotype of *Lactobacillus reuteri* DSM 17938, as well as the substitution of glutamine (Q) with leucine (L) at position 479 (Rosander *et al*., 2008). In addition, the alanine (A) at position 526 of the PBP2x sequence characterized sensitive *L. reuteri* strain, while threonine (T) was linked to the resistance phenotype (Rosander *et al*., 2008). The sequence analysis of the PBPs identified in the *Leuconostoc* type strains revealed the presence of a conserved aspartic acid at position 399 of PBP2a and a conserved glycine at position 526 of PBP2x sequence. Interestingly, the amino acid at position 479 of PBP2a showed variability, but no strain displayed the presence of leucine, which was demonstrated to correlate with β-lactam resistance. Therefore, protein structure analysis combined with affinity test for the penicillin compounds should be performed to clarify the involvement of these PBPs in the ampicillin resistance phenotype observed for eight *Leuconostoc* type strains

Regarding trimethoprim, the genome analysis revealed the presence of the genes *dfr* in all strains examined, even though *L. mesenteroides* subsp. *dextranicum* LMG 6908^T and *L. inhae* CECT 7026^T showed susceptibility to this antibiotic. Generally, trimethoprim resistance is associated to mutations in the DHFR sequence resulting in a lower affinity for the antibiotic (Sköld, 2001). In particular, trimethoprim resistance was associated with the replacement of phenylalanine at position 98 with tyrosine in the amino acid sequence of the DHFR enzyme of *Staphylococcus aureus* (Dale *et al*., 1997). The alignment of the DHFR amino acid sequence retrieved from the 11 *Leuconostoc* strains revealed the presence of a conserved tyrosine residue at the position 98, expect for *L. pseudomesenteroides* LMG 11482^T which displayed a phenylalanine at this position. However, this observation is in contrast with the phenotype showed by the *Leuconostoc* strains. However, the trimethoprim resistance phenotypes revealed for those strains may be due to the presence of antagonist components in the medium used for phenotypic determination of

susceptibility, such as thymidine in MRS medium (Klare *et al*., 2005), which may interfere with the trimethoprim activity, thus the test may not be coherent (EFSA, 2008; Danielsen *et al*., 2004).

Among the putative AR determinants identified through the genome sequence analysis, the manual annotation of the genes *van* revealed that they code for ligase and dehydrogenase enzymes which are not linked straight to the vancomycin resistance. The synthesis of D-Ala-D-Lac dipeptide, that characterizes the peptidoglycan of *Leuconostoc* spp. and confers intrinsic resistance toward vancomycin, is mediated by the D-Ala-D-Ala ligase (LmDdl2) enzyme (Park and Walsh, 1997). The replacement of tyrosine (Y) 261 with phenylalanine (F) causes a change in the substrate affinity of the enzyme, thus making it able to insert D-Lactate instead of D-Alanine, and synthesizes only the precursor D-Ala-D-Lac (Park and Walsh, 1997). Interestingly, all the D-Ala-D-Ala ligase sequences retrieved from the genomes of the 11 type strains showed a phenylalanine at position 261 (Figure 4.3), suggesting that such enzymes are involved in the vancomycin resistance displayed by *Leuconostoc* strains. The analysis of the flanking region did not reveal an organization in operon for these vancomycin resistance genes.

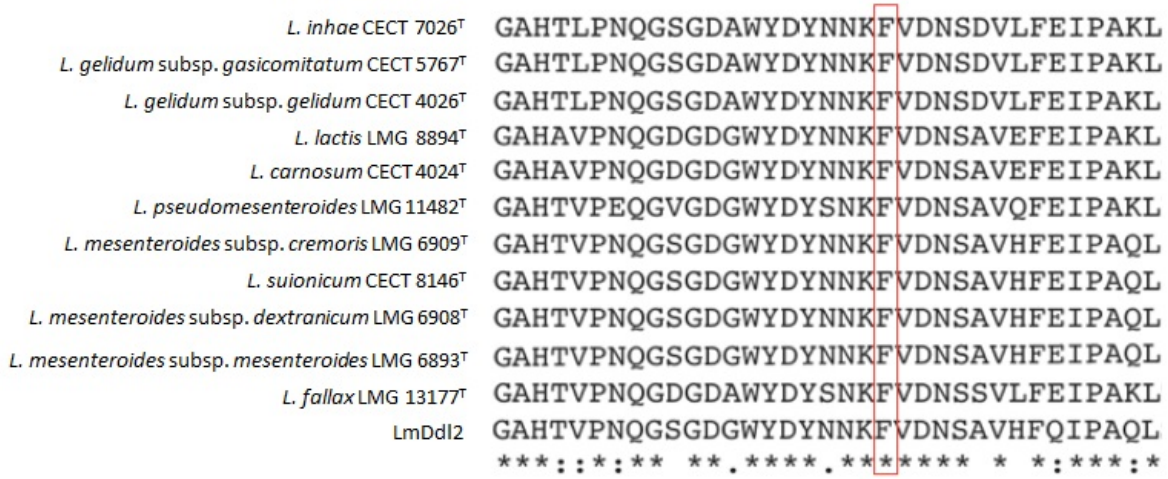

Figure 4.3. Alignment of the amino acid sequence of D-Ala-D-Ala ligase of the 11 *Leuconostoc* strains and the sequence of LmDdl2 of *L. mesenteroides* ATCC 8293^T reported by Park and Walsh (1997). The phenylalanine (F) at position 216 essential for the synthesis of D-Ala-D-Lac precursor is highlighted in red.

Concerning tetracycline, the sequence of OtrC protein found in *L. pseudomesenteroides* LMG 11482^T was aligned against the NR database of NCBI in order to confirm its function as tetracycline ABC transporter (Yu *et al*., 2012). However, this analysis revealed that this gene sequence codes for a generic ABC transporter with no specificity for tetracycline and this is in accordance with the tetracycline susceptible phenotype displayed by all the *Leuconostoc* strains.

Regards the MLS phenotype including erythromycin, clindamycin, and streptogramins, such as quinupristin-dalfopristin, the manual annotation of the putative AR genes identified through the genome sequence analysis revealed that the genes *mac*B and *car*A, formerly associated to erythromycin resistance, and *lmr*B/C/D, linked to clindamycin resistance, code for ABC transporters with no specificity to those antibiotics. Whereas, the genes *erm*D and *mef*A/B, formerly associated to erythromycin resistance, encoded for KsgA methyltransferase and MFS multidrug transporter, respectively. Therefore, these determinants may be associated to antibiotic resistance, but the affinity for a specific antimicrobial class should be evaluated in further studies.

In contrast, the annotation of all the genes associated to the streptogramin resistance was confirmed, except for *vga*E coding for an ABC transporter. In particular, the annotation of the genes *vat* identified in *L. gelidum* subsp. *gasicomitatum* CECT 5767^T, *L. gelidum* subsp. *gelidum* CECT 4026^T, and *L. inhae* CECT 7026^T was confirmed. In detail, they code for an O-acetyltransferase of the Vat family (Roberts, 2008), showing high sequence similarity with the genes *vat* previously found in *Lactobacillus paralimentarius* and *Clostridium* sp. MSTE9. These Vat enzymes catalyse the acetylation of the O18 residue of streptogramin A compounds decreasing their affinity for the ribosomal binding site (Stogios *et al*., 2014). However, the strains CECT 5767^T, CECT 4026^T, and CECT 7026^T showed phenotypic susceptibility toward streptogramin, and this highlights that the involvement of the genes *vat* in *Leuconostoc* strains should be deeper analysed.

In addition, the gene *lsa*A found in the genome of *L. fallax* LMG 13177^T and *L. pseudomesenteroides* LMG 11482^T was manually annotated as an ABC transporter involved in the resistance to clindamycin and quinupristin-dalfopristin. For both strains the amino acid sequence of the LsaA protein shows high similarity to that of *E. faecalis* V583 (Singh *et al*., 2002). The alignment of the LsaA amino acid sequence of the two *Leuconostoc* strains and *E. faecalis* revealed the presence of conserved Walker A and B motifs, which are peculiar of ABC transporter, and they are involved in the binding and hydrolysis of ATP (Singh *et al*., 2002) (Figure 4.4). This observation suggests that the gene *lsa*A code for functioning transporter for clindamycin and quinupristin-dalfopristin in *L. fallax* LMG 13177^T, which showed a resistance phenotype toward both antibiotics. However, *L. pseudomesenteroides* LMG 11482^T was classified as resistant to clindamycin, but sensitive to quinupristin-dalfopristin based on the reference ECOFFs. Indeed, all strains showed MIC values lower or equal to 1 μg/mL, except for *L. fallax* and *L. pseudomesenteroides* which were characterized respectively by MICs equal to 8 and 2 μg/mL for quinupristin-dalfopristin (Table 4.3). The discrepancy between genotype and

phenotype toward streptogramins for *L. pseudomesenteroides* may be due to the lack of the xpression of the gene *lsa*A, or the ECOFF proposed for the *Leuconostoc* genus, 4 μg/mL, is not appropriate to distinguish resistant from susceptible strains belonging to the *L. pseudomesenteroides* species. Therefore, an update of the quinupristin-dalfopristin ECOFF should be performed, analysing the MIC values of a larger number of *L. pseudomesenteroides* isolates.

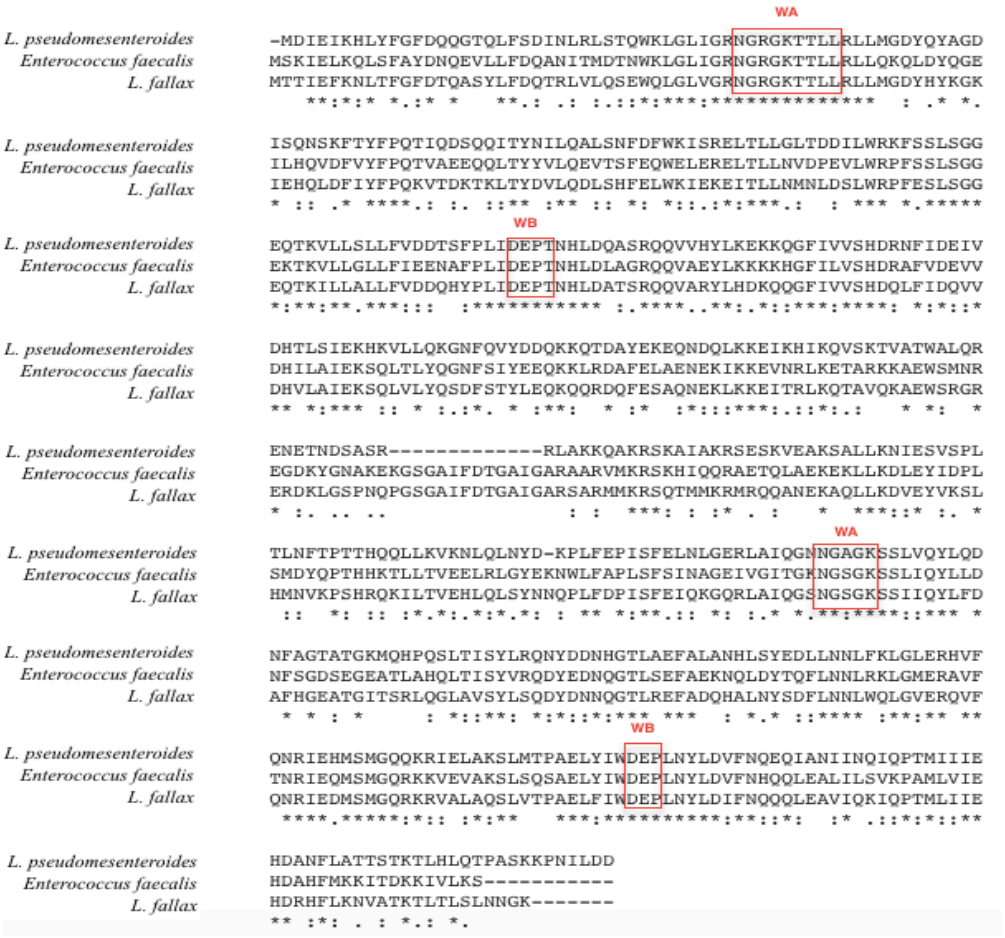

Figure 4.4. Alignment of LsaA amino acid sequence of *L. pseudomesenteroides* LMG 11482^T, *L. fallax* LMG 13177^T and *E. faecalis* V583. The red boxes highlight the Walker A (WA) and Walker B (WB) domains conserved in the three sequences.

Sequence surrounding the gene *lsa*A were analysed by retrieving those contigs carrying this antibiotic determinant from the available genome sequence of LMG 13177^T and LMG 11482^T. In detail, *lsa*A is located in the contig AEIZ01000026.1 (274,324 bp) and AEOQ01000036.1 (56,435 bp) for *L. fallax* LMG 13177^T and *L. pseudomesenteroides* LMG 11482^T, respectively. The up- and downstream regions flanking *lsa*A in LMG 11482^T are characterized by the presence of genes coding for ABC transporters, methyltransferases and ligases (Figure 4.5 A).

While, downstream of *lsa*A in *L. fallax* LMG 13177^T sequences coding for oxide reductase, glucosidase and permease enzymes, in addition to transporters and membrane proteins, were found (Figure 4.5 B). Therefore, the surrounding regions of *lsa*A do not display any mobile genetic elements in both strains, suggesting that this AR determinant cannot be transferred to any other microorganism, thus *L. fallax* LMG 13177^T and *L. pseudomesenteroides* LMG 11482^T may not represent vectors for the spread of clindamycin and quinupristin-dalfopristin resistance in the food chain.

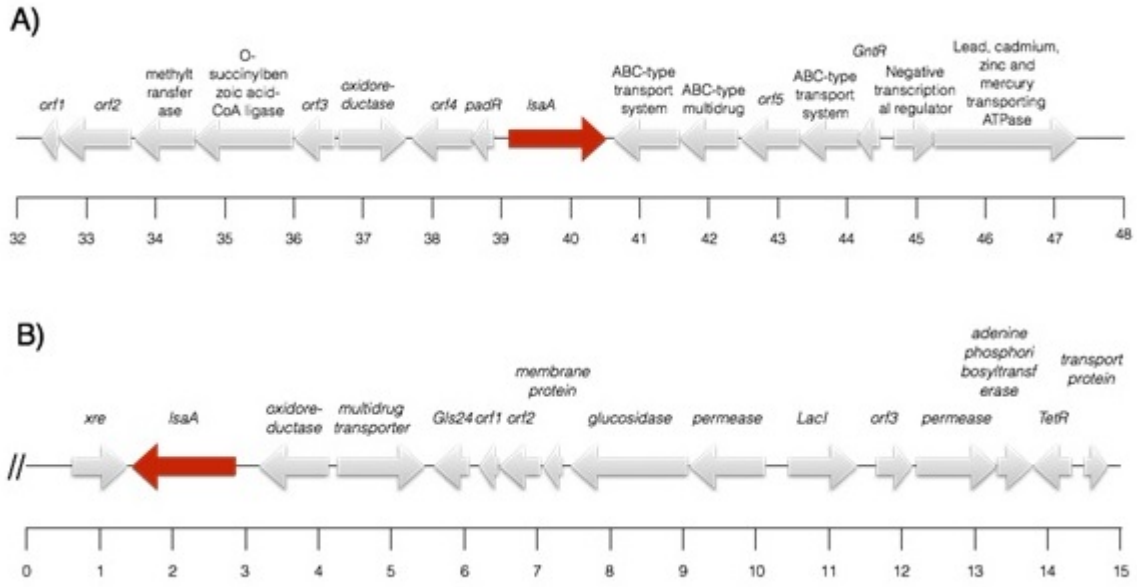

Figure 4.5. Diagram showing the genetic organization of the region surrounding the gene *lsa*A identified in the genome of *L. pseudomesenteroides* LMG 11482^T (A) and *L. fallax* LMG 13177^T (B) and. Color code of genes and open reading frames (orfs): antibiotic resistance genes are in red, and in grey, all orfs annotated though RAST.

Since in *E. faecalis*, the gene *lsa*A is associated with resistance to clindamycin and quinupristin-dalphopristin (Singh *et al*., 2002), it is necessary to clarify its role in *L. pseudomesenteroides* LMG 11482^T, which was resistant to clindamycin, but susceptible to quinupristin-dalfopristin. For this reason, the relative quantification of *lsa*A expression was performed in the presence and absence of these antimicrobial substances, using the 16S rRNA gene as internal control. This analysis revealed an increased expression of *lsa*A in the presence of both antibiotics either in the exponential (OD_{600} = 0.2) and stationary (OD_{600} = 0.8) growth phase of *L. pseudomesenteroides* LMG 11482^T (Figure 4.6). Therefore, these results confirm the involvement of *lsa*A in the clindamycin resistance phenotype showed by *L. pseudomesenteroides* LMG 11482^T, thus suggesting that the ECOFF value for quinupristin-dalfopristin should be updated for this species.

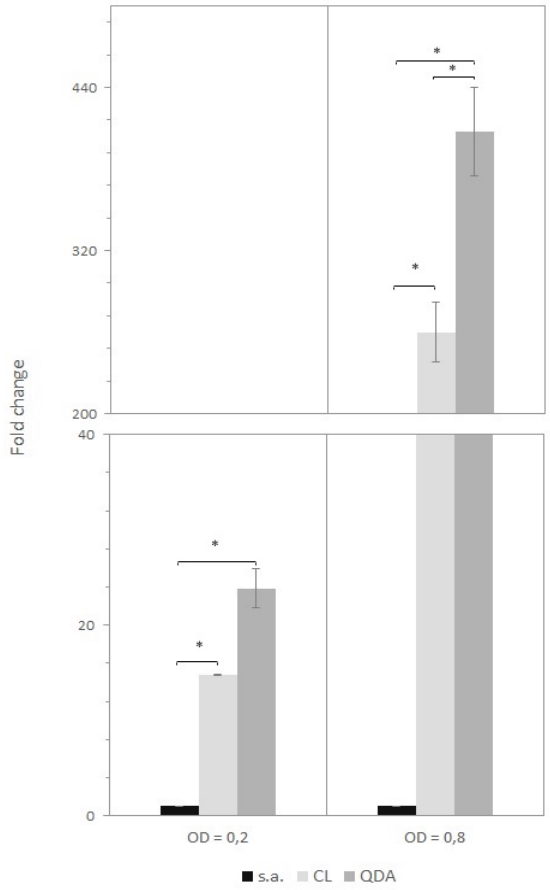

Figure 4.6. Relative quantification of the gene *lsa*A expression for *L. pseudomesenteroides* LMG 11482^T grown in free-antibiotic culture (sa) in the presence of clindamycin 4 µg/mL (CL) and quinupristin-dalphopristin 1 µg/mL (QDA). The cells were collected in the exponential and stationary growth phases, respectively 0.2 and 0.8 OD_{600}. The expression of *lsa*A was normalized based on the expression of the 16S rRNA, and calculated using the $2^{-\Delta\Delta ct}$ method.

Therefore, the manual annotation of the putative AR genes initially identified through the genome sequence analysis confirmed the actual involvement in the resistance of five determinants, which were linked to streptogramin resistance, i.e. the gene *vat* of *L. gelidum* subsp. *gasicomitatum* CECT 5767^T, *L. gelidum* subsp. *gelidum* CECT 4026^T, and *L. inhae* CECT 7026^T; and the gene *lsa*A of *L. fallax* LMG 13177^T and *L. pseudomesenteroides* LMG 11482^T. The latter gene is even involved in the resistance to clindamycin, to which both strains are resistant. In addition, the gene encoding the D-Ala-D-Ala ligase enzyme associated with vancomycin resistance was identified in all *Leuconostoc* genomes analysed. Its amino acid sequence showed the presence of a phenylalanine at position 261, which allows the insertion of a D-Lactate residue in the dipeptide of the peptidoglycan rather than D-Alanine. Moreover, all the

type strains of the collection carried gene sequences coding for drug efflux pumps, probably involved in AR mechanisms.

Finally, the resistance genotype correlates with the phenotype for the 74% of the cases analysed (Figure S4.2), the remaining percentage of observed discordance may be due to the presence of unknown resistance mechanisms, which are not detectable through the use of database for the genome sequence analysed. This result points out that genomic analysis for AR prediction in LAB is not as accurate as it is for some pathogenic bacteria (McDermott *et al*., 2016; Liu *et al*., 2015). Therefore, further studies are necessary to increase the current knowledge about AR mechanisms in LAB in order to enrich the number of AR determinants included in the database. These researches will improve the effectiveness of the genome sequence analysis as a tool for the prediction of AR characters even in food borne bacteria, such as LAB.

4.4 Conclusions

The genome sequence analysis of 11 type strains of the genus *Leuconostoc* were carried out to improve the current knowledge about the AR features of this genus. This analysis revealed the presence of 192 gene sequences putatively associated with the resistance to the main important antibiotics used in medicine, including tetracycline, erythromycin, clindamycin, penicillins, vancomycin, streptogramins, and trimethoprim. However, the manual annotation of these putative AR determinants confirmed the actual involvement in the AR of only five gene sequences, which are linked to the resistance toward streptomycin and clindamycin. In particular, the gene *lsa*A was found for the first time in *L. pseudomesenteroides* LMG 11482^T and *L. fallax* LMG 13177^T and the absence of mobile genetic elements in the flanking regions reduces the possibility to be horizontally transfer to other bacterial species. In addition, the role of LsaA in the clindamycin and quinupristin-dalfopristin resistance was confirmed for *L. pseudomesenteroides*, through the relative quantification of the gene expression.

Moreover, the sequence of the gene encoding for the enzyme D-Ala-D-Ala ligase was identified in the 11 *Leuconostoc* genomes investigated and, the analysis of the their amino acid sequences revealed the presence of a phenylalanine at position 261 conferring the vancomycin resistance phenotype for the genus *Leuconostoc*. The antibiotic susceptibility testing was performed for several antibiotics to confirm the resistance features predicted through the genome sequences analysis. Such analysis revealed a positive correlation between genotype and phenotype for the 74% of the cases examined. However, the presence of some resistance phenotypes not associated

with particular genetic determinants emphasizes the requirement of deeper studies focused on the identification of novel genes involved in AR for LAB.

In conclusion, the application of the genomic approach for the characterization of AR in LAB may provide: (i) an important initial contribution to the identification of genes potentially associated with resistance; and (ii) relevant information about the possibility of AR genes to be spread along the food chain. Moreover, the decrease of sequencing costs and the improvement of AR algorithms and databases will support the worth application of the genomic approach as a tool of choice for detection and characterization of antimicrobial resistance.

Lact-1	1	2	3	4	5	6	7	8	9	10	11	12
A	P	Gm 0.5	1	2	4	8	16	32	64	128	256	N
B	P	Km 2	4	8	16	32	64	128	256	512	1024	N
C	P	Sm 0.5	1	2	4	8	16	32	64	128	256	N
D	P	Nm 0.5	1	2	4	8	16	32	64	128	256	N
E	P	Tc 0.12	0.25	0.5	1	2	4	8	16	32	64	N
F	P	Em 0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	N
G	P	Cl 0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	N
H	P	Cm 0.12	0.25	0.5	1	2	4	8	16	32	64	N

Lact-2	1	2	3	4	5	6	7	8	9	10	11	12
A	P	Am 0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	N
B	P	Pc 0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	N
C	P	Va 0.25	0.5	1	2	4	8	16	32	64	128	N
D	P	Qda 0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	N
E	P	Lz 0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	N
F	P	Tm 0.12	0.25	0.5	1	2	4	8	16	32	64	N
G	P	Ci 0.25	0.5	1	2	4	8	16	32	64	128	N
H	P	Ri 0.12	0.25	0.5	1	2	4	8	16	32	64	N

Figure S4.1. Diagram of the 96-well microtiter plates used for the determination of MIC values. Each well in the scheme reported the concentration of the corresponding antibiotic.
GM: Gentamicin; KM: Kanamycin; SM: Streptomycin; NM: Neomycin; TC: Tetracycline; EM: Erythromycin; CL: Clindamycin; CM: Chloramphenicol; AM: Ampicillin; PC: Penicillin; VA: Vancomycin; QD: Quinupristin/dalfopristin; LZ: Linezolid; TM: Trimethoprim; CI: Ciprofloxacin; RI: Rifampicin.

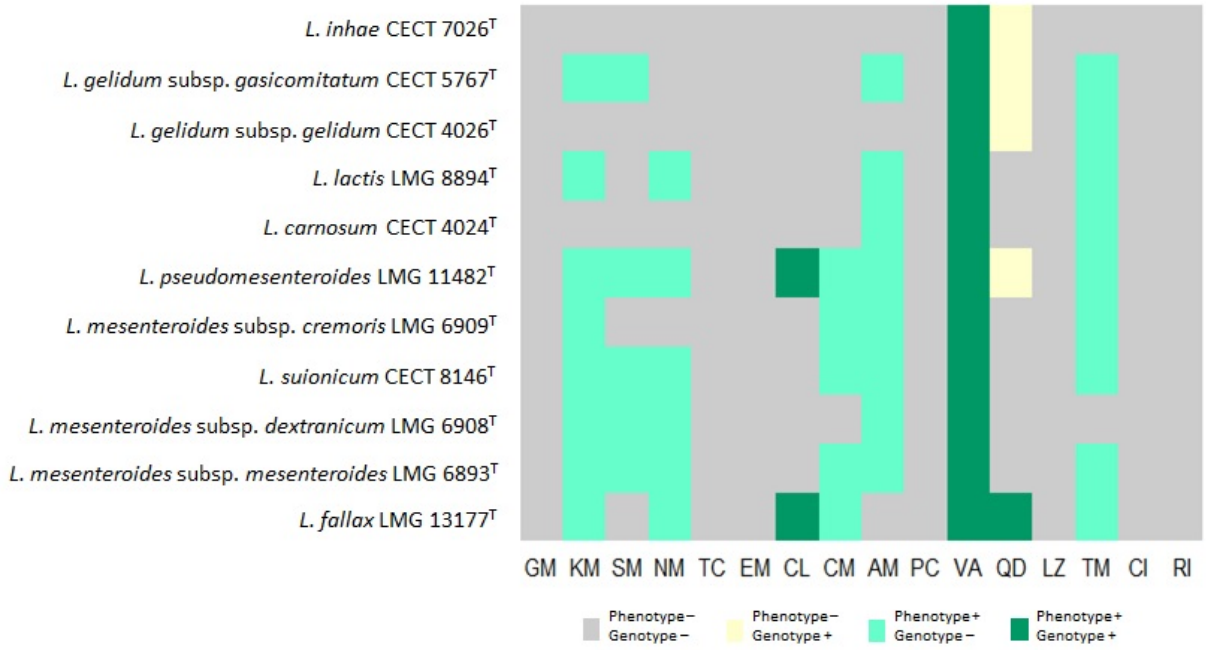

Figure S4.2. Genotype-phenotype correlation for the 11 type strains of the genus *Leuconostoc* after manual annotation of each putative AR genes identified through the genome sequence analysis. Positive correlations between genomic data and phenotypes observed are reported in green and grey, whereas negative correlation are indicated in yellow and blue. Antimicrobial abbreviations: GM, Gentamicin; KM, Kanamycin; SM, Streptomycin; NM, Neomycin; TC, Tetracycline; EM, Erythromycin, CL, Clindamycin; CM, Chloramphenicol; AM, Ampicillin; PC, Penicillin; VA, Vancomycin; QD, Quinupristin-dalfopristin; LZ, Linezolid; TM, Trimethoprim; CI, Ciprofloxacin; RI, Rifampicin.

PART II

Chapter 5

Antibiotic susceptibility profiles and analysis of the resistance genetic basis of the whole genus *Lactobacillus*

5.1 Introduction

The genus *Lactobacillus* is currently composed by over 200 properly described species and subspecies that have been isolated from different sources where substrates rich in carbohydrates are available (Sun *et al*., 2015; Salvetti *et al*., in preparation). In particular, lactobacilli are found on mucosal membranes, gastrointestinal tract (GIT), oral cavity and vagina of humans and animals (Papizadeh *et al*., 2017), on plants or material of plant origin, and in man–made products as fermented foods (Liu *et al*., 2011, Herve-Jimenez *et al*., 2009).

The economic and scientific impact of this genus of lactic acid bacteria (LAB) is unquestionable: *Lactobacillus*, in fact, includes many strains commonly used as probiotics (defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"; Hill *et al*., 2014) and can be found in the market as probiotic cosmetics, drug supplements or medical devices (Papizadeh *et al*., 2017). Further, *Lactobacillus* species are probably the most widely used as starter cultures for industrial and agriculture applications, such as fermented foods, also due to their long history of safe and technological use (Venema and Meijerink, 2015; Devirgiliis *et al*., 2013). *De facto*, lactobacilli are generally considered to be non–pathogenic (Sanders *et al*., 2010), as only few cases have been reported in which *Lactobacillus* spp. have been identified as the infection agents in patients that already suffered from highly debilitating illnesses and/or were significantly immunodepressed (Ricci *et al*., 2017). According to this, the European Food Safety Authority (EFSA) mentioned that the daily consumption of large quantities of lactobacilli in a variety of fermented foods by people of all ages and health statuses apparently does not have ill effects (EFSA, 2007).

Despite their safety status, a considerable number of antibiotic resistant lactobacilli has been reported (Abriouel *et al*., 2015; Gueimonde *et al*., 2013; Devirgiliis *et al*., 2013), in which vancomycin–resistant phenotype was perhaps the best–characterized intrinsic resistance

(Goldstein *et al*., 2015). Regarding their antibiotic resistance (AR) patterns, most *Lactobacillus* species are mostly intrinsically resistant to aminoglycosides (gentamycin, kanamycin, streptomycin, and neomycin), ciprofloxacin, and trimethoprim; and they are susceptible to the cell wall–targeting penicillin and β–lactams, chloramphenicol, tetracycline, erythromycin, linezolid, and quinupristin/dalfopristin (Abriuel *et al*., 2015). However, acquired resistance to tetracycline, erythromycin, clindamycin and chloramphenicol has been detected in lactobacilli isolated from fermented foods (Casado Muñoz *et al*., 2014; Thumu and Halami, 2012a; Thumu and Halami, 2012b; Comunian *et al*., 2010). Thus, given the broad use of these species in fermented food production and healthcare system, also lactobacilli could act as donors or *reservoirs* for AR genes, with the potential risk of transferring the genes to pathogenic bacteria in food matrices as well as in the GIT (Salvetti and O'Toole, 2017). Therefore, the safety of *Lactobacillus* species needs to be assessed, even though more than 35 species meet the criteria of qualified presumption of safety (QPS) proposed by the EFSA (Ricci *et al*., 2017).

To tackle this issue, in 2012 EFSA provides a method to identify resistance to antimicrobials of human and veterinary importance in *Lactobacillus* strains intended for use as food and feed additives, based on the determination of antibiotic susceptibility profiles and analysis of the genetic basis of the resistance. The absence of acquired or transferable resistance factors should be determined prior to considering these strains safe for human and animal consumption (EFSA, 2012).

Resistance can occur by either spontaneous mutation of the genes encoding for antimicrobial targets or for drug transport systems or through the capture of resistance genes from other bacteria via horizontal gene transfer (HGT) (Crofts *et al*., 2017). HGT is the main factor that contributes to the spread of AR from commensal and environmental strains to pathogens. In fact, it can occur between closely or distantly related species and in different environments, including the GIT and food (von Wintersdorff *et al*., 2016; Huddleston, 2014, Verraes *et al*., 2013). Molecular mechanisms of acquired resistance include: production of insensitive antimicrobial targets, blockage of antimicrobial penetration into the cell, transport of antimicrobials out of the cell and expression of enzymes that modify antimicrobials to inactive forms (Blair *et al*, 2015; Andersson and Hughes, 2010).

Considering the importance to assess the AR mechanisms from a genomic viewpoint, the availability of the genome sequence of almost all the type strains of *Lactobacillus* (Sun *et al*., 2015, Zheng *et al*., 2015) offers an unprecedented additional advantage for the safety assessment of the genus *Lactobacillus* and for the surveillance of AR genes as well as their potential transfer to other microorganisms. In fact, whole genome sequencing potentially allows to uncover all

possible genetic determinants of antimicrobial resistance in a single microbial genome (Chan, 2016).

Moreover, the implementation of high–throughput sequencing methods has resulted in a massive increase in the number of available AR gene sequences, which are catalogued in specific databases (Martinez *et al*., 2015).

In this context, the aim of this study was to determine the antibiotic susceptibility patterns of 197 type strains representing the whole *Lactobacillus* genus from the phenotypic and genotypic point of view, using the genome sequence analysis as a tool for the identification of AR determinants and for the characterization of their genetic make–up. The parallel execution of phenotypic assays on lactobacilli and the accurate analysis of the genome sequences will allow the first robust genotype–phenotype resistance correlation for the genus *Lactobacillus*, improving the current knowledge on the distribution, origin and mechanisms of AR in the whole genus.

5.2 Materials and Methods

Bacterial strains and growth conditions. The 197 type strains of the *Lactobacillus* genus used in this study are listed in Table S5.1 and they were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia), BCCM/LMG Bacteria Collection (Ghent, Belgium), the Spanish Type Culture Collection (CECT, Valencia, Spain), the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the Korean Collection for Type Cultures (KCTC, Jeollabuk–do, Korea), the Japan Collection of Microorganisms (JCM, Koyadai Tsukuba, Japan) and the NITE Biological Resource Centre (NBRC, Nishihara, Japan). *Lactobacillus* strains were grown in de Man–Rogosa–Sharpe (MRS, Thermo Fisher Scientific, Waltham, USA) medium under specific conditions reported in Table S5.1 and kept in liquid cultures with 20% (w/vol) glycerol at –80°C for long term storage.

Antimicrobial susceptibility testing. This analysis was performed at the Teagasc (Agriculture and Food Development Authority) Food Research Centre (Fermoy, Ireland), where the minimum inhibitory concentration (MIC) of several antibiotics was determined using microdilution broth methods according to the Clinical and Laboratory Standard Institute (CLSI; www.clsi.org), the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) and ISO standard. In particular, VetMIC plates (National Veterinary Institute, Uppsala, Sweden) for LAB were used containing serial two–fold dilutions of 16 antibiotics (ampicillin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, linezolid, neomycin,

penicillin, quinupristin–dalfopristin, rifampicin, streptomycin, tetracycline, trimethoprim, and vancomycin). MICs were evaluated in LAB susceptibility test medium (LSM) (Klare *et al*., 2005), a mixed formulation containing Iso–Sensitest broth (90%) and MRS Difco broth (10%) supplemented with 0.05% w/v cysteine as described in ISO 10932 IDF 223 document and recommended by EFSA (2012). *L. paracasei* LMG 12586 was used as control strain. Briefly, individual *Lactobacillus* strains were grown on MRS agar (24–48 h depending on the strain) and a 1 μL loop with material from at least 3–5 colonies was suspended in 4 mL sterile saline solution to obtain a concentration of about 3×10^8 cfu/mL. This suspension was diluted 1:1000 in LSM broth (final concentration 3×10^5 cfu/mL) and then 100 μL was added to each well of the VetMIC plate. This test was performed in triplicate for each strain of the collection. Plates were incubated under anaerobic conditions at 28°C for 48 h. MICs were read as the lowest concentration of an antimicrobial agent at which visible growth was inhibited. Epidemiological cut–off (ECOFF) values were retrieved from EFSA (2012). Breakpoints for antibiotic not covered by EFSA were adopted from Ammor *et al*. (2007), and Danielsen and Wind (2003).

Identification of resistance genes. The annotated sequences of the available genomes for the type strains of the genus *Lactobacillus* (Sun *et al*., 2015) were downloaded from NCBI using the Accession Number reported in Table S5.1. These sequences were employed to query the Comprehensive Antibiotic Resistance Database (CARD, version 1.0.6, http://arpcard.mcmaster.ca) (McArthur *et al*., 2013) through the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov) in order to identify all AR genes involved in the resistance phenotypes observed. A gene was annotated as putative AR determinant according to its best BLASTP hit in CARD with a threshold of amino acid sequence identity > 30% and query coverage > 70%. In addition, the amino acid sequences of all AR genes retrieved from CARD, resulting in a reference dataset of 2,163 amino acid sequences, were aligned against the annotated genome sequences of the collection and the best BLASTP hits were filtered as described above. In order to minimise putative false negative or false positive outputs, only the putative AR determinants obtained from both approaches were considered for subsequent analyses. In detail, each putative AR determinant was manually annotated querying the NCBI non–redundant (NR) protein database to verify its actual function in the resistome and to determine its involvement in acquired phenotypes.

Phenotype–genotype correlation. Considering the nine antibiotics for which EFSA defined reference ECOFFs (EFSA, 2012) and the genome sequence available for 161 lactobacilli strains, a total of 1,449 phenotypic tests were considered for the phenotype–genotype correlation. Each interpretation of resistant or susceptible phenotype to a given antimicrobial agent was compared

with the presence or absence of a known corresponding resistance gene(s) manually annotated and/or structural gene mutations identified through the genome sequence analysis (McDermott *et al*., 2016; Zhao *et al*., 2016; Tyson *et al*., 2015). The overall correlation between phenotype and genotype was classified as positive when genomic data agreed with phenotypic testing, thus resistance and susceptible phenotypes correlated respectively with presence and absence of one or more AR genes. Otherwise, the correlation was considered negative.

Flanking regions of the AR genes. The genetic make–up of upstream and downstream sequences flanking tetracycline and erythromycin resistance genes were characterized performing a BLASTN and BLASTX alignment of the contigs carrying the AR genes against the NCBI NR database. This analysis was carried out for *Lactobacillus ingluviei* DSM 15946^T, *Lactobacillus amylophilus* DSM 20533^T, and *Lactobacillus amylotrophicus* DSM 20534^T and allowed to identify mobile genetic elements which could be involved in the spread of AR determinants.

5.3 Results and Discussion

5.3.1 Determination of the Minimum Inhibitory Concentration. The MIC values of 16 antibiotics belonging to the most important antimicrobial classes used in human and veterinary medicine were determined through broth microdilution vetMIC plates for 197 *Lactobacillus* strains representing the whole *Lactobacillus* genus. The MIC distribution profiles were obtained for 182 strains (as 15 strains did not grow in the vetMIC medium) and analysed based on the *Lactobacillus* phylogroups described by Sun *et al*. (2015) (Table S5.2). A wide range of MIC values was exhibited by all phylogroups for most antibiotics analysed, except for linezolid, quinupristin–dalfopristin and chloramphenicol. In particular, unimodal MIC distribution was generally observed for the latter antibiotics which covered 4 two–fold dilution for most strains analysed (89% for quinupristin–dalfopristin and 96% for chloramphenicol and linezolid), ranging from 2 to 16 µg/mL for chloramphenicol, 1 to 8 µg/mL for linezolid, and 0.5 to 4 µg/mL for quinupristin–dalfopristin. This last range has been recently reported for 11 *Lactobacillus helveticus* strains isolated from dairy products (Guo *et al*., 2017). Regarding chloramphenicol, the MIC distribution observed in this study is consistent with data reported by Nawaz *et al*. (2011), where 74 *Lactobacillus* strains isolated from tradition fermented foods in China were characterized by MIC values ranging from 1 to 8 µg/mL, except for *Lactobacillus animalis* and *Lactobacillus salivarius* strains whose MIC was equal to 32 µg/mL.

High MIC values were observed for trimethroprim and vancomycin, ≥ 64 and 128 μg/mL respectively, in almost all 182 strains analysed (81 and 73%, respectively). The insensitivity to high concentrations of trimethoprim is frequently observed in lactobacilli (Guo *et al*., 2017; Casado Muñoz *et al*., 2014; Klare *et al*., 2007). Interestingly, strains belonging to the *Lactobacillus delbreuckii* group showed susceptibility to low concentration of vancomycin (concentrations lower than 1 μg/mL inhibited the growth of 92% of strains), despite the growth of *Lactobacillus* strains is supposed to be not inhibited by the presence of this antibiotic.

The MICs of the rest of antibiotics showed variability. The obtained MIC values for aminoglycosides covered more than nine two–fold dilution steps; ranging from 2 to > 1,024 μg/mL for kanamycin, and from 0.2, 0.5, 2 to > 256 μg/mL for gentamicin, neomycin and streptomycin, respectively. Bimodal MIC distribution were observed in *Lactobacillus alimentarius*, *Lactobacillus collinoides*, *Lactobacillus fructivorans*, *Lactobacillus plantarum*, and *Lactobacillus reuteri–vaccinostercus* groups for all aminoglycosides tested. Moreover, strains of *L. reuteri–vaccinostercus* group showed MIC values distributed at the whole concentration range tested for gentamicin (0.5–256 μg/mL), kanamycin (2–1,024 μg/mL) and neomycin (0.5–256 μg/mL). A wide range of MICs for aminoglycosides has been previously observed for different *Lactobacillus* species isolated from chickens (Dec *et al*., 2017), wild boar intestines (Klose *et al*., 2014), fermented foods, and human gut (Ma *et al*., 2017). *L. alimentarius*, *L. collinoides*, *L. delbrueckii*, *L. fructivorans*, *L. plantarum*, and *L. reuteri–vaccinostercus* groups displayed bimodal distribution also for tetracycline, erythromycin and clindamycin, which notably affect the function of ribosomal subunits. As previously described by Mayrhofer *et al*. (2010), the tetracycline MIC distribution is bimodal for species belonging to the *L. delbrueckii* group, such as *Lactobacillus amylovorus*, *Lactobacillus crispatus* and *Lactobacillus johnsonii* , which splits the population in two subgroups, one characterized by low MICs (2–16 μg/mL) and the another with higher MIC values (3–>128 μg/mL).

An unimodal MIC distribution was also observed for β–lactams, including ampicillin and penicillin, except for few strains belonging to the *Lactobacillus brevis* (*Lactobacillus spicheri* DSM 15429^T, *Lactobacillus zymae* DSM 19395^T), *L. collinoides* (*Lactobacillus similis* DSM 23365^T), *L. plantarum* (*Lactobacillus pentosus* DSM 20314^T), *L. salivarius* (*Lactobacillus ghanensis* DSM 18630^T) and *other* (*Lactobacillus selangorensis* ATCC BAA66^T) groups which displayed MIC values higher than 16 μg/mL. Even though lactobacilli are usually susceptible to low concentration of β–lactams (Goldstein *et al*., 2015), atypical insensitivity to high concentration of these cell wall inhibitors has been reported for some strains isolated from healthy chickens, belonging to the species *L. crispatus* and *L. johnsonii* (Dec *et al*., 2017).

The distribution of rifampicin and ciprofloxacin MIC values was broad: they were particularly positioned at the low–end concentration range for rifampicin (0.12–16 μg/mL), except for *Lactobacillus jensenii* DSM 20557^T and *Lactobacillus oris* DSM 4864^T belonging to the *L. delbrueckii* and *L. reuteri–vaccinostercus* group, respectively, which showed MIC higher than 64 μg/mL. The common capability of rifampicin to inhibit the growth of different *Lactobacillus* species, even at low concentrations, has been reported in different studies (Guo *et al*., 2017; Botina *et al*., 2011; D'Aimmo *et al*., 2007).

5.3.2 Identification of resistance phenotypes. Phenotypic resistance was interpreted based on the epidemiological cut–off (ECOFF) values reported by EFSA (2012), Ammor *et al*. (2007), Danielsen and Wind (2003), classifying a strain as resistant when the MIC value for a specific antibiotic was higher than the corresponding ECOFF. When not defined at the species level, the ECOFFs described for each *Lactobacillus* fermentation metabolism phenotype were considered (EFSA, 2012), which are based on the type of fermented sugars and fermentation products [obligately homofermentative (OHO), facultatively heterofermentative (FHE) and obligately heterofermentative (OHE)] (Table S5.1).

Trimethoprim resistance was the most common phenotype observed, and most of the *Lactobacillus* strains were not susceptible to vancomycin (77%, 141/182) and kanamycin (61%, 111/181) (Figure 5.1). Vancomycin–resistance phenotype is perhaps the best–characterized resistance in lactobacilli (Goldstein *et al*., 2015). Vancomycin comes in to contact with peptidoglycan precursors on the cell wall side of cytoplasmic membrane and binds to the terminal D–Alanine–D–Alanine dipeptide, preventing polymerization of peptidoglycan precursors. The synthesis of modified cell wall peptidoglycan precursors containing terminal D–lactate residue instead of D–Alanine prevents vancomycin binding resulting in the resistance to this glycopeptide (Gueimonde *et al*., 2013). Resistance of *Lactobacillus* species to vancomycin is considered as intrinsic (Ammor et al., 2007), except for *L. delbrueckii*, *Lactobacillus acidophilus, L. johnsonii* and *L. crispatus* in which the vancomycin susceptible phenotype has been associated to the presence of Y–type D–ala–D–ala ligase enzyme (Kleerebezem *et al*., 2010). This observation is in accordance to the data reported in this study. Indeed, vancomycin susceptible strains were mainly represented by almost all members of the *L. delbreuckii* phylogroup.

Trimethoprim inhibits dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate representing a key product for DNA synthesis (Houvinen, 2001). Folate auxotrophic lactobacilli have been reported as intrinsic resistant to trimethoprim (Katla *et al*., 2001). This resistance is associated to cell wall impermeability, alternative

metabolic pathway, the presence or overproduction of a DHFR insensitive to trimethoprim (Abriouel *et al*., 2015). However, the presence of antagonist components in the medium used for phenotypic determination of susceptibility, such as thymidine in MRS medium, may interfere with the trimethoprim activity, and the test may not be coherent (EFSA, 2008; Danielsen *et al*., 2004).

Multi–drug resistance, defined as resistance to three or more different antimicrobials, was observed in 152 strains (84%). These multiple phenotypes could be the consequence of continued selective pressure by different drugs that results in additional forms of resistance mechanisms, such as novel penicillin–binding proteins (PBPs), enzymatic mechanisms of drug modification, mutated drug targets, enhanced efflux pump expression, and altered membrane permeability (Alekshun and Levy, 2007). Interestingly, *Lactobacillus thailandensis* DSM 22698^T showed resistance to all 16 antibiotics tested (Figure 5.1). This strain was isolated from fermented tea leaves, a traditional fermented product in the northern part of Thailand. Young tea leaves are fermented in containers for 4–7 days, or 1 year for mature tea leaves and subsequently are consumed as a snack (Tanasupawat *et al*., 2007). The natural microbiota present on the surface of tea leaves are directly involved in the fermentation as well as in the synthesis of products with inhibitory effects against food–borne bacteria (Mo *et al*., 2008). Moreover, some components of tea extracts, such as catechins, promise for having antimicrobial effects (Reygaert, 2014). Therefore, this environment may exert on bacteria, including *L. thailandensis*, a selective pressure leading to the development of multidrug resistance features. However, HGT could also explain the resistance phenotype showed by DSM 22698^T, in fact co–selection of resistance to more than one antibiotic is a common feature of resistance acquired by HGT (Andersson and Hughes, 2010).

In contrast, *Lactobacillus sanfranciscensis* LMG 16002^T and *Lactobacillus pobuzihii* NBRC 103219^T were identified as susceptible to all 16 antibiotics, including vancomycin. They were respectively isolated from sourdough (Kline and Sugihara, 1971) and from a traditional fermented food in Taiwan called Pobuzihi (Chen *et al*., 2010). On the other hand, *Lactobacillus ozensis* DSM 23829^T, *Lactobacillus equigenerosi* DSM 18793^T, *Lactobacillus capillatus* DSM 19910^T, and *Lactobacillus vini* DSM 20605^T showed resistance only towards vancomycin. This high antimicrobial susceptibility could be due to the absence of a significant antibiotic exposure or the lack of the ability to acquire AR genes from the environment or from other microorganisms.

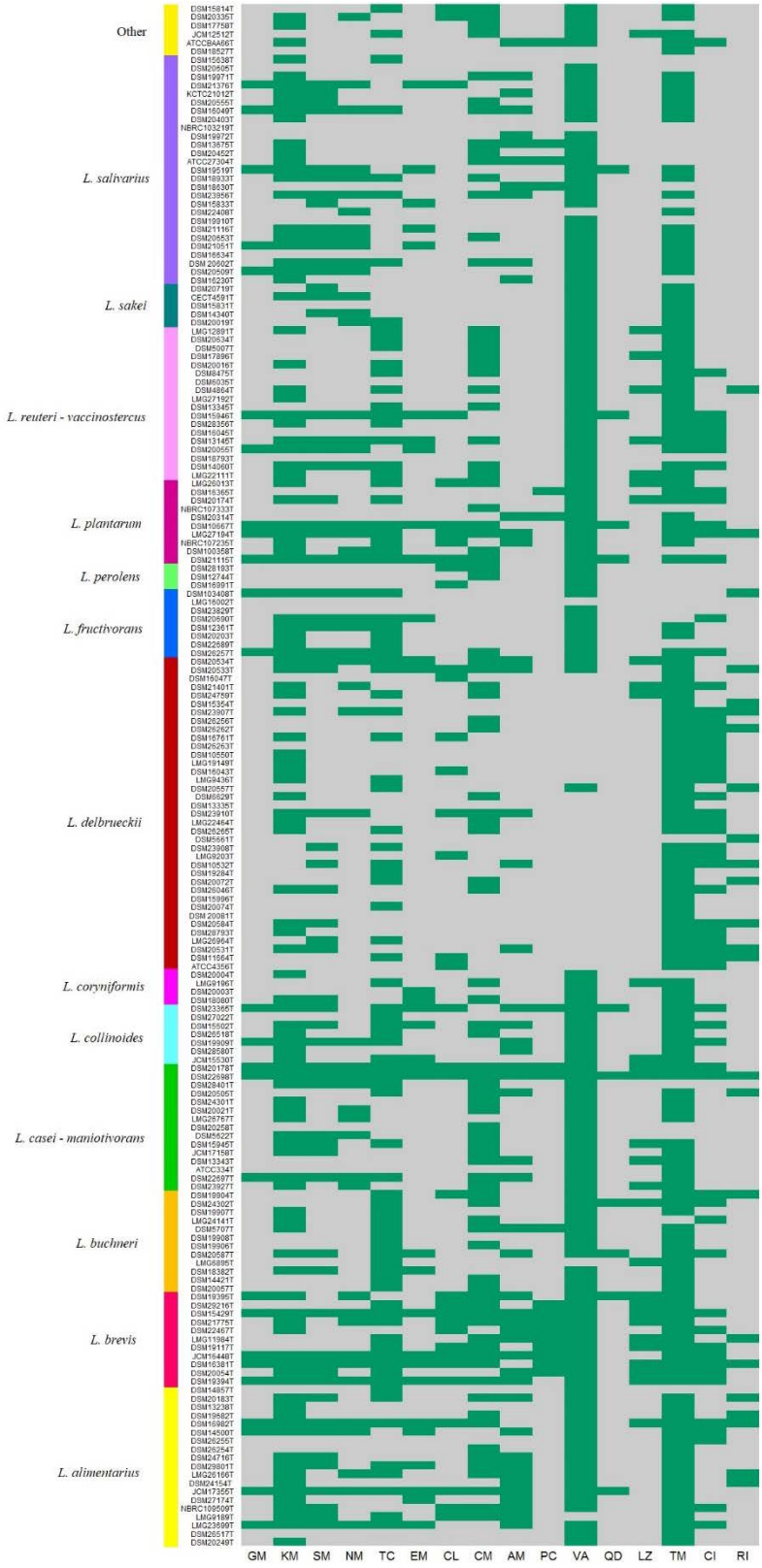

Figure 5.1. Resistance profiles of 182 type strains of the genus *Lactobacillus* compared with epidemiological cut–off values provided by EFSA (2012), Ammor *et al*. (2007), and Danielsen and Wind (2003). Resistant strains with MIC values higher than the ECOFF are indicated in green, whereas sensitive strains are reported in grey. Strains are clustered by phylogroups reported by Sun *et al*. (2015) and are demarcated by coloured bar on the left part of the heat plot GM: Gentamicin; KM: Kanamycin; SM: Streptomycin; NM: Neomycin; TC: Tetracycline; EM: Erythromycin; CL: Clindamycin; CM: Chloramphenicol; AM: Ampicillin; PC: Penicillin; VA: Vancomycin; QD: Quinupristin/dalfopristin; LZ: Linezolid; TM: Trimethoprim; CI: Ciprofloxacin; RI: Rifampicin.

Overall, the 182 type strains analysed showed high susceptibility to gentamicin, erythromycin, penicillin, quinupristin–dalfopristin, linezolid, and rifampicin (Figure 5.1). In fact, only 5% of the strains investigated (10 out of 182) were resistant to quinupristin–dalfopristin. As a matter of fact, the inhibitory action of this antibiotic on lactobacilli has been previously reported by Sharma *et al*. (2016), where some *Lactobacillus* strains isolated from various clinical specimens in Taiwan exhibited high rates of nonsusceptibility to quinupristin–dalfopristin (Luh *et al*., 2000).

Linezolid is a synthetic oxazolidinone, generally employed for the treatment of Gram–positive infections (Ager and Gould, 2012). The common inhibitory effect of this antibiotic on *Lactobacillus* species growth has been reported in several research studies (Sharma *et al*., 2016; Mayrhofer *et al*., 2010; Klare *et al*., 2007), as well as for rifampicin (Zhou *et al*., 2005). Indeed, low resistance levels towards rifampicin were observed in lactobacilli isolated from human GIT (Botina *et al*., 2011), traditional dairy products (Guo *et al*., 2017), and in probiotic strains in marketed foods and drugs (Liu *et al*., 2009). Interestingly, no lactobacilli isolated from Parmigiano Reggiano cheese showed resistance to rifampicin (Coppola *et al*., 2005).

Generally, *Lactobacillus* species are sensitive to cell wall inhibitor agents, such as penicillin and ampicillin (Abriouel *et al*., 2015), even though resistance towards penicillin G was found to be widespread among *L. plantarum* strains used as probiotics (Sharma *et al*., 2016) or identified as part of the natural microbiota of spontaneous fermentation of vegetable products (Pérez Pulido *et al*., 2005). In contrast, the resistance levels towards ampicillin as revealed in this study were higher than those identified for penicillin and they are mainly detected in members of the phylogroups *L. brevis* (73%), *L. alimentarius* (58%), and *L. collinoides* (57%) (Figure 5.2). This observation corroborates data reported in previous studies, in which the ampicillin resistance phenotype has been detected for lactobacilli strains isolated from several fermented foods, such as fish (Sornplang *et al*., 2011), milk (Lavanya *et al*., 2011), and sausages (Pan *et al*., 2011).

Commonly, most *Lactobacillus* species are susceptible to antibiotics that inhibit protein synthesis, including erythromycin, tetracycline, clindamycin, and chloramphenicol (Abriouel *et al*., 2015). However, 50 and 49% of the type strains examined in this study were resistant to tetracycline and chloramphenicol, respectively. Tetracycline resistance phenotypes were mainly observed in species of the phylogroups *Lactobacillus buchneri*, *L. collinoides*, *L. plantarum*, *L. reuteri–vaccinostercus*, *L. fructivorans* and *L. brevis*; while members of the phylogroups *L. brevis*, *Lactobacillus casei–manihotivorans*, and *Lactobacillus perolens* showed the highest resistance to chloramphenicol (Figure 5.2). As for clindamycin and streptomycin, the resistance levels were low, representing respectively the 20 and 18% of the 182 *Lactobacillus* strains

analysed. The resistance to those antibiotics is usually reported as acquired phenotypes for lactobacilli (Thumu and Halami, 2012a; Nawaz *et al*., 2011; Gevers *et al*., 2003). This type of resistance is mediated by several mechanisms, which are generally classified into three main processes: the minimization of intracellular concentration of the antibiotic through the alteration of the membrane permeability; the modification of the antibiotic target by genetic mutation or post–translational modification of the target; and the inactivation of the antibiotic by hydrolysis or enzymatic modification (Blair *et al*., 2015).

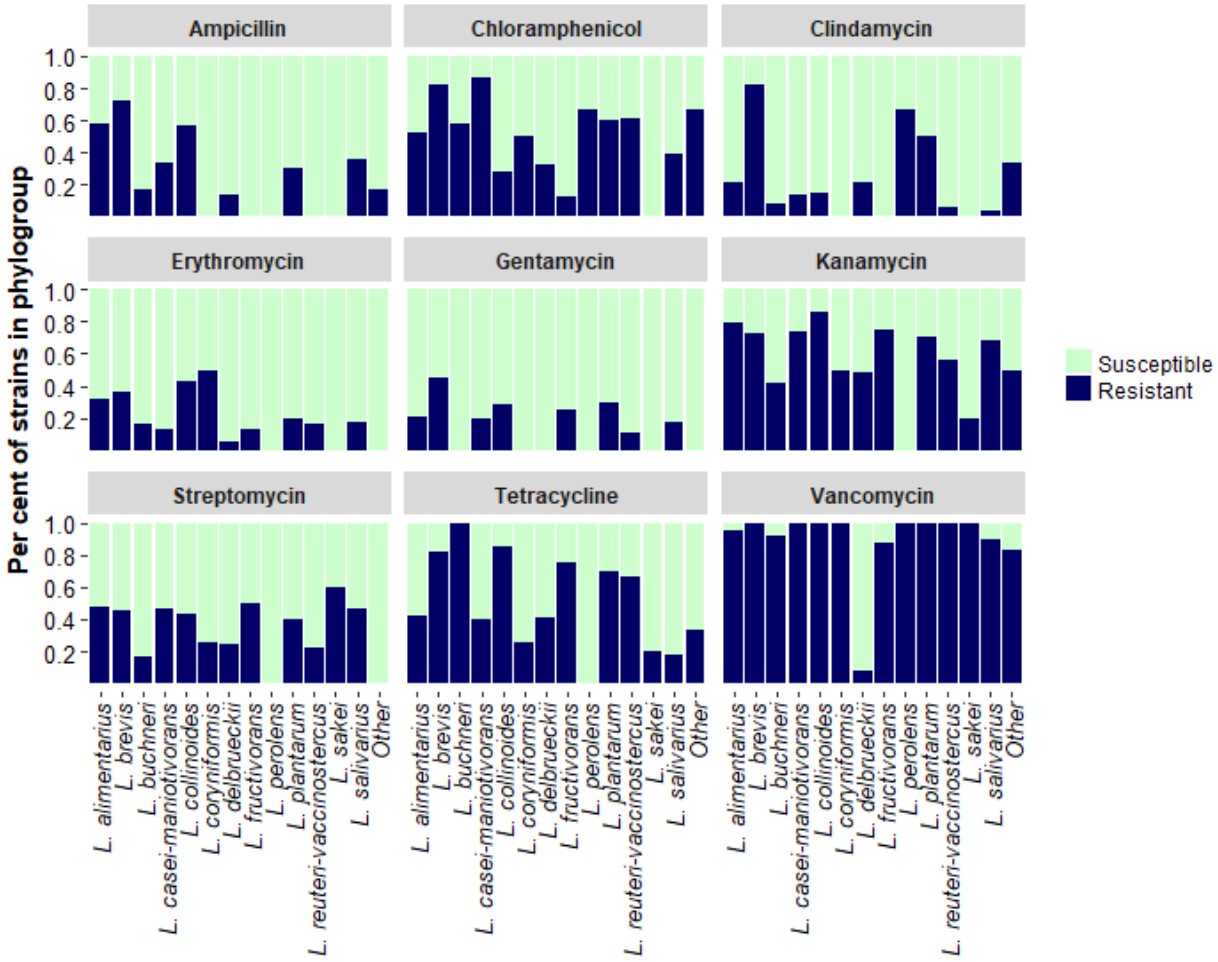

Figure 5.2. Prevalence of antibiotic resistant (blue) and susceptible (green) strains within the *Lactobacillus* phylogroups examined for antimicrobial agents reported by EFSA (2012), including inhibitors of cell wall synthesis (ampicillin and vancomycin), inhibitors of protein synthesis (erythromycin, clindamycin, chloramphenicol, and tetracycline) and aminoglycosides (gentamicin, kanamycin, and streptomycin).

Regarding the aminoglycosides, the kanamycin resistance was reported in at least the 50% of the members of each phylogroup of *Lactobacillus*, except for *L. perolens* that showed high susceptibility towards this antibiotic. As for streptomycin, resistance phenotypes were observed

among 35% of the strains examined. In fact, the resistance against Gram–negative spectrum antibiotics, such as kanamycin and streptomycin, is frequently observed in lactobacilli (Abriouel *et al*., 2015, Devirgiliis *et al*., 2013), and this may be due to the high rate of spontaneous chromosomal mutations conveying resistance to these antibiotics or related to membrane impermeability (Mayrhofer *et al*., 2011). The resistance to aminoglycosides has been described as an intrinsic resistance for some *Lactobacillus* species (i.e. *Lactobacillus rhamnosus*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, and *L. helveticus*) (Hummel *et al*., 2007b, Coppola *et al*., 2005) due to the lack of cytochrome–mediated drug transport (Gueimonde *et al*., 2013). Whereas the high susceptibility towards gentamicin is probably linked to the better ability of this antibiotic to cross the membrane compared to other aminoglycosides (Elkins and Mullis, 2004).

5.3.3 Identification of AR genes. EFSA established that the resistance phenotypes for *Lactobacillus* strains towards gentamicin, kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin and vancomycin should be investigated under the genetic point of view to verify the absence of acquired and transferable AR determinants (EFSA, 2012). Thus, genome sequences for 161 out of the 182 type strains (21 genome sequences were not available at the time of the study) tested for phenotypic resistance, were aligned against the protein sequences of AR genes in CARD to reveal the genes relevant to AR phenotypes observed for the nine antibiotics mentioned. Based on the selection criteria and the manually annotation, a total of 146 gene sequences were identified among the type strains analysed, which encode for resistance to aminoglycosides (20 sequences), tetracycline (18), erythromycin (6), clindamycin (60), and chloramphenicol (42) (Figure 5.3). Regarding ampicillin resistance, this feature has been reported for *L. reuteri* as the result of point mutations in the genes encoding for the penicillin binding proteins (PBPs), which represent the target of β–lactams (Rosander *et al*., 2008). However, the amino acid sequence analysis of the PBPs for the 161 type strains revealed the presence of conserved amino acid residues in the binding site for the antibiotics.

As expected for vancomycin, the amino acid sequence of the Ddl ligase, involved in the synthesis of peptidoglycan, was found in all the genomes investigated. Sequence analysis has shown that the specificity of the Ddl ligase for D–Ala–D–Ala or D–Ala–D–Lac is associated with a tyrosine (Y–type) or phenylalanine (F–type) residue, respectively, at position 261 (Kleerebezem *et al*., 2010), as also reported in *Leuconostoc mesenteroides* (Park and Walsh, 1997).

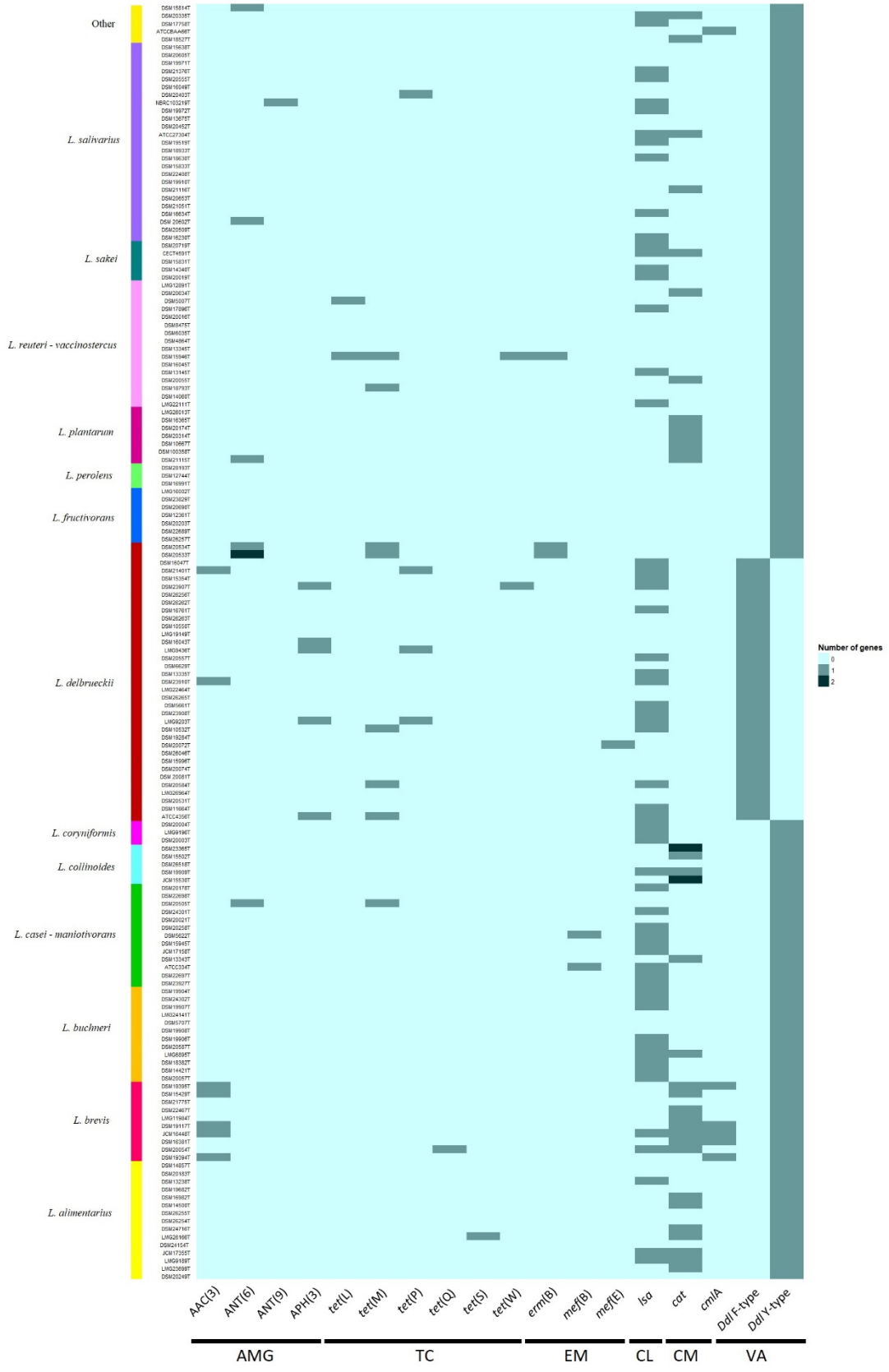

Figure 5.3. Distribution of AR genes for 161 *Lactobacillus* strains, for which the sequence of the genome was available at the time of the study. The heat plot is the result of BLASTP alignment of genome sequences against the Comprehensive Antibiotic Resistance Database (CARD; McArthur *et al.*, 2013). The AR genes identified were grouped according to the resistance they confer (AMG: aminoglycosides, TC: tetracycline, EM: erythromycin; CL: clindamycin; CM: chloramphenicol; VA: vancomycin). On the left, the *Lactobacillus* phylogroups as described by Sun *et al*. (2015).

Interestingly, the Ddl enzyme of all vancomycin resistant type strains examined was of F–type, while all members of the phylogroup *L. delbrueckii*, which were susceptible to vancomycin, was characterized by the presence of Y–type enzyme, with the exception of the strains *L. jensenii* DSM 20557^T, *L. amylophilus* DSM 20533^T, *L. amylotrophicus* DSM 20534^T, *L. sanfraciscensis* LMG 16002^T, *Lactobacillus hilgardii* LMG 6895^T, *Lactobacillus composti* DSM 18527^T, *L. pobuzihii* NBRC 103219^T, *Lactobacillus farciminis* LMG 9189^T, *Lactobacillus ceti* DSM 22408^T, and *Lactobacillus algidus* DSM 15638^T (Figure S5.1).

However, *L. jensenii* DSM 20557^T, *L. amylophilus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T carried Ddl Y–type even though it exhibited resistance to vancomycin. The latter two strains harboured specific D–Alanine–D–Lactate ligase sequences in their genome. This finding could explain the vancomycin-resistance phenotypes shown by *L. amylophilus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T. In contrast, *L. sanfraciscensis* LMG 16002^T, *L. hilgardii* LMG 6895^T, *L. composti* DSM 18527^T, *L. pobuzihii* NBRC 103219^T, *L. farciminis* LMG 9189^T, *L. ceti* DSM 22408^T, and *L. algidus* DSM 15638^T were characterized by the presence of Ddl ligase of F–type although their susceptibility to vancomycin. This inconsistency between phenotype and genotype for vancomycin could be due to the presence of alternative resistance mechanisms or to the alteration of gene expression. Therefore, these observations emphasize the requirement of deeper studies aiming at the analysis of the actual contribution of Ddl ligase enzymes in vancomycin-resistance in the genus *Lactobacillus* and at the identification of new genetic determinants encoding for resistance.

Aminoglycoside resistance genes. The 20 gene sequences identified among the 161 *Lactobacillus* genomes encode for aminoglycoside modifying enzymes (Figure 5.3) and they are mainly acetyltransferases (AACs) (7 sequences), nucleotidyltranferases (ANTs) (8 sequences), or phosphotransferases (APHs) (5 sequences) (Ramirez and Tolmasky, 2010). In particular, the *aac*(3) gene was found in five and two members of the phylogroups *L. brevis* and *L. delbrueckii*, respectively. All these strains showed resistance to kanamycin and some of them were resistant also towards streptomycin, such as *Lactobacillus acidifarinae* DSM 19394^T, *Lactobacillus koreensis* JCM 16448^T, *L. spicheri* DSM 15429^T, and *L. hominis* DSM 23910^T. Moreover, *L. zymae* DSM 19395^T showed resistance to gentamicin, whereas *Lactobacillus namurensis* DSM 19117^T was susceptible to aminoglycosides although the presence of the gene *aac*(3). This is the first time, to our knowledge, that *aac*(3) is detected in *Lactobacillus* strains.

Gene sequences coding for nucleotidyltranferase enzymes, such as *ant*(6) and *ant*(9), were identified in 7 type strains: *L. amilophylus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T (*L. delbrueckii* phylogroup), *Lactobacillus fabifermentas* DSM 21115^T (*L. plantarum* phylogroup),

L. animalis DSM 20602^T and *L. pobuzihii* NBRC 103219^T (*L. salivarius* phylogroup), *Lactobacillus sharpeae* DSM 20505^T (*L. casei–manihotivorans* phylogroup), and *Lactobacillus rossiae* DSM 15814^T ("other" phylogroup). In particular, the gene *ant*(9) was found only in the genome of *L. pobuzihii* NBRC 103219^T, although its phenotypic susceptibility towards aminoglycosides, as previously observed. Similarly, the gene *ant*(6) was found in *L. sharpeae* DMS 20505^T and *L. rossiae* DSM 15814^T, which were susceptible to aminoglycosides. Conversely, the presence of this AR determinant in *L. amylophilus* DSM 20533^T, *L. amylotrophicus* DSM 20534^T, *L. fabifermentas* DMS 21115^T, and *L. animalis* DSM 20602^T could be at the basis of their resistance toward kanamycin and streptomycin. This observation is in accordance with the high affinity for streptomycin showed by ANT6 nucleotidyltransferases (Ramirez *et al*., 2010). Moreover, the gene *ant*(6) found in the genome of *L. animalis* DSM 20602^T and *L. amylophilus* DSM 20533^T shared 99% similarity with the ANT6 aminoglycoside nucleotidyltransferase of *Streptococcus suis* (Accession number: WP_044770667.1) and *Clostridium difficile* (AN: WP_077726164.1). Strains of these species are usually associated to the animal GIT (Ferrando *et al*., 2015; Miller *et al*., 2014; Keessen *et al*., 2011), where the high cell density leads microbial interactions and facilitates HGT events (Martínez *et al*., 2015; Huddleston *et al*., 2014). These findings suggest that *ant*(6) could represent an acquired character for DSM 20602^T and DSM 20533^T, thus exposing them as potential vectors for AR genes in the food chain.

The presence of the gene *ant*(6) has been already reported for some *Lactobacillus* species isolated from dairy product (Devirgiliis *et al*., 2013), wine (Rojo–Bezares *et al*., 2006), and used as probiotics (Wong *et al*., 2015). Moreover, the presence of this gene has been recently reported for *L. salivarius*, *L. reuteri*, and *L. ingluviei* strains isolated from chickens and showing wide resistance to streptomycin (Dec *et al*., 2017).

Regarding phosphotransferases, the gene *aph*(3) was found in five members of the *L. delbrueckii* phylogroup (*L. acidophilus* ATCC 4356^T, *Lactobacillus gasseri* LMG 9203^T, *L. johnsonii* LMG 9436^T, *Lactobacillus kalixensis* DMS 16043^T, and *Lactobacillus pasteurii* DSM 23907^T) which showed resistance to kanamycin except for *L. acidophilus* ATCC 4356^T and *L. gasseri* LMG 9203^T. *Aph*(3) is generally involved in kanamycin resistance (Ramirez and Tolmasky, 2010). The same aminoglycoside resistance determinant has been detected in some *Lactobacillus* species, such as *Lactobacillus murinus* strains of animal origin (Klose *et al*., 2014), and in *Lactobacillus casei* and *Lactobacillus paracasei* strains isolated from human samples (Ouoba *et al*., 2008).

The genes *ant*(6) and *aph*(3) are of greatest clinical importance since they are usually found in plasmids or transposons, increasing the risk of resistance dissemination along the food chain (van Hoek *et al*., 2011).

Clindamycin resistance genes. The gene *lsa* encoding for a lincosamide efflux protein (Roberts, 2008) was found in 60 strains, 13 of which displayed resistance to clindamycin. This gene has been previously identified in *Enterococcus faecium*, where it plays a key role in the resistance to clindamycin and quinupristin–dalfopristin (Singh *et al*., 2002). The genetic make–up in *E. faecium* is characterized by the presence of a leader peptide surrounding the gene *lsa* which is involved in the post–transcriptional regulation of *lsa* expression. (Singh *et al*., 2002). Thus, further studies are necessary to determine the genetic make–up of this clindamycin resistance gene in order to clarify its actual role in the resistance for the genus *Lactobacillus* . This is the first time, to our knowledge, that the gene *lsa* is detected in lactobacilli. However, Kastner *et al*. (2006) revealed the presence of the lincosamide resistance *lnu*(A) gene in a *L. reuteri* strain isolated from food additives in Switzerland, which encodes for a lincomycin nucleotidyltransferase enzyme. The same gene was even found in two plasmids of a *L. reuteri* commercial strain (Rosander *et al*., 2008). In contrast, this AR determinant was not found in *L. reuteri* DSM 20016^{T}.

Chloramphenicol resistance genes. Among the 161 type strains of the genus *Lactobacillus*, 36 chloramphenicol resistance sequences were found, which code for chloramphenicol acetyltransferase, such as the genes *cat*, and for specific membrane–associated transporters like *cml*A genes. In detail, *cat* gene was detected in 34 lactobacilli, two of which (*Lactobacillus kimchicus* JCM15530^{T} and *L. similis* DSM23365^{T}) carried two copies of this gene. Moreover, *Lactobacillus hammesii* DMS 16381^{T}, *L. koreensis* JCM 16448^{T}, *L. namurensis* DSM 19117^{T}, and *L. zymae* DSM 19395^{T} (belonging to the phylogroup *L. brevis*) are characterized by the presence of either *cml*A and *cat* genes. Conversely, *L. acidifarinae* DSM 19394^{T} and *L. selangorensis* ATCC BAA66^{T} displayed only the presence of *cml*A. This chloramphenicol resistance determinant has been identified in Gram–negative bacteria and is inducible expressed by a translational attenuator (Roberts and Schwarz, 2009). Therefore, the presence of *cml*A in lactobacilli is unusual and is revealed for the first time in this study.

On the other hand, *cat* genes are one of the most common resistance determinants found in lactobacilli and they have been detected in several *Lactobacillus* species of different origin, such as *L. acidophilus*, *L. johnsonii*, *L. delbreuckii*, *L. reuteri*, and *L. plantarum* (Gueimonde *et al*., 2013).

Although *cat* gene has been frequently found on plasmids associated to various *Lactobacillus* spp., more recent genome sequence analysis located this gene also in the chromosome of *Lactobacillus* strains isolated from fermented vegetables (*L. brevis*, *L. plantarum* and *Lactobacillus sakei* strains), dairy products (*Lactobacillus fermentum* and *L. plantarum* strains) and fermented sausages (*L. sakei* strains) (Abriouel *et al*., 2015).

Tetracycline resistance genes. The 18 gene sequences found among lactobacilli strains code for ribosomal protection proteins [*tet*(M), *tet*(S), *tet*(Q), *tet*(W)] and efflux pumps [*tet*(L), *tet*(P)]. In detail, the gene *tet*(L) was found in the tetracycline resistant *Lactobacillus suebicus* DSM 5007^T and *L. ingluviei* DSM 15946^T.The latter strain was also characterized by the presence of *tet*(W) and *tet*(M). Interestingly, *tet*(M) in DSM 15946^T exhibited 99% similarity with the corresponding sequence of *Enterococcus faecalis* (AN: WP_049098680.1), *E. faecium* (AN: WP_010777232.1), *Streptococcus pneumoniae* PT814 and *Staphylococcus epidermidis* (AN: WP_002403674.1). Similarly, the sequence of *tet*(L) displayed 99% similarity with that carried by *E. faecalis* (AN: WP_002387933.1), *E. faecium* (AN: WP_096541192.1), and *Streptococcus agalactiae* (AN: WP_041974946.1). *Tet*(W) showed 99% similarity with the sequence of *Trueperella pyogenes* OX9, *Bifidobacterium longum* subsp. *longum* F21, and *C. difficile* CI7 (Wang *et al*., 2017; Spigaglia *et al*., 2011; Billington and Jost, 2006). Simultaneous presence of *tet*(L) *tet*(M), and *tet*(W) has been previously reported for two *L. johnsonii* strains (van Hoek *et al*., 2008), but the genetic organization of these tetracycline resistance determinants was not investigated.

Tet(W) was also identified in *L. pasteurii* DSM 23907^T; while, *tet*(M) was found in *L. sharpeae* DSM 20505^T (*L. casei–manihotivorans* group), *L. acidophilus* ATCC 4356^T, *L. crispatus* DSM 20584^T, *L. gallinarum* DSM 10532^T, *L. amylophilus* DSM 20533^T, *L. amylotrophicus* DSM 20534^T (*L. delbrueckii* group) and *L. equigenerosi* DSM 18793^T (*L. reuteri–vaccinostercus* group). Strains DSM 20584^T, ATCC 4356^T, and DSM 18793^T showed susceptibility to tetracycline. Regarding other ribosomal protection proteins, *tet*(Q) and *tet*(S) genes were respectively found in *L. brevis* DSM 20054^T and *L. heilongjiangensis* LMG 26166^T, both resistant to tetracycline; while *tet*(P) was found in *L. gasseri* LMG 9203^T, *L. taiwanensis* DSM 21401^T, *L. ruminis* DSM 20403^T, and *L. johnsonii* LMG 9436^T. Only LMG 9436^T showed phenotypic resistance towards tetracycline. *Tet*(P) is unusual because it consists of two gene sequences: *tetA*(P), encoding for a functional efflux protein, and *tetB*(P), which appears to code for a ribosomal protection protein (Chopra and Roberts, 2001). Therefore, the absence of this genetic conformation in LMG 9203^T, DSM 21401^T and DSM 20403^T could result in the lack of resistance phenotype.

Tetracycline resistance determinants are the most common resistance genes found in lactobacilli (Gueimonde *et al*., 2013), where the most widespread resistance genes are represented by *tet*(M) and *tet*(S) for food-borne and probiotic bacteria (Abriouel *et al*., 2015; Devirgiliis *et al*., 2013, Ouoba *et al*., 2008). This is due to the frequent association of *tet*(M) with conjugative transposons, like Tn*916* (Roberts and Schwarz, 2009). In particular, *tet*(M) was the main tetracycline resistance gene detected among *Lactobacillus* strains isolated from Italian fermented dry sausages, belonging to *L. rhamnosus*, *L. curvatus*, *L. sakei* and *L. plantarum* species, followed by *tet*(W) and *tet*(S) (Zonenschain *et al*., 2009). Similarly, *tet*(W), *tet*(O) and *tet*(M) were found in lactobacilli isolated from fermented foods of Indian origin (Thumu and Halami, 2012b). Even though *tet*(Q) was the first described *tet* gene in Gram–negative bacteria and can be expressed also in Gram–positive bacteria (Chopra and Roberts, 2001), a previous AR analysis in *Lactobacillus* spp. isolated from faeces of healthy chickens through molecular methods retrieved no positive results (Dec *et al*., 2017).

Erythromycin resistance genes. Among the 161 *Lactobacillus* genomes, six gene sequences involved in erythromycin resistance were found, which include the gene *erm*(B) coding for rRNA methylase and two variants of the gene *mef*, *mef*(E) and *mef*(B), encoding for macrolide efflux pumps (Roberts, 2008). These genes were identified in three erythromycin susceptible strains, such as *L. delbrueckii* subsp. *lactis* DSM 20072^{T} [*mef*(E)], *L. casei* DSM 20041^{T} and *L. paracasei* subsp. *paracasei* DSM 5622^{T} [*mef*(B)]. *Erm*(B) was found in erythromycin resistant *L. amylophilus* DSM 20533^{T}, *L. amylotrophicus* DSM 20534^{T}, and *L. ingluviei* DSM 15946^{T}, which also showed either *tet*(W). This simultaneous presence of tetracycline and erythromycin resistance genes has been previously reported for *L. paracasei* and *Lactococcus lactis* strains isolated from traditional Italian fermented foods (Devirgiliis *et al*., 2010; Comunian *et al*., 2010). The presence of *erm*(B) in *Lactobacillus* species from different sources has been reported in several studies (Thumu and Halami, 2012b; Nawaz *et al*., 2011; Belletti *et al*., 2009; Zonenschain *et al*., 2009; Ammor *et al*., 2007; Delgado *et al*., 2005). Moreover, *erm*(B), which encodes a rRNA methylase acting on the 23S ribosomal subunit, has been also detected in lactobacilli isolated from pharmaceutical products (Gad *et al*., 2014) and in one *L. salivarius* strain used as probiotic (Hummel *et al*., 2007b). The rRNA methylases are the largest group of acquired erythromycin resistance genes with *erm*(B) found to have a broad host range, including both Gram–positive and Gram–negative bacteria (Roberts, 2008). This may be due to the generally association of this gene to mobile genetic elements. In fact, *erm*(B) has been generally found in lactobacilli in conjugative transposons located in chromosomes, in plasmids, but also even in nonconjugative transposons, such as Tn*917* and Tn*551* (Gueimonde *et al*., 2013). In

contrast, *mef* genes are common in streptococci (Roberts *et al*., 1999). In fact, PCR assays used to detect the presence of *mef*A/E in 88 *Lactobacillus* strains isolated from chickens retrieved no positive results (Dec *et al*., 2017).

5.3.4 Analysis of ORFs in regions flanking the tetracycline and erythromycin resistance genes. Up– and down–stream regions of the tetracycline and erythromycin resistance genes were further investigated in *L. ingluviei* DSM 15946^T, *L. amylophilus* DMS 20533^T, and *L. amylotrophicus* DSM 20534^T.

The sequence analysis of *L. ingluviei* genome revealed that *tet*(M) and *tet*(L) genes were inserted in a *Tn916*–like transposon. It is 17,073 bp–long and contained 16 ORFs, which have the same direction of transcription (Figure 5.4). Sequence alignment showed that this transposon is essentially identical to the Tn*5251* of *S. pneumoniae* DP1322 (Santoro *et al*., 2010), sharing 99% similarity, except for the presence of *tet*(L). In fact, part of the regulation module of the Tn*916* transposon, including *orf*6, *orf*9, *orf*10, *orf*7, *orf*8, *orf*5 and the gene *xis* (Roberts and Mullany, 2009), was substituted by *tet*(L) in *L. ingluviei*, as shown in Figure 5.4. However, the sequence of *orf*12 coding for the leader peptide and the conjugation region, essential for replication and transfer of the transposon (Wright and Grossman, 2016), were conserved in DSM 15946^T.

Tn916–like transposon was also found in *L. sakei* strain isolated from Italian Sola cheese made from raw milk (Ammor *et al*., 2008), and in other *Lactobacillus* species of different fermented food origin (Abriouel *et al*., 2015). However, this is the first time, to our knowledge, that Tn*916*–like transposon has been identified in *L. ingluviei* and the transferability of this element should be verified in further studies.

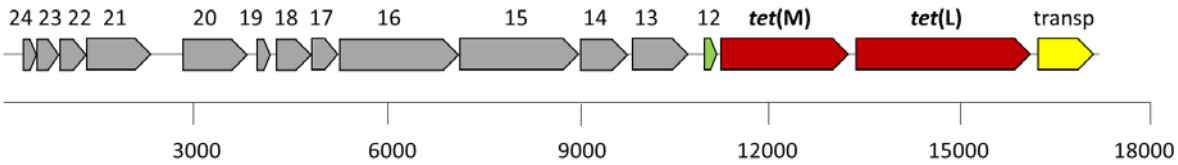

Figure 5.4. Diagram showing the genetic organization of the Tn*916*–like transposon identified in *L. ingluviei* DSM 15946^T. Color codes: red – AR genes; yellow – genes involved in the genetic transfer; grey – the ORFs involved in the conjugation process; green – regulatory sequences.

Tet(W) and *erm*(B) were located in the same genomic region and shared 99% similarity with the corresponding sequences of the strain *S. suis* SsCA (Palmieri *et al*., 2011). Moreover, these AR

determinants were flanked by regions with high similarity (99%) to replication proteins and to the integrase of the plasmid pLR581 of *L. reuteri* SD2112 (Accession number: CP002845.1) (Figure 5.5). The presence of megaplasmids has been previously detected in *L. ingluviei* DSM 15946^T and *L. ingluviei* DSM 14792 isolated from pigeon crop (Li *et al*., 2007), but no evidence of AR genes were reported. This finding suggests that *erm*(B) and *tet*(W) may be located in a plasmid of DSM 15946^T strain. Plasmid–borne resistance genes are commonly associated to HGT, causing the spread of resistance characters among microbial communities (Crofts *et al*., 2017). This aspect could be supported by the fact that: i) *L. ingluviei* and *L. reuteri* strains may be found in the same ecological niches as strains of both species are isolated in the GIT of chickens (Gravett *et al*., 2013; Kobierecka *et al*., 2017) and ii) the GC content of this region is more similar to that of *L. reuteri* SD2112 than the GC% of *L. ingluviei* DSM 15946^T (40.71, 38.63 and 49.88%, respectively).

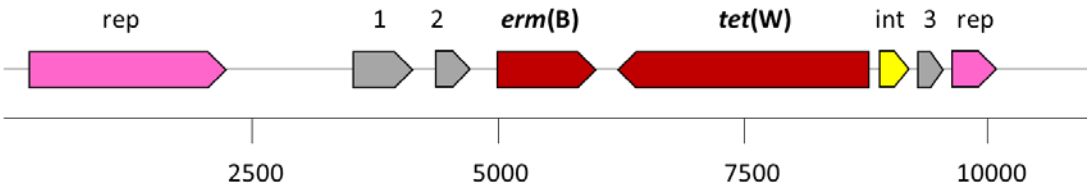

Figure 5.5. Diagram showing the genetic organization of sequences surrounding the *tet*(W) and *erm*(B) genes identified in *L. ingluviei* DSM 15946^T. Color codes: red – AR genes; yellow – genes involved in the genetic transfer; pink – genes encoding plasmid–associated replication proteins; grey – gene coding for hypothetical proteins.

L. amylophilus and *L. amylotrophicus* harboured the *tet*(M) and *erm*(B) genes in two different genomic regions and they shared 99% similarity respectively with the corresponding sequence of *S. agalactiae* SG–M4 and *Staphylococcus hyicus* HW17, respectively. Interestingly, the flaking region structure of these AR determinants was identical in the two type strains, this probably due to the phylogenetic association between *L. amylophilus* and *L. amylotrophicus*. In fact, the latter species has been described from the reclassification of some *L. amylophilus* strains (Naser *et al*., 2006). In particular, the up– and downstream sequences surrounding *tet*(M) were characterized by the presence of several genes encoding for conjugation proteins and transposases (Figure 5.6 A). Therefore, this tetracycline determinant could be involved in genetic transfer events resulting in the improvement of the spread of AR elements. As for *erm*(B), the upstream region showed a 83–bp sequence corresponding to a 27–amino acid leader peptide. Upstream the leader peptide sequence, a gene encoding for a mobilization protein was found, which shared 99% similarity

with the corresponding sequence of the *S. hyicus* plasmid pSTE1 (Accession number: HE662694.1) (Figure 5.6 B). Unfortunately, the location of *erm*(B) at the 3'–end of the contig did not allow the characterization of the downstream region.

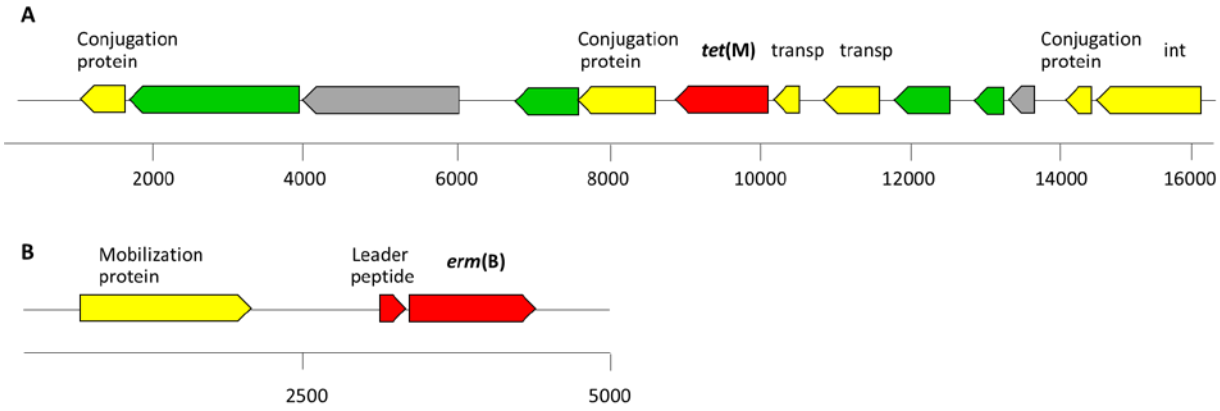

Figure 5.6. Diagram showing the genetic organization of sequences surrounding the *tet*(M) (A) and *erm*(B) (B) genes identified in *L. amylophilus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T. Color code: red – AR genes; yellow – genes involved in the genetic transfer; green – genes encoding regulatory proteins; grey – gene coding for hypothetical proteins.

These observations suggest that *L. ingluviei*, *L. amylophilus* and *L. amylotrophicus* could act as dissemination vectors for tetracycline and erythromycin resistance. In fact, swine wastes represent the natural habitat of *L. amylophilus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T (Naser *et al*., 2006), which are considered as a potential hotspot for promoting the dissemination of AR genes in the environment. In particular, these determinants carried by bacteria could contaminate the environment via discharge of animal wastes, thus resulting in the spread of AR in soil, water, and food (He *et al*., 2016). *L. ingluviei* strains are able to colonize the GIT of animals and to manipulate the gut microbiota resulting in weight gain (Million *et al*., 2012). The close proximity of bacterial cells in such environment could favour the transfer of genetic material, including AR genes, among microorganisms (Aarts and Margolles, 2015). The transfer of *erm*(B) and *tet*(M) from lactobacilli to other microorganisms has been revealed in previous studies through *in vitro* filter mating experiments (Nawaz *et al*., 2011, Ouoba *et al*., 2008, Gevers *et al*., 2003b). In particular, Gevers *et al*. (2003b) revealed the transfer of plasmid–borne *tet*(M) from 14 different *Lactobacillus* strains, including *L. plantarum*, *L. curvatus*, *L. alimentarius*, *L. sakei* subsp. *sakei*, *L. sakei* subsp. *carnosus*, to *E. faecalis* JH2–2 at relatively high frequency (10^{-4}–10^{-6} transconjugants per recipient). However, transfer of macrolide resistance from *Lactobacillus* to enterococci *in vivo* has been reported by Jacobsen *et al*. (2006) indicating that *Lactobacillus* spp. may play a role in the spread of antimicrobial resistance.

5.3.5 Phenotype–genotype correlation. Overall, phenotypic resistance correlated for the 67% of the cases examined with genomic data. In detail, genotype was in accordance with the phenotype for 892 out of 1,449 phenotypic tests investigated, which included 782 cases representing susceptible phenotype towards a specific antibiotic linked to the absence of AR determinants, and 110 cases for which the resistance phenotype correlated with the presence of one or more AR genes.

On the other hand, the inconsistency between phenotypic and genetic data was mostly represented by strains with resistance phenotypes, but no AR genes were identified. This may be linked to the development of novel or unusual microbial resistance mechanisms, which cannot be revealed through only sequence analysis based on database searching, or may be due to the modification of the membrane structure resulting in a reduced permeability which prevent the access to the target (Blair *et al*., 2015). Therefore, further studies focused on the identification of new AR determinants should be performed, which combine data derived from the various processes related to DNA sequence, gene expression, and protein function (Werner, 2010).

In contrast, the presence of susceptible strains carrying AR determinants (reported in yellow in Figure 5.7) may be associated to the absence of gene expression depending to the existence of a functional promoter or inducer, or depending to the post–transcriptional modification (Depardieu *et al*., 2007).

Figure 5.7. Phenotype–genotype correlation for the 161 type strains of the genus *Lactobacillus*. Positive correlations between genomic data and phenotypes observed are reported in green and grey, whereas negative correlation are indicated in yellow and blue. Antimicrobial abbreviations: GM, Gentamicin; KM, Kanamycin; SM, Streptomycin; TC, Tetracycline; EM, Erythromycin, CL, Clindamycin; CM, Chloramphenicol; AM, Ampicillin; VA, Vancomycin.

5.4 Conclusions

Lactobacillus species are probably the most widely used as probiotics and starter cultures in a variety of foods. They can be found as components of the natural microbiota of several environments, including the GIT. However, official guidelines for the safety assessment of all these relevant strains are not available. Therefore, the parallel execution of resistance phenotypic assays and accurate analysis of the genome sequence of the whole genus *Lactobacillus* was performed in this study, providing the complete estimation of the AR for this genus.

In particular, resistance towards different antibiotics was detected among 182 *Lactobacillus* type strains, revealing a wide distribution of resistance phenotypes with 84% of the strains showing multidrug resistance. The resistance towards trimethoprim, vancomycin and kanamycin represented the most common phenotypes observed among lactobacilli. The genome sequencing analysis revealed the presence of a Y–type Ddl ligase in the vancomycin susceptible strains belonging to the *L. delbrueckii* group. Instead, almost all vancomycin-resistance strains carried a Ddl ligase of the F–type, which is involved in the synthesis of peptidoglycan precursors contained a D–Ala–D–Lac residue.

Moreover, the combination of the homology–based method and the manual annotation used for the analysis of 161 genome sequences of the genus *Lactobacillus* identified the presence of genes encoding for resistance to aminoglycosides (20 sequences), tetracycline (18), erythromycin (6), clindamycin (60), and chloramphenicol (42). In particular, the gene *aac*(3), *lsa* and *cml*(A) involved in the resistance towards aminoglycoside, clindamycin and chloramphenicol, respectively, were found for the first time in *Lactobacillus* strains.

In addition, acquired determinants coding for tetracycline and erythromycin resistance were simultaneously detected in *L. ingluviei* DSM 15946^T, *L. amylophilus* DSM 20533^T, and *L. amylotrophicus* DSM 20534^T. The analysis of the surrounding regions highlighted the presence of sequences involved in the mobilization of genetic elements, revealing the potential of these AR genes to be horizontally transferred to other microorganisms.

Finally, the correlation between phenotype and genotype was found to be positive for the 67% of the cases examined. However, the presence of some resistance phenotypes not associated to particular genetic determinants emphasizes the requirement of deeper studies focused on the identification of novel genes involved in AR for lactobacilli. This investigation not only can provide AR gene sequences for the development of specific databases for the safety assessment of lactobacilli, but it can also used as a template for the molecular analysis of other lactic acid bacteria.

In conclusion, the results reported in this study may be utilized as a starting point for the generation of new and more focused scientific protocols and regulatory procedures for the safety assessment of lactobacilli employed as starter cultures, food preservatives or probiotic by food and probiotic stakeholders.

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Table S5.1. Features of the 197 type strains of the genus *Lactobacillus* analysed, including genome accession number and growth condition applied for the determination of MIC values. The phylogroups correspond to those described by Sun *et al*. (2015).

Species	Strain ID	Metabolism Phenotype	Phylogroup	Genome AN	Source	Niche category	Growth conditions	Temp (°C)	Medium
Lactobacillus acetotolerans	DSM 20749^T	FHE	*L. delbrueckii*	AYZC00000000	Fermented Vinegar Broth	Food	Anaerobic	30	MRS + 0.05% cysteine
Lactobacillus acidifarinae	DSM 19394^T	OHE	*L. brevis*	AZDV00000000	Artisanal wheat sourdough	Food	Microaerophilic	30	MRS + 0.05% cysteine, pH 5.2
Lactobacillus acidipiscis	DSM 15836^T	FHE	*L. salivarius*	AZFI00000000	Fermented fish	Food	Microaerophilic	37	MRS
Lactobacillus acidophilus	ATCC 4356^T	OHO	*L. delbrueckii*	AZCS00000000	Human	Animal	Microaerophilic	37	MRS
Lactobacillus agilis	DSM 20509^T	FHE	*L. salivarius*	AYYP00000000	Municipal sewage	Environment	Microaerophilic	37	MRS + 0.05% cysteine
Lactobacillus algidus	DSM 15638^T	FHE	*L. salivarius*	AZDI00000000	Vacuum–packed beef	Food	Anaerobic	20	MRS, pH 5.7
Lactobacillus alimentarius	DSM 20249^T	FHE	*L. alimentarius*	AZDQ00000000	Marinated fish product	Food	Aerobic	30	MRS
Lactobacillus amylolyticus	DSM 11664^T	OHO	*L. delbrueckii*	AZEP00000000	Acidified beer wort	Wine product	Preferably anaerobic	37	MRS
Lactobacillus amylophilus	DSM 20533^T	OHO	*L. delbrueckii*	AYYS00000000	Swine waste–corn fermentation	Animal	Aerobic	30	MRS + 1% glucose
Lactobacillus amylotrophicus	DSM 20534^T	OHO	*L. delbrueckii*	AZCV00000000	Swine waste–corn fermentation	Animal	Aerobic	30	MRS
Lactobacillus amylovorus	DSM 20531^T	OHO	*L. delbrueckii*	AZCM00000000	Cattle waste–corn fermentation	Animal	Microaerophilic–anaerobic	37	MRS
Lactobacillus animalis	DSM 20602^T	OHO	*L. salivarius*	AYYW00000000	Dental plaque of baboon	Animal	Aerobic	37	MRS
Lactobacillus antri	LMG 22111^T	OHE	*L. reuteri – vaccinostercus*	AZDK00000000	Gastric biopsies, Human stomach mucosa	Animal	Anaerobic	37	MRS
Lactobacillus apinorum	DSM 26257^T	OHE	*L. fructivorans*	JXCT00000000	Honey stomach of honeybee	Animal	Anaerobic	37	MRS + 2% fructose
Lactobacillus apis	LMG 26964^T	OHO	*L. delbrueckii*	JXLG00000000	Stomachs of honeybees	Animal	Anaerobic	37	MRS
Lactobacillus apodemi	DSM 16634^T	OHE	*L. salivarius*	AZFT00000000	Faeces of wild Japanese wood mouse	Animal	Microaerophilic	37	MRS
Lactobacillus aquaticus	DSM 21051^T	OHO	*L. salivarius*	AYZD00000000	Surface of a eutrophic freshwater pond	Environment	Microaerophilic	37	MRS
Lactobacillus aviarius subsp. araffinosus	DSM 20653^T	OHO	*L. salivarius*	AYYZ00000000	Intestine of chicken	Animal	Microaerophilic	37	MRS + 0.05% cysteine–hydrochloride
Lactobacillus aviarius subsp. aviarius	DSM 20655^T	OHO	*L. salivarius*	AYZA00000000	Faeces of chicken	Animal	Microaerophilic	37	MRS + 0.05% cysteine
Lactobacillus backii	DSM 18080^T	OHO	*L. coryniformis*	n.a.	Orchardgrass silage	Plant	Preferably anaerobic	28	MRS
Lactobacillus bifermentans	DSM 20003^T	FHE	*L. coryniformis*	AZDA00000000	Blown cheese	Food	Anaerobic	30	MRS
Lactobacillus bombi	DSM 26517^T	FHE	*L. alimentarius*	n.a.	Digestive tracts of bumblebee queens	Animal	Anaerobic	37	MRS

Lactobacillus bombicola	DSM 28793^T	FHE	*L. delbrueckii*	n.a.	Bumble bee gut	Animal	Strictly anaerobic	37	MRS + 0.05% cysteine–hydrochloride
Lactobacillus brantae	DSM 23927^T	FHE	*L. casei – manihotivorans*	AYZQ00000000	Faeces of Canada goose	Animal	Microaerophilic	37	MRS
Lactobacillus brevis	DSM 20054^T	OHE	*L. brevis*	AZCP00000000	Faeces	Animal	Microaerophilic	30	MRS
Lactobacillus buchneri	DSM 20057^T	OHE	*L. buchneri*	AZDM00000000	Tomato pulp	Plant	Microaerophilic	37	MRS
Lactobacillus cacaonum	DSM 21116^T	FHE	*L. salivarius*	AYZE00000000	Cocoa bean heap fermentation	Plant	Microaerophilic	30	MRS
Lactobacillus camelliae	DSM 22697^T	OHO	*L. casei – manihotivorans*	AYZJ00000000	Fermented tea leaves (miang)	Plant	Microaerophilic	37	MRS
Lactobacilllus capillatus	DSM 19910^T	FHE	*L. salivarius*	AZEF00000000	Fermented brine used for stinky tofu production	Food	Microaerophilic	30	MRS
Lactobacillus casei	DSM 20011^T	FHE	*L. casei – manihotivorans*	AZCO00000000	Cheese	Food	Microaerophilic	30	MRS
Lactobacillus ceti	DSM 22408^T	FHE	*L. salivarius*	JQBZ00000000	Lungs of a beaked whale	Animal	Strictly anaerobic	37	MRS
Lactobacillus colehominis	DSM 14060^T	FHE	*L. reuteri – vaccinostercus*	AZEW00000000	Human vagina	Animal	Microaerophilic	37	MRS
Lactobacillus collinoides	DSM 20515^T	OHE	*L. collinoides*	AYYR00000000	Fermenting apple juice	Food	Anaerobic	26	MRS
Lactobacillus composti	DSM 18527^T	FHE	Other	AZGA00000000	Composting material of distilled shochu residue	Wine product	Microaerophilic	30	MRS
Lactobacillus concavus	DSM 17758^T	OHO	Other	AZFX00000000	Walls of a distilled spirit fermenting cellar	Environment	Microaerophilic	30	MRS
Lactobacillus coryniformis subsp. *coryniformis*	LMG 9196^T	FHE	*L. coryniformis*	AZCN00000000	Silage	Plant	Microaerophilic	30	MRS
Lactobacillus coryniformis subsp. *torquens*	DSM 20004^T	FHE	*L. coryniformis*	AZDC00000000	Air of cow shed	Environment	Microaerophilic	30	MRS
Lactobacillus crispatus	DSM 20584^T	OHO	*L. delbrueckii*	AZCW00000000	Eye	Animal	Preferably anaerobic	37	MRS
Lactobacillus crustorum	LMG 23699^T	OHO	*L. alimentarius*	JQCK00000000	Wheat sourdough	Food	Aerobic	30	MRS
Lactobacillus curieae	JCM 18524^T	OHE	*L. buchneri*	CP018906	Tofu brine	Food	Facultatively anaerobic	30	MRS
Lactobacillus curvatus	DSM 20019^T	FHE	*L. sakei*	AZDL00000000	Milk	Food	Microaerophilic	30	MRS
Lactobacillus delbrueckii subsp. *bulgaricus*	DSM 20081^T	OHO	*L. delbrueckii*	JQAV00000000	Bulgarian yoghourt	Food	Microaerophilic	37	MRS
Lactobacillus delbrueckii subsp. *delbrueckii*	DSM 20074^T	OHO	*L. delbrueckii*	AZCR00000000	Sour grain mash	Food	Microaerophilic	37	MRS
Lactobacillus delbrueckii subsp. *indicus*	DSM 15996^T	OHO	*L. delbrueckii*	AZFL00000000	Traditional dairy fermented product (Dahi type)	Food	Microaerophilic	37	MRS
Lactobacillus delbrueckii subsp. *jakobsenii*	DSM 26046^T	OHO	*L. delbrueckii*	JQCG00000000	Dolo wort (Alcholic fermented beverage)	Wine product	Anaerobic	37	MRS
Lactobacillus delbrueckii subsp. *lactis*	DSM 20072^T	OHO	*L. delbrueckii*	AZDE00000000	Emmental cheese	Food	Microaerophilic	37	MRS

Lactobacillus dextrinicus	DSM 20335^T	OHE	Other	AYYK00000000	Silage	Plant	Aerobic	30	MRS
Lactobacillus diolivorans	DSM 14421^T	OHE	*L. buchneri*	AZEY00000000	Maize silage	Plant	Aerobic	30	MRS
Lactobacillus equi	DSM 15833^T	OHO	*L. salivarius*	AZFH00000000	Faeces of horses	Animal	Microaerophilic	37	MRS
Lactobacillus equicursoris	DSM 19284^T	OHO	*L. delbrueckii*	AZDU00000000	Healthy thoroughbred racehorse	Animal	Microaerophilic	37	MRS
Lactobacillus equigenerosi	DSM 18793^T	OHE	*L. reuteri – vaccinostercus*	AZGC00000000	Faeces of thoroughbred horse	Animal	Microaerophilic	37	MRS
Lactobacillus fabifermentans	DSM 21115^T	FHE	*L. plantarum*	AYGX00000000	Cocoa bean heap fermentation	Plant	Microaerophilic	30	MRS
Lactobacillus faecis	DSM 23956^T	OHO	*L. salivarius*	n.a.	Animal faeces	Animal	Microaerophilic	37	MRS
Lactobacillus farciminis	LMG 9189^T	OHO	*L. alimentarius*	AZDR00000000	Sausage	Food	Microaerophilic	30	MRS
Lactobacillus farraginis	DSM 18382^T	FHE	*L. buchneri*	AZFY00000000	Composting material of distilled shochu residue	Wine product	Microaerophilic	30	MRS
Lactobacillus fermentum	DSM 20055^T	OHE	*L. reuteri – vaccinostercus*	JQAU00000000	Human saliva	Animal	Aerobic	30	MRS
Lactobacillus floricola	DSM 23037^T	OHO	*L. delbrueckii*	AYZL00000000	Flower of Caltha palustris	Plant	Aerobic	30	MRS
Lactobacillus florum	DSM 22689^T	OHE	*L. fructivorans*	AYZI00000000	Peony (Paeonia suffruticosa)	Plant	Anaerobic	28	MRS + 1% fructose
Lactobacillus formosensis	NBRC 109509^T	OHO	*L. alimentarius*	n.a.	Fermented soybean	Food	Anaerobic	37	MRS, pH 6.2
Lactobacillus fornicalis	JCM 12512^T	?	Other	n.a.	Human vagina	Animal	Aerobic	37	MRS
Lactobacillus fructivorans	DSM 20203^T	OHE	*L. fructivorans*	AZDS00000000	N/A	Unknown	Microaerophilic	30	MRS
Lactobacillus frumenti	DSM 13145^T	OHE	*L. reuteri – vaccinostercus*	AZER00000000	Rye–bran sourdough	Food	Anaerobic	40	MRS, pH 6.2
Lactobacillus fuchuensis	DSM 14340^T	FHE	*L. sakei*	AZEX00000000	Vacuum–packaged beef	Food	Aerobic	20	MRS
Lactobacillus furfuricola	DSM 27174^T	OHO	*L. alimentarius*	n.a.	Rice bran paste	Plant	Microaerophilic	30	MRS
Lactobacillus futsaii	JCM 17355^T	OHO	*L. alimentarius*	AZDO00000000	Fu–tsai, a traditional fermented mustard product	Food	Aerobic	30	MRS
Lactobacillus gallinarum	DSM 10532^T	OHO	*L. delbrueckii*	AZEL00000000	Chicken crop	Animal	Microaerophilic	37	MRS
Lactobacillus gasseri	LMG 9203^T	OHO	*L. delbrueckii*	NC_008530	Human	Animal	Preferably anaerobic	30	MRS
Lactobacillus gastricus	DSM 16045^T	OHE	*L. reuteri – vaccinostercus*	AZFN00000000	Gastric biopsies, Human stomach mucosa	Animal	Anaerobic	37	MRS
Lactobacillus ghanensis	DSM 18630^T	OHO	*L. salivarius*	AZGB00000000	Cocoa fermentation	Plant	Anaerobic	30	MRS
Lactobacillus gigeriorum	DSM 23908^T	OHO	*L. delbrueckii*	AYZO00000000	Chicken crop	Animal	Microaerophilic	37	MRS
Lactobacillus ginsenosidimutans	DSM 24154^T	OHO	*L. alimentarius*	CP012034	Kimchi	Food	Anaerobic	30	MRS

Lactobacillus gorillae	DSM 28356^T	FHE	*L. reuteri – L. vaccinostercus*	n.a.	Gorilla faeces	Animal	Microaerophilic	37	MRS
Lactobacillus graminis	DSM 20719^T	FHE	*L. sakei*	AYZB00000000	Grass silage	Plant	Microaerophilic	30	MRS
Lactobacillus hammesii	DSM 16381^T	FHE	*L. brevis*	AZFS00000000	Wheat sourdough	Food	Microaerophilic	30	MRS + 1% maltose + 0.5% yeast extract
Lactobacillus hamsteri	DSM 5661^T	FHE	*L. delbrueckii*	AZGI00000000	Faeces of hamster	Animal	Anaerobic	37	MRS
Lactobacillus harbinensis	DSM 16991^T	FHE	*L. perolens*	AZFW00000000	Chinese traditional fermented vegetable	Food	Microaerophilic	37	MRS
Lactobacillus hayakitensis	DSM 18933^T	OHO	*L. salivarius*	AZGD00000000	Faeces of thoroughbred horse	Animal	Microaerophilic	37	MRS
Lactobacillus heilongjiangensis	LMG 26166^T	OHO	*L. alimentarius*	CP012559	Chinese pickle	Plant	Microaerophilic	28	MRS
Lactobacillus helsingborgensis	DSM 26265^T	OHO	*L. delbrueckii*	JXJR00000000	Honey stomach of honeybee	Animal	Anaerobic	35	MRS + Fructose (20 g/L)
Lactobacillus helveticus	LMG 22464^T	OHO	*L. delbrueckii*	JQCJ00000000	Malt whiskey fermentation	Wine product	Anaerobic	37	MRS
Lactobacillus herbarum	DSM 100358^T	OHE	*L. plantarum*	LFEE00000000	Fermented white radish	Food	Microaerophilic	25	MRS
Lactobacillus hilgardii	LMG 6895^T	OHE	*L. buchneri*	AZDF00000000	Wine	Wine product	Preferably anaerobic	37	MRS
Lactobacillus hokkaidonensis	DSM 26202^T	OHE	*L. reuteri – vaccinostercus*	JQCH00000000	timothy grass silage	Plant	Microaerophilic	25	MRS
Lactobacillus hominis	DSM 23910^T	OHO	*L. delbrueckii*	AYZP00000000	Human intestine	Animal	Microaerophilic	37	MRS
Lactobacillus homohiochii	DSM 20571^T	FHE	*L. fructivorans*	JQBN00000000	Spoiled sake	Wine product	Microaerophilic	26	MRS
Lactobacillus hordei	DSM 19519^T	OHO	*L. salivarius*	AZDX00000000	Malted barley	Plant	Anaerobic	37	MRS
Lactobacillus iners	DSM 13335^T	OHO	*L. delbrueckii*	AZET00000000	Human urine	Animal	Anaerobic	37	MRS
Lactobacillus ingluviei	DSM 15946^T	OHE	*L. reuteri – vaccinostercus*	AZFK00000000	Pigeon, crop	Animal	Microaerophilic	37	MRS
Lactobacillus insicii	DSM 29801^T	OHO	*L. alimentarius*	n.a.	Fermented raw meat	Animal	Microaerophilic	30	MRS
Lactobacillus intestinalis	DSM 6629^T	FHE	*L. delbrueckii*	AZGN00000000	Intestine of rat	Animal	Aerobic	37	MRS
Lactobacillus iwatensis	DSM 26942^T	OHO	*L. coryniformis*	n.a.	Orchardgrass silage	Plant	Microaerophilic	30	MRS
Lactobacillus jensenii	DSM 20557^T	FHE	*L. delbrueckii*	AYYU00000000	Human vaginal discharge	Animal	Microaerophilic	37	MRS
Lactobacillus johnsonii	LMG 9436^T	OHO	*L. delbrueckii*	AZCY00000000	Human blood	Animal	Preferably anaerobic	30	MRS
Lactobacillus kalixensis	DSM 16043^T	OHO	*L. delbrueckii*	AZFM00000000	Gastric biopsies, Human stomach mucosa	Animal	Microaerophilic	37	MRS
Lactobacillus kefiranofaciens subsp. *kefiranofaciens*	LMG 19149^T	OHO	*L. delbrueckii*	AZGG00000000	Kefir grains	Plant	Anaerobic	30	MRS
Lactobacillus kefiranofaciens subsp. *kefirgranum*	DSM 10550^T	OHO	*L. delbrueckii*	AZEM00000000	Kefir grains	Plant	Anaerobic	30	MRS

Lactobacillus kefiri	DSM 20587^T	OHE	*L. buchneri*	AYYV00000000	Kefir grains	Plant	Aerobic	30	MRS
Lactobacillus kimbladii	DSM 26263^T	FHE	*L. delbrueckii*	JXLH00000000	Honey stomach of honeybee	Animal	Anaerobic	30	MRS
Lactobacillus kimchicus	JCM 15530^T	FHE	*L. collinoides*	AZCX00000000	Kimchi	Food	Aerobic	37	MRS
Lactobacillus kimchiensis	DSM 24716^T	OHO	*L. alimentarius*	JQCF00000000	Kimchi	Food	Microaerophilic	25	MRS
Lactobacillus kisonensis	DSM 19906^T	OHE	*L. buchneri*	AZEB00000000	Sunki, a Japanese traditional pickle	Food	Microaerophilic	30	MRS + Maltose (10 g/L) + L–Arabinose (10 g/L)
Lactobacilus kitasatonis	DSM 16761^T	OHO	*L. delbrueckii*	AZFU00000000	Chicken intestine	Animal	Microaerophilic	37	MRS
Lactobacillus koreensis	JCM 16448^T	OHE	*L. brevis*	AZDP00000000	Cabbage Kimchi	Food	Aerobic	30	MRS pH 5.5
Lactobacillus kullabergensis	DSM 26262^T	FHE	*L. delbrueckii*	JXBY00000000	Honey stomach of honeybee	Animal	Strictly anaerobic	30	MRS
Lactobacillus kunkeei	DSM 12361^T	OHE	*L. fructivorans*	AZCK00000000	Commercial grape wine	Wine product	Aerobic	30	MRS + 0.05% cysteine, pH 5.2
Lactobacillus lindneri	DSM 20690^T	OHE	*L. fructivorans*	JQBT00000000	Spoit beer	Wine product	Aerobic	30	MRS + 0.05% cysteine, pH 5.2
Lactobacillus malefermentans	LMG 11455^T	OHE	*L. collinoides*	AZGJ00000000	Sour beer	Wine product	Microaerophilic	30	MRS
Lactobacillus mali	ATCC 27304^T	OHO	*L. salivarius*	JQAR00000000	Wine must	Wine product	Aerobic	30	MRS
Lactobacillus manihotivorans	DSM 13343^T	OHO	*L. casei–manihotivorans*	AZEU00000000	Cassava sour starch fermentation	Plant	Aerobic	30	MRS
Lactobacillus mellifer	DSM 26254^T	FHE	*L. alimentarius*	JXJQ00000000	Honey stomach of honeybee	Animal	Anaerobic	35	MRS + Fructose (20 g/L)
Lactobacillus mellis	DSM 26255^T	FHE	*L. alimentarius*	JXBZ00000000	Honey stomach of honeybee	Animal	Strictly anaerobic	30	MRS + Fructose (20 g/L)
Lactobacillus melliventris	DSM 26256^T	FHE	*L. delbrueckii*	JXLI00000000	Honey stomach of honeybee	Animal	Strictly anaerobic	30	MRS + Fructose (20 g/L)
Lactobacillus mindensis	DSM 14500^T	OHO	*L. alimentarius*	AZEZ00000000	Sourdough	Food	Microaerophilic	30	MRS + 0.05% cysteine–hydrochloride, pH 5.2
Lactobacillus mixtipabuli	DSM 28580^T	OHE	*L. collinoides*	n.a.	Silage	Plant	Microaerophilic	30	MRS
Lactobacillus modestisalitolerans	NBRC 107235^T	OHO	*L. plantarum*	n.a.	Fermented fish	Food	Facultatively anaerobic	30	MRS
Lactobacillus mucosae	DSM 13345^T	OHE	*L. reuteri – vaccinostercus*	AZEQ00000000	Pig small intestine	Animal	Preferably anaerobic	37	MRS
Lactobacillus mudanjiangensis	LMG 27194^T	OHO	*L. plantarum*	n.a.	Pickle	Plant	Microaerophilic	28	MRS
Lactobacillus murinus	DSM 20452^T	FHE	*L. salivarius*	AYYN00000000	Intestine of rat	Animal	Microaerophilic	37	MRS
Lactobacillus nagelii	DSM 13675^T	OHO	*L. salivarius*	AZEV00000000	Partially fermented wine	Wine product	Anaerobic	30	MRS

Lactobacillus namurensis	DSM 19117^T	OHE	*L. brevis*	AZDT00000000	Sourdough	Food	Microaerophilic	30	MRS + 0.05% cysteine + 0.7 % maltose, pH 5.2
Lactobacillus nantensis	DSM 16982^T	FHE	*L. alimentarius*	AZFV00000000	Wheat sourdough	Food	Microaerophilic	30	MRS + 0.05% cysteine–hydrochloride + 1% maltose + 0.5% fresh yeast extract
Lactobacillus nasuensis	JCM 17158^T	OHO	*L. casei – manihotivorans*	AZDJ00000000	Sudangrass silage sample	Plant	Anaerobic	30	MRS
Lactobacillus nenjiangensis	LMG 27192^T	OHE	*L. reuteri – vaccinostercus*	n.a.	Pickle	Plant	Microaerophilic	28	MRS
Lactobacillus nodensis	DSM 19682^T	FHE	*L. alimentarius*	AZDZ00000000	Japanese pickles	Food	Microaerophilic	30	MRS
Lactobacillus odoratitofui	DSM 19909^T	OHE	*L. collinoides*	AZEE00000000	Fermented brine used for stinky tofu production	Food	Microaerophilic	30	MRS
Lactobacillus oeni	DSM 19972^T	OHO	*L. salivarius*	AZEH00000000	Bobal wine	Wine product	Microaerophilic	30	MRS
Lactobacillus oligofermentans	DSM 15707^T	OHE	*L. reuteri – vaccinostercus*	AZFE00000000	Broiler leg	Animal	Microaerophilic	25	MRS
Lactobacillus oris	DSM 4864^T	OHE	*L. reuteri – vaccinostercus*	AZGE00000000	Human saliva	Animal	Anaerobic	37	MRS
Lactobacillus oryzae	DSM 26518^T	OHE	*L. collinoides*	BBJM00000000	Fermented rice grain	Food	Anaerobic	30	MRS
Lactobacillus otakiensis	DSM 19908^T	OHE	*L. buchneri*	AZED00000000	Sunki, a Japanese traditional pickle	Food	Microaerophilic	30	MRS
Lactobacillus ozensis	DSM 23829^T	OHE	*L. fructivorans*	AYYQ00000000	Chrysanthemum, Oze National Park	Plant	Microaerophilic	30	MRS
Lactobacillus panis	DSM 6035^T	OHE	*L. reuteri – vaccinostercus*	AZGM00000000	Sourdough	Food	Aerobic	37	MRS
Lactobacillus pantheris	DSM 15945^T	OHO	*L. casei – manihotivorans*	AZFJ00000000	Jaguar faeces	Animal	Microaerophilic	37	MRS
Lactobacillus parabrevis	LMG 11984^T	OHE	*L. brevis*	JQCI00000000	Wheat	Food	Anaerobic	30	MRS
Lactobacillus parabuchneri	DSM 5707^T	OHE	*L. buchneri*	AZGK00000000	Human saliva	Animal	Aerobic	30	MRS
Lactobacillus paracasei subsp. paracasei	DSM 5622^T	FHE	*L. casei – manihotivorans*	AZGH00000000	N/A	Unknwon	Microaerophilic	30	MRS
Lactobacillus paracasei subsp. tolerans	DSM 20258^T	FHE	*L. casei – manihotivorans*	AYYJ00000000	Pasteurized milk	Food	Aerobic	30	MRS
Lactobacillus paracollinoides	DSM 15502^T	OHE	*L. collinoides*	AZFD00000000	Brewery environment	Environment	Anaerobic	25	MRS, pH 5.8
Lactobacillus parafarraginis	LMG 24141^T	FHE	*L. buchneri*	AZFZ00000000	Composting material of distilled shochun residue	Wine product	Microaerophilic	28	MRS

Lactobacillus parakefiri	DSM 10551^T	OHE	*L. buchneri*	AZEN00000000	Kefir grain	Plant	Anaerobic	30	MRS
Lactobacillus paralimentarius	DSM 13238^T	FHE	*L. alimentarius*	AZES00000000	Sourdough	Food	Microaerophilic	30	MRS
Lactobacillus paraplantarum	DSM 10667^T	FHE	*L. plantarum*	AZEO00000000	Beer contaminant	Wine product	Aerobic	30	MRS
Lactobacillus pasteurii	DSM 23907^T	FHE	*L. delbrueckii*	AYZN00000000	N/A	Unknwon	Microaerophilic	37	MRS
Lactobacillus paucivorans	DSM 22467^T	FHE	*L. brevis*	JQCA00000000	Yeast storage tank containing lager beer	Wine product	Anaerobic	28	MRS + 0.05% cysteine–hydrochloride + 1% fructose, pH 5.8
Lactobacillus pentosus	DSM 20314^T	FHE	*L. plantarum*	AZCU00000000	N/A	Unknwon	Microaerophilic	30	MRS
Lactobacillus perolens	DSM 12744^T	FHE	*L. perolens*	AZEC00000000	Orange lemonade	Food	Anaerobic	30	MRS
Lactobacillus plajomi	NBRC 107333^T	FHE	*L. plantarum*	n.a.	Fermented fish	Food	Facultatively anaerobic	30	MRS
Lactobacillus plantarum (former: Lactobacillus arizonensis)	DSM 20174^T	FHE	*L. plantarum*	AZEJ00000000	Pickled cabbage	Food	Aerobic	37	MRS
Lactobacillus plantarum subsp. argentoratensis	DSM 16365^T	FHE	*L. plantarum*	AZFR00000000	Fermented cassava roots (fufu)	Plant	Microaerophilic	30	MRS
Lactobacillus pobuzihii	NBRC 103219^T	FHE	*L. salivarius*	JQCN00000000	Pobuzih (fermented cummingcordia), Cordia dichotoma	Plant	Microaerophilic	37	MRS + 5% NaCl
Lactobacillus pontis	DSM 8475^T	OHE	*L. reuteri – vaccinostercus*	AZGO00000000	Rye sourdough	Food	Aerobic	30	MRS
Lactobacillus porcinae	LMG 26767^T	FHE	*L. casei – manihotivorans*	n.a.	Nem chua (fermented meat)	Food	Microaerophilic	28	MRS
Lactobacillus psittaci	DSM 15354^T	OHE	*L. delbrueckii*	AZFB00000000	Lung of parrott	Animal	Anaerobic	37	MRS
Lactobacillus rapi	DSM 19907^T	OHE	*L. buchneri*	AZEI00000000	Sunki, a Japanese traditional pickle	Food	Microaerophilic	30	MRS
Lactobacillus rennini	DSM 20253^T	FHE	*L. coryniformis*	AYYI00000000	Rennin	Animal	Microaerophilic	30	MRS
Lactobacillus reuteri	DSM 20016^T	OHE	*L. reuteri – vaccinostercus*	AZDD00000000	Intestine of adult	Animal	Aerobic	37	MRS
Lactobacillus rhamnosus	DSM 20021^T	FHE	*L. casei – manihotivorans*	AZCQ00000000	N/A	Unknown	Anaerobic	37	MRS
Lactobacillus rodentium	DSM 24759^T	OHO	*L. delbrueckii*	n.a.	Digestive tract of rodents	Animal	Microaerophilic	37	MRS
Lactobacillus rossiae	DSM 15814^T	OHE	Other	AZFF00000000	Wheat sourdough	Food	Microaerophilic	30	MRS + 1% maltose + 1% yeast extract, pH 5.6
Lactobacillus ruminis	DSM 20403^T	OHO	*L. salivarius*	AYYL00000000	Bovine rumen	Animal	Anaerobic	37	MRS + 2% glucose
Lactobacillus saerimneri	DSM 16049^T	OHO	*L. salivarius*	AZFP00000000	Pig faeces	Animal	Aerobic	37	MRS

Lactobacillus sakei subsp. *carnosus*	DSM 15831^T	FHE	*L. sakei*	AZFG00000000	Fermented meat product	Food	Microaerophilic	37	MRS
Lactobacillus sakei subsp. *sakei*	CECT 4591^T	FHE	*L. sakei*	AZDN00000000	"Moto" starter of sake	Wine product	Aerobic	30	MRS
Lactobacillus salivarius	DSM 20555^T	FHE	*L. salivarius*	AYYT00000000	Saliva	Animal	Microaerophilic	37	MRS
Lactobacillus sanfranciscensis	LMG 16002^T	OHE	*L. fructivorans*	AYYM00000000	Sourdough	Food	Anaerobic	28	MRS + 1% fructose, pH 5.5
Lactobacillus saniviri	DSM 24301^T	FHE	*L. casei – manihotivorans*	JQCE00000000	Faeces of a Japanese healthy adult male	Animal	Microaerophilic	37	MRS
Lactobacillus satsumensis	DSM 16230^T	OHO	*L. salivarius*	AZFQ00000000	Shochu mash	Wine product	Microaerophilic	30	MRS
Lactobacillus secaliphilus	DSM 17896^T	FHE	*L. reuteri – vaccinostercus*	JQBW00000000	Sourdough	Food	Microaerophilic	37	MRS
Lactobacillus selangorensis	ATCC BAA66^T	OHO	Other	JQAT00000000	Chili bo	Food	Aerobic	30	MRS
Lactobacillus senioris	DSM 24302^T	FHE	*L. buchneri*	AYZR00000000	Faeces of a healthy 100–year–old Japanese female	Animal	Microaerophilic	37	MRS
Lactobacillus senmaizukei	DSM 21775^T	FHE	*L. brevis*	AYZH00000000	Senmaizuke, a Japanese pickle	Food	Microaerophilic	30	MRS
Lactobacillus sharpeae	DSM 20505^T	OHO	*L. casei – manihotivorans*	AYYO00000000	Municipal sewage	Environment	Microaerophilic	30	MRS + 0.05% cysteine
Lactobacillus shenzhenensis	DSM 28193^T	OHE	*L. perolens*	AVAA00000000	Fermented dairy beverage	Food	Microaerophilic	37	MRS
Lactobacillus sicerae	KCTC 21012^T	OHO	*L. salivarius*	n.a.	Spanish natural cider	Food	Anaerobic	37	MRS
Lactobacillus silagei	DSM 27022^T	OHE	*L. collinoides*	n.a.	Orchardgrass silage	Plant	Microaerophilic	30	MRS
Lactobacillus siliginis	DSM 22696^T	OHE	Other	JQCB00000000	Wheat sourdough	Food	Anaerobic	37	MRS
Lactobacillus similis	DSM 23365^T	OHE	*L. collinoides*	AYZM00000000	Fermented cane molasses at alcohol plants	Wine product	Microaerophilic	37	MRS
Lactobacillus songhuajiangensis	DSM 28401^T	OHO	*L. casei – manihotivorans*	n.a.	Sourdough	Food	Microaerophilic	28	MRS
Lactobacillus spicheri	DSM 15429^T	FHE	*L. brevis*	AZFC00000000	Rice sourdough	Food	Microaerophilic	30	MRS, pH 5.8
Lactobacillus sucicola	DSM 21376^T	OHO	*L. salivarius*	AYZF00000000	Sap of an Oak tree	Plant	Microaerophilic	30	MRS
Lactobacillus suebicus	DSM 5007^T	OHE	*L. reuteri – vaccinostercus*	AZGF00000000	Apple mash	Food	Microaerophilic	30	MRS
Lactobacillus sunkii	DSM 19904^T	OHE	*L. buchneri*	AZEA00000000	Sunki, a Japanese traditional pickle	Food	Microaerophilic	30	MRS
Lactobacillus taiwanensis	DSM 21401^T	OHO	*L. delbrueckii*	AYZG00000000	Silage cattle feed	Plant	Microaerophilic	37	MRS
Lactobacillus thailandensis	DSM 22698^T	OHO	*L. casei – manihotivorans*	AYZK00000000	Fermented tea leaves (miang)	Plant	Microaerophilic	30	MRS

Lactobacillus tucceti	DSM 20183^T	OHO	*L. alimentarius*	AZDG00000000	Sausage	Food	Aerobic	30	MRS
Lactobacillus ultunensis	DSM 16047^T	OHO	*L. delbrueckii*	AZFO00000000	Gastric biopsies, Human stomach mucosa	Animal	Anaerobic	37	MRS
Lactobacillus uvarum	DSM 19971^T	OHO	*L. salivarius*	AZEG00000000	Must of Bobal grape variety	Plant	Microaerophilic	30	MRS
Lactobacillus vaccinostercus	DSM 20634^T	OHE	*L. reuteri – vaccinostercus*	AYYY00000000	Cow dung	Animal	Microaerophilic	30	MRS
Lactobacillus vaginalis	LMG 12891^T	OHE	*L. reuteri – vaccinostercus*	AZGL00000000	Vaginal swab	Animal	Anaerobic	28	MRS
Lactobacillus versmoldensis	DSM 14857^T	OHO	*L. alimentarius*	AZFA00000000	Poultry salami	Food	Microaerophilic	30	MRS
Lactobacillus vespulae	DSM 103408^T	OHE	*L. fructivorans*	n.a.	Gut of queen wasp	Animal	Microaerophilic	30	MRS
Lactobacillus vini	DSM20605^T	FHE	*L. salivarius*	AYYX00000000	Must of grape	Plant	Aerobic	37	MRS
Lactobacillus wasatchensis	LMG 28678^T	OHE	*L. reuteri – vaccinostercus*	AWTT00000000	Cheddar cheese	Food	Anaerobic	25	MRS
Lactobacillus xiangfangensis	LMG 26013^T	FHE	*L. plantarum*	JQCL00000000	Pickles	Food	Aerobic	30	MRS
Lactobacillus yonginensis	DSM 29216^T	FHE	*L. brevis*	n.a.	Kimchi	Food	Microaerophilic	30	MRS
Lactobacillus zeae	DSM 20178^T	FHE	*L. brevis*	AZCT00000000	Corn steep liquor	Wine product	Anaerobic	37	MRS
Lactobacillus zymae	DSM 19395^T	OHE	*L. casei – manihotivorans*	AZDW00000000	Artisanal wheat sourdough	Food	Microaerophilic	30	MRS + 0.05% cysteine, pH 5.2

OHO: obligately homofermentative; FHE: facultatively heterofermentative; OHE: obligately heterofermentative.

n.a.: not available.

Table S5.2. Distribution of antibiotic MIC values for the 182 type strains of the genus *Lactobacillus* based on the phylogroups described by Sun *et al*. (2015). Areas with white backgrounds indicate the range of dilutions tested for each antibiotic. MICs above the range exceed the highest concentration tested and they are reported in bold.

Antibiotic	Phylogroup	No. of strains with the following MICs (µg/mL)																	
		0.016	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**>1024**	**Total**
Gentamycin	*L. alimentarius*						4	2	3	4	2				1		**3**			19
	L. brevis							2	1	1	1	1	1	1	2	1				11
	L. buchneri						4	4	1	2		1								12
	L. casei–manihotivorans							2	2	6		1	2	1		1				15
	L. collinoides						1		3			1	2							7
	L. coryniformis								2	2										4
	L. delbrueckii						4	11	10	10	1	1								37
	L. fructivorans							1	2			3	1			1				8
	L. perolens								3											3
	L. plantarum						1	1	1	2	2		1				**2**			10
	L. reuteri–vaccinostercus						4	3	5	2	1	1				1	**1**			18
	L. sakei						1			1		3								5
	L. salivarius						3	5	4	1	4	5	2	1	1		**1**			27
	Other						2		1	2		1								6
	All						24	31	38	33	11	18	9	3	4	4	**7**			182
Kanamycin	*L. alimentarius*										1	3	2	2	5	2			**4**	19
	L. brevis											1	2		1	1	2	4		11
	L. buchneri									1	2	2	2	2	2		1			12
	L. casei–manihotivorans											2	2	2	5	2	1	1		15
	L. collinoides											1		2	1	1	1		**1**	7
	L. coryniformis												1	2	1					4
	L. delbrueckii									3	3	9	6	7	8		1			37
	L. fructivorans												2	1	1	1	2		**1**	8

	L. perolens							2	1								3
	L. plantarum							1	2	2	1	1	1		**2**		10
	L. reuteri–vaccinostercus				1		1	4	1	4	3		2		**2**		18
	L. sakei				1			1	1	1	1						5
	L. salivarius				1	1	1	1	8	1	2	1	7	3	**1**		27
	Other							1	2	2			1				6
	All				3	5	8	28	32	28	31	9	19	8	**11**		182
Streptomycin	*L. alimentarius*						2	4	6	1	2		**4**				19
	L. brevis							3		2	1	2	**3**				11
	L. buchneri				2	1	2	4	1				**2**				12
	L. casei–manihotivorans							5	2	4	3	1					15
	L. collinoides						1	1	2		2		**1**				7
	L. coryniformis							1	2		1						4
	L. delbrueckii		2	7	6	7	5	3	2		1		**4**				37
	L. fructivorans					1	2		1	1	2		**1**				8
	L. perolens			1		2											3
	L. plantarum					2	1	3	1	1			**2**				10
	L. reuteri–vaccinostercus			1		1	6	5	1	2			**2**				18
	L. sakei				1			1		3							5
	L. salivarius			1	4	1	6	1	6	1	1		**6**				27
	Other					1	4	1									6
	All		2	12	12	20	42	27	18	17	7		**25**				182
Neomycin	*L. alimentarius*	1	1	4	4	1	3	1					**4**				19
	L. brevis		1		2	1	1	1		1	4						11
	L. buchneri	2	3	2	2	3											12
	L. casei–manihotivorans		1	1	1	2	2	1	4	1	2						15
	L. collinoides	1		1	2	1	1		1								7
	L. coryniformis				2		2										4
	L. delbrueckii	2	2	6	9	7	7	2	1	1							37

	L. fructivorans					1		1	1	1	2		1		**1**		8
	L. perolens							2		1							3
	L. plantarum						2	2		2	1	1		1	**1**		10
	L. reuteri–vaccinostercus				2	4	2	2	3	1		2		1	**1**		18
	L. sakei				1					1	2	1					5
	L. salivarius				2	3	1	4	3	3	2	6	2		**1**		27
	Other						2		2	1	1						6
	All				11	16	21	33	24	26	13	16	6	8	**8**		182
Tetracycline	*L. alimentarius*							8	7	3			**1**				19
	L. brevis								1	2	8						11
	L. buchneri									2	4	4	**2**				12
	L. casei–manihotivorans					2	6		3	3		1					15
	L. collinoides								1	5			**1**				7
	L. coryniformis				1	1	1				1						4
	L. delbrueckii				1	1	8	11	10	2		1	**3**				37
	L. fructivorans								2		3	2	**1**				8
	L. perolens			1		2											3
	L. plantarum							1	1	2	5	1					10
	L. reuteri–vaccinostercus					1	2		3	2	9		**1**				18
	L. sakei				1		1	2		1							5
	L. salivarius				2	5	11	4	1	1	1	1	**1**				27
	Other					1	2	1			1	1					6
	All			1	5	13	31	27	29	23	32	11	**10**				182
Erythromycin	*L. alimentarius*	1	6	2	1	3	1		4	**1**							19
	L. brevis		1	1	2	2	1	1	1	**2**							11
	L. buchneri		3	3	3	1		2									12
	L. casei–manihotivorans	1		6	6	1			1								15
	L. collinoides		2	2			2		1								7
	L. coryniformis			1		1		1	1								4

	L. delbrueckii	1	3	11	18	1	1		1			**1**				37
	L. fructivorans			1	4		1	1	1							8
	L. perolens				1		2									3
	L. plantarum					5	2	1			1	**1**				10
	L. reuteri–vaccinostercus	1			6	4	3	1		2		**1**				18
	L. sakei			1	3	1										5
	L. salivarius			1	6	8	5	2		1	1	**3**				27
	Other				2	2		2								6
	All	2	3	16	52	36	26	15	6	7	10	**9**				182
Clindamycin	*L. alimentarius*			5	4		3	3	2	1			**1**			19
	L. brevis					1		1	1	1	1	1	**5**			11
	L. buchneri		4	3	1		1	2	1							12
	L. casei–manihotivorans			1	4	5	3		1			1				15
	L. collinoides		1	2			2	1	1							7
	L. coryniformis			1	2	1										4
	L. delbrueckii			8	9	7	5		3	3	1		**1**			37
	L. fructivorans		1	4	1	2										8
	L. perolens				1				1			1				3
	L. plantarum		1			2	1		1	2		1	**2**			10
	L. reuteri–vaccinostercus		4	5	3	3	2						**1**			18
	L. sakei		1	1	1	1	1									5
	L. salivarius			7	10	4	5		1							27
	Other			1	1	1		1		1	1					6
	All		12	38	37	27	23	8	12	8	3	4	**10**			182
Chloramphenicol	*L. alimentarius*								2	7	9		1			19
	L. brevis								1	1	6	3				11
	L. buchneri									5	6	1				12
	L. casei–manihotivorans									2	8	4		1		15
	L. collinoides									2	3	2				7

	L. coryniformis				1				1		2							4
	L. delbrueckii								3	22	11	1						37
	L. fructivorans							1	1	5	1							8
	L. perolens									1	2							3
	L. plantarum									2	7	1						10
	L. reuteri–vaccinostercus						1		2	4	9	2						18
	L. sakei								3	2								5
	L. salivarius				1				2	14	8	2						27
	Other								1	1	2	2						6
	All				2		1	1	16	68	74	18	1	1				182
Ampicillin	*L. alimentarius*				1		1	1	12	3	1							19
	L. brevis								1	4	5		**1**					11
	L. buchneri					1		4	5	2								12
	L. casei–manihotivorans						4	3	2	4	1	1						15
	L. collinoides							3		2	1		**1**					7
	L. coryniformis					1	1	2										4
	L. delbrueckii			2	2	7	6	15	4	1								37
	L. fructivorans		1			1	3	2	1									8
	L. perolens					2	1											3
	L. plantarum				1		3	1	4				**1**					10
	L. reuteri–vaccinostercus		1			1	7	7	2									18
	L. sakei							1	3	1								5
	L. salivarius		1		1	3	4	5	6	2	4	1						27
	Other				1	1	1	2				1						6
	All		3	2	6	17	31	46	40	19	12	3	**3**					182
Penicillin G	*L. alimentarius*					1	5	10	3									19
	L. brevis								1		7	1	**2**					11
	L. buchneri			1		2	3	3	1	1	1							12
	L. casei–manihotivorans					4	3	4	3			1						15

	L. collinoides					2	2	2				**1**						7
	L. coryniformis				2	1	1											4
	L. delbrueckii		6	5	18	4	2	1	1									37
	L. fructivorans	1	2	2	1	1	1											8
	L. perolens			1	2													3
	L. plantarum				1	2	2	1	2	1		**1**						10
	L. reuteri–vaccinostercus	1			5	5	3	3	1									18
	L. sakei					1	3	1										5
	L. salivarius	1	2	2	4	2	5	6	2	2		**1**						27
	Other				2	2	1					**1**						6
	All	3	11	10	42	31	37	22	7	11	2	**6**						182
Vancomycin	*L. alimentarius*											1			**18**			19
	L. brevis												1		**10**			11
	L. buchneri														**12**			12
	L. casei–manihotivorans												1	3	**11**			15
	L. collinoides														**7**			7
	L. coryniformis								1						**3**			4
	L. delbrueckii				1	16	17					1	1		**1**			37
	L. fructivorans				1										**7**			8
	L. perolens													1	**2**			3
	L. plantarum														**10**			10
	L. reuteri–vaccinostercus										1			1	**16**			18
	L. sakei												2		**3**			5
	L. salivarius						1		1		1	1		4	**19**			27
	Other						1								**5**			6
	All				2	16	19		2		2	3	5	9	**124**			182
Quinupristin–dalfopristin	*L. alimentarius*					2	8	5	3	1								19
	L. brevis						1	5	5									11
	L. buchneri						2	4	4	2								12

	L. casei–manihotivorans					6	3	4	2						15
	L. collinoides				1	1	3	1	1						7
	L. coryniformis					3		1							4
	L. delbrueckii			4	6	17	7	3							37
	L. fructivorans	1		1	2	3		1							8
	L. perolens				1	1	1								3
	L. plantarum					2	4	2	2						10
	L. reuteri–vaccinostercus			1	1	6	5	4		**1**					18
	L. sakei					2	3								5
	L. salivarius			1	2	16	3	4	1						27
	Other			1		2	3								6
	All	1		8	15	70	46	32	9	**1**					182
Linezolid	*L. alimentarius*					1	11	6	1						19
	L. brevis						1	1	9						11
	L. buchneri						2	8	2						12
	L. casei–manihotivorans						4	6	3	2					15
	L. collinoides					1	2	3	1						7
	L. coryniformis					1	2		1						4
	L. delbrueckii				1	5	13	15	3						37
	L. fructivorans		1		1		4	2							8
	L. perolens						2	1							3
	L. plantarum						6	2	2						10
	L. reuteri–vaccinostercus			1		2	3	7	5						18
	L. sakei					1	4								5
	L. salivarius				1	8	17	3							27
	Other					1	1	3	1						6
	All		1	1	3	20	70	57	28	2					182
Trimethoprim	*L. alimentarius*					1						**18**			19
	L. brevis											**11**			11

	L. buchneri						1				1		**10**						12
	L. casei–manihotivorans						2		1			2	**10**						15
	L. collinoides												**7**						7
	L. coryniformis						1					1	**2**						4
	L. delbrueckii			1						1	1	6	**28**						37
	L. fructivorans		2			2	1					1	**2**						8
	L. perolens		2			1													3
	L. plantarum							1	1				**8**						10
	L. reuteri–vaccinostercus			1								1	**16**						18
	L. sakei											1	**4**						5
	L. salivarius		2		1		1	4	3	1		1	**14**						27
	Other							1		1		1	**3**						6
	All		6	1	2	4	6	6	5	3	2	14	**133**						182
Ciprofloxacin	*L. alimentarius*							3	4	2	5	2	2	**1**					19
	L. brevis								2	1		1		**7**					11
	L. buchneri								1	2	5	3		**1**					12
	L. casei–manihotivorans						2	4	5		3		1						15
	L. collinoides									4		2	1						7
	L. coryniformis				1			2		1									4
	L. delbrueckii					1		1	3	4	4	14	9	**1**					37
	L. fructivorans			1					1	1	3	1		**1**					8
	L. perolens					1	2												3
	L. plantarum									3	2	1	2	**2**					10
	L. reuteri–vaccinostercus				1				1	1	8	3	3	**1**					18
	L. sakei								1	4									5
	L. salivarius			1	2	3	9	5	2	4	1								27
	Other						1	2	1		1		1						6
	All			2	4	5	14	17	21	27	32	27	19	**14**					182

Rifampicin	*L. alimentarius*		5	5	3		1				3	2			19
	L. brevis				5	3	1	1		1					11
	L. buchneri		1	4	4	1	1	1							12
	L. casei–manihotivorans		1	4	5	1	2	1				1			15
	L. collinoides		3		2		2								7
	L. coryniformis		1		1	2									4
	L. delbrueckii			2	4	11	9	6	2	1		1	**1**		37
	L. fructivorans		1	1	3	1	1	1							8
	L. perolens		1	1	1										3
	L. plantarum		1	1	1	5	1	1							10
	L. reuteri–vaccinostercus		4	4	3	4	2						**1**		18
	L. sakei		2	3											5
	L. salivarius		2	3	9	10	2	1							27
	Other		1		2	2	1								6
	All		23	28	43	40	23	12	2	2	3	4	**2**		182

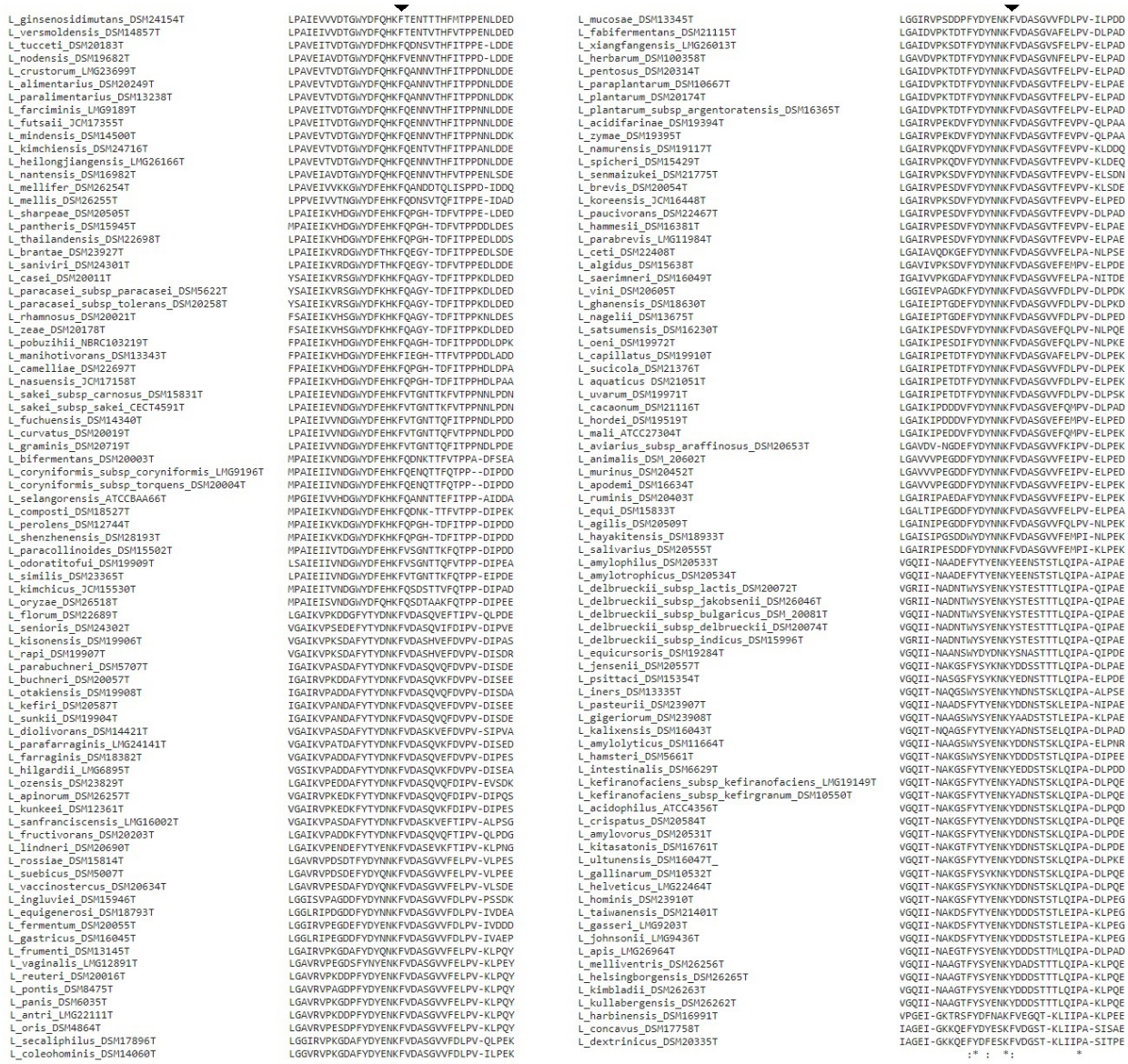

Figure S5.1. Alignment of the amino acid sequence of D–Ala–D–Ala ligase of the 161 *Lactobacillus* strains. The substitution of the tyrosine (Y) at position 261, indicated with the arrow, with a phenylalanine (F) results in the vancomycin-resistance phenotype.

Chapter 6

Overall conclusions

The ever-increasing magnitude of antibiotic resistance (AR) is a global public health challenge. Indeed, the overprescribing of antibiotics has tremendously raised the emergence and spread of antibiotic resistant bacteria in the food chain (Verraes *et al*., 2013). Food may act as a vector for the transfer of AR bacteria and resistance genes to humans. The issue of AR in foodstuffs is addressed either as a direct or an indirect hazard. The first is associated to the presence in food of resistant pathogenic bacterium, which can be transmitted to people and cause infectious illness by ingestion or handling contaminated food. While, the indirect hazard to human health is linked to the horizontal transfer of mobile genetic elements from non-pathogenic bacteria, such as commensal, probiotic and technological strains, to pathogenic bacteria (EFSA, 2008). These events can occur anywhere throughout the food chain, including environment, food producing animals, food-industry surfaces, in foods or in the human body (Capita and Calleja, 2011).

For these reasons, the absence of acquired resistance factors in a candidate probiotic or starter culture should be determined prior to approval for Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (EFSA, 2012).

Lactic acid bacteria (LAB) have been extensively used as probiotics and starter cultures due to their long history of safe use and several strains having the QPS status (Casado Muñoz *et al*., 2016; Kechagia *et al*., 2013), which include members of the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* (Ricci *et al*., 2017).

However, very limited information on the antimicrobial susceptibility profiles of *Leuconostoc* spp. is available, as well as their possible involvement in the dissemination of AR determinants between bacteria. Regarding the genus *Lactobacillus*, even though more than 35 species meet the criteria of QPS proposed by EFSA, a considerable number of resistant lactobacilli has been reported.

In the present study, the antimicrobial susceptibility profiles and the genetic basis of the resistance were investigated for both the genera *Leuconostoc* and *Lactobacillus* through the

application of either standard methods, such as phenotypic testing and molecular techniques, and whole-genome sequencing (WGS)-based approaches.

In the first part of the dissection, resistance to different antibiotics was detected among 32 strains of the genus *Leuconostoc* isolated from traditional Italian and Spanish cheeses, of which three *L. mesenteroides* strains exhibited multidrug resistance (MDR). In detail, these strains displayed atypical resistance to erythromycin and clindamycin (LbE15), kanamycin, streptomycin, tetracycline and virginiamycin (LbE16), and tetracycline (LbT16). The application of conventional molecular techniques, such as PCR, allowed to identify the presence of the genes *erm*(B) and *tet*(S) in the erythromycin resistant LbE15 strain and in the tetracycline resistant LbE16 strain, respectively. Moreover, conjugation experiments *in vitro* and in cheese provide the first evidence of the erythromycin resistance transfer between *L. mesenteroides* and *E. faecalis*, supplying novel proof that AR LAB can act as a reservoir of acquired AR genes.

However, the genes *erm*(B) and *tet*(S) can only partially explain the MDR phenotypes showed by these three *L. mesenteroides* strains.

Therefore, a WGS-based analysis was performed for these strains, which revealed the presence of genes coding for aminoglycoside resistance, such as *aad6*, *sat*4 and *aphA*-3, and for streptogramin A resistance, as *vat*E, in the genome sequence of LbE16. Thus, these genes represented the genetic basis of the resistance towards kanamycin, streptomycin and virginiamycin displayed by LbE16.

Moreover, the WGS approach revealed for the first time the presence of a *erm*(B)-bearing *Tn*917 transposon in the genome of *L. mesenteroides* subsp. *dextranicum* LbE15, which showed high similarity (99%) to the nucleotide sequence of the corresponding transposon of *E. faecalis*, *B. subtilis*, *S. aureus*, and several species of *Streptococcus*.

Therefore, the WGS appear to be more informative than conventional molecular techniques, providing data about any resistance gene or mutation present in a single microbial genome (Chan, 2016).

To verify the effectiveness of the WGS as a tool for surveillance of AR, genomic analysis was performed for the available genome sequences of the type strains of the genus *Leuconostoc*.

This analysis revealed the presence of 192 gene sequences putatively associated with the resistance to the main important antibiotics used in medicine. Since, the prediction of gene function through automatic annotation increases the number of false positives AR genes in growing databases, a manual annotation of the putative AR determinants identified was carried out, confirming the actual involvement in the AR of only five gene sequences, which code for the resistance towards streptomycin and clindamycin. In particular, the gene *lsa*A was found for

the first time in *L. pseudomesenteroides* LMG 11482^T and *L. fallax* LMG 13177^T and its role in the clindamycin and quinupristin-dalfopristin resistance phenotype characterizing *L. pseudomesenteroides* strains was confirmed through the relative quantification of the gene expression. Moreover, the sequence analysis of the gene coding for the enzyme D-Ala-D-Ala ligase revealed the presence of a phenylalanine at position 261, which was previously linked to the peculiar intrinsic vancomycin resistance characterizing all members of the genus *Leuconostoc*.

The antibiotic susceptibility testing was performed for several antibiotics to confirm the resistance features predicted through the genome sequences analysis, revealing a positive correlation between genotype and phenotype for the 74% of the cases examined. However, the presence of some resistance phenotypes not associated with particular genetic determinants emphasizes the requirement of deeper studies focused on the identification of novel genes involved in AR for LAB.

Therefore, in the last part of the dissection, the WGS-based analysis of the AR was performed for the whole genus *Lactobacillus*, which has a crucial economic and scientific impact in food productions and human health as probiotics and starter cultures. Moreover, the availability of the genome sequence of almost all the type strains of the genus *Lactobacillus* (Sun *et al*., 2015, Zheng *et al*., 2015) offers an unprecedented advantage for the safety assessment of this genus.

In particular, the combination of phenotypic susceptibility testing and genome-based analysis revealed a positive correlation between phenotype and genotype for the 67% of the cases examined. Where, the resistance towards trimethoprim, vancomycin and kanamycin represented the most common phenotypes observed among lactobacilli. While, the analysis of the genome sequences identified the presence of genes encoding for resistance to aminoglycosides (20 sequences), tetracycline (18), erythromycin (6), clindamycin (60), and chloramphenicol (42). In particular, the genes *aac*(3), *lsa* and *cml*(A) involved in the resistance towards aminoglycoside, clindamycin and chloramphenicol, respectively, were found for the first time in *Lactobacillus* strains. In addition, acquired determinants coding for tetracycline and erythromycin resistance were simultaneously detected in *L. amylophilus* DSM 20533^T and *L. amylotrophicus* DSM 20534^T. Whereas, *L. ingluviei* DSM 15946^T harboured the *Tn*916-like transposon carrying the genes *tet*(M) and *tet*(L), highlighting the potential of these AR genes to be horizontally transferred to other microorganisms.

The results reported in this study may be utilized as a starting point for the generation of new and more focused scientific protocols and regulatory procedures based on WGS approaches for the

safety assessment of *Leuconostoc* and *Lactobacillus* strains employed as starter cultures, food preservatives or probiotic by food and probiotic stakeholders.

The implementation of AR genes available for LAB could result in a paradigm shift from phenotype- to genotype-based assessment of the resistance not only for pathogens but also for food-borne and technological bacteria. However, sequenced-based prediction of phenotypic resistance should be made with caution: manual curation and eventual re-annotation of the results obtained after homology-based methods, such as BLAST, against a set of AR reference sequences has a relevant importance in the minimisation of putative false negative or false positive outputs, thus getting the best correlation between phenotypic data and detection of AR genes. In addition, due to the increase number of AR database, the selection of the reference database represents another fundamental step of the WGS-based analysis. In this study, CARD was selected as the most complete and manually curated database.

Moreover, the absence of resistance gene does not preclude the possibility of susceptibility to a specific antibiotic, as any new resistances that are not included in AR database might have been missed. Therefore, data generated from WGS-based analysis still need to be confirmed with phenotypic susceptibility testing in order to distinguish between sensitive and resistant bacteria. On the other hand, functional studies aiming at identifying new genetic determinants for resistance, combining data derived from analysing DNA sequence, gene expression, and protein function should be performed to enrich AR databases. Moreover, RNAseq technologies can reveal the genetic expression of any determinants involved in the resistance, representing a novel technique for the discovery of resistance mechanisms for specific antibiotics, which may also allow to develop new strategies to over-come the AR crisis.

In conclusion, WGS-based approaches can be used as a tool for the surveillance of the emergence and spread of AR determinants in bacteria, providing: (i) an important initial contribution for the identification of genes potentially associated with resistance; and (ii) relevant information about the possibility of AR genes to be spread along the food chain.

References

Aarts H and Margolles A. 2015. Antibiotic resistance genes in food and gut (non-pathogenic) bacteria. Bad genes in good bugs. Front Microbiol. 5, 754

Abriouel H, Casado Muñoz MDC, Lavilla Lerma L, Pérez Montoro B, Bockelmann W, Pichner R, Kabisch J, Cho GS, Franz CMAP, Gálvez A, Benomar N. 2015. New insights in antibiotic resistance of *Lactobacillus* species from fermented foods. Food Res Int. 78, 465–481

Acar J and Röstel B. 2001. Antimicrobial resistance: an overview. Rev Sci Tech. 20, 797–810

Adimpong DB, Nielsen DS, Sørensen KI, Derkx PM, Jespersen L. 2012. Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. BMC Microbiol. 12, 1–12

Adu-Oppong B, Gasparrini AJ, Dantas G. 2017. Genomic and functional techniques to mine the microbiome for novel antimicrobials and antimicrobial resistance genes. Ann NY Acad Sci. 1388, 42–58

Ager S and Gould K. 2012. Clinical update on linezolid in the treatment of Gram-positive bacterial infections. Infect Drug Resist. 5, 87–102

Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. 1992. DNA diversity among clinical isolates of Helicobacter pylori detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. 20, 5137–5142

Albanese A, Spanu T, Sali M, Novegno F, D'Inzeo T, Santangelo R, Mangiola A, Anile C, Fadda G. 2006. Molecular identification of *Leuconostoc mesenteroides* as a cause of brain abscess in an immunocompromised patient. J Clin Microbiol. 44, 3044–3045

Alegría Á, Delgado S, Flórez AB, Mayo B. 2013. Identification, typing, and functional characterization of *Leuconostoc* spp. strains from traditional, starter-free cheeses. Dairy Sci & Technol. 93, 657–673

Alekshun MN and Levy SB. 2007. Molecular mechanisms of antibacterial multidrug resistance. Cell. 128, 1037–1050

Ali Y, Kot W, Atamer Z, Hinrichs J, Vogensen FK, Heller KJ, Neve H. 2013. Classification of lytic bacteriophages attacking dairy *Leuconostoc* starter strains. Appl Environ Microbiol. 79, 3628–3636

Alifano P, Palumbo C, Pasanisi D, Talà A. 2015. Rifampicin-resistance, *rpo*B polymorphism and RNA polymerase genetic engineering. J Biotechnol. 202, 60–77

Aminov R. 2017. History of antimicrobial drug discovery: Major classes and health impact. Biochem Pharmacol. 133, 4–19

Aminov RI. 2011. Horizontal gene exchange in environmental microbiota. Front Microbiol. 2, 1–19

Ammor MS, Gueimonde M, Danielsen M, Zagorec M, van Hoek A, de los Reyes-Gavilán CG, Mayo B, Margolles A. 2008. Two Different Tetracycline Resistance Mechanisms, Plasmid-Carried *tet*(L) and Chromosomally Located Transposon-Associated *tet*(M), Coexist in *Lactobacillus sakei* Rits 9. Appl Environ Microbiol. 74, 1394–1401

Ammor MS, Flórez AB, Mayo B. 2007. Antibiotic resistance in nonenterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24, 559–570

Andersson DI and Hughes D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol. 8, 260–271

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics. 9, 1–15

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 6, 71–79

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 19, 455–477

Barletta J, Estrada T, Rolón MJ, Erbin M, Kaufman S, Pérez H. 2017. Meningitis due to *Leuconostoc mesenteroides* associated with central nervous system tuberculosis: a case report. Ann Clin Case Rep. 2, 1228

Bautista-Gallego J, Arroyo-López FN, Rantsiou K, Jiménez-Díaz R, Garrido-Fernández A, Cocolin L. 2013. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. Food Res Int. 50, 135–142

Beganović J, Pavunc AL, Gjuračić K, Špoljarec M, Šušković J, Kos B. 2011. Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. J Food Sci. 76, 124–129

Bellanger X, Payot S, Leblond-Bourget N, Guédon G. 2014. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. FEMS Microbiol Rev. 38, 720–760

Belletti N, Gatti M, Bottari B, Neviani E, Tabanelli G, Gardini F. 2009. Antibiotic resistance of lactobacilli isolated from two italian hard cheeses. J Food Prot. 72, 2162–2169

Benmechernene Z, Chentouf HF, Yahia B, Fatima G, Quintela-Baluja M, Calo-Mata P, Barros-Velázquez J. 2013. Technological Aptitude and Applications of *Leuconostoc mesenteroides* Bioactive Strains Isolated from Algerian Raw Camel Milk. BioMed Research International. 2013, 1–14

Billington SJ and Jost BH. 2006. Multiple Genetic Elements Carry the Tetracycline Resistance Gene *tet*(W) in the Animal Pathogen *Arcanobacterium pyogenes*. Antimicrob Agents Chemother. 50, 3580–3587

Björkroth J and Holzapfel W. 2006. Genera *Leuconostoc*, *Oenococcus* and *Weissella*. In: The Prokaryotes: a Handbook on the Biology of Bacteria. 3rd ed. Dworkin M, Falkow S, Rosemberg E, Schleifer KH, Stackebrandt E, New York, USA, Springer. 4, 267–319

Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. 2015. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 13, 42–51

Blanco P, Hernando-Amado S, Reales-Calderon JA, Corona F, Lira F, Alcalde-Rico M, Bernardini A, Sanchez MB, Martinez JL. 2016. Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. Microorganisms. 4, 1–19

Botina SG, Poluektova EU, Glazova AA, Zakharevich NV, Koroban NV, Zinchenko VV, Babykin MM, Zhilenkova OG, Amerkhanova AM, Danilenko VN. 2011. Antibiotic

resistance of potential probiotic bacteria of the genus *Lactobacillus* from human gastrointestinal microbiome. Microbiology. 80, 164

Breidt F. 2004. A genomic study of *Leuconostoc mesenteroides* and the molecular ecology of sauerkraut fermentations. J Food Sci. 69, 30–32

Bryan LE and Kwan S. 1981. Mechanisms of aminoglycoside resistance of anaerobic bacteria and facultative bacteria grown anaerobically. J Antimicrob Chemother. 8, SD1-SD8

Bou G, Luis Saleta J, Sáez Nieto JA, Tomás M, Valdezate S, Sousa D, Lueiro F, Villanueva R, Jose Pereira M, Llinares P. 2008. Nosocomial Outbreaks Caused by *Leuconostoc mesenteroides* subsp. *mesenteroides*. Emerg Infect Dis. 14, 968–971

Capita R and Alonso-Calleja C. 2013. Antibiotic-resistant bacteria: a challenge for the food industry. Crit Rev Food Sci Nutr. 53, 11–48

Carver T, Thomson N, Bleasby A, Berriman M, Parkhill J. 2009. DNAPlotter: circular and linear interactive genome visualization. Bioinformatics. 25, 119–120

Casado Muñoz MC, Benomar N, Lavilla Lerma L, Knapp CW, Gálvez A, Abriouel H. 2016. Biocide tolerance, phenotypic and molecular response of lactic acid bacteria isolated from naturally-fermented Aloreña table to different physico-chemical stresses. Food Microbiol. 60, 1–12

Casado Muñoz MC, Benomar N, Lerma LL, Gálvez A, Abriouel H. 2014. Antibiotic resistance of *Lactobacillus pentosus* and *Leuconostoc pseudomesenteroides* isolated from naturally–fermented Aloreña table olives throughout fermentation process. Int J Food Microbiol. 172, 110–118

Cassir N, Rolain JM, Brouqui P. 2014. A new strategy to fight antimicrobial resistance: the revival of old antibiotics. Front Microbiol. 5, 1–15

Chan K. 2016. Whole-genome sequencing in the prediction of antimicrobial resistance. Expert Review of Anti-infective Therapy. 14

Chen YS, Miyashita M, Suzuki K, Sato H, Hsu JS, Yanagida F. 2010. *Lactobacillus pobuzihii* sp. nov., isolated from pobuzihi (fermented cummingcordia). Int J Syst Evol Microbiol. 60, 1914–1917

Chopra I and Roberts M. 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol Mol Biol Rev. 65, 232–260

Chun BH, Kim KH, Jeon HH, Lee SH, Jeon CO. 2017. Pan-genomic and transcriptomic analyses of *Leuconostoc mesenteroides* provide insights into its genomic and metabolic features and roles in kimchi fermentation. Sci Rep. 7, 1–16

Cibik R, Lepage E, Talliez P. 2000. Molecular diversity of *Leuconostoc mesenteroides* and *Leuconostoc citreum* isolated from traditional french cheeses as revealed by RAPD fingerprinting, 16S rDNA sequencing and 16S rDNA fragment amplification. Syst Appl Microbiol. 3, 267–278

Clewell DB, Flannagan SE, Jaworski DD. 1995. Unconstrained bacterial promiscuity: the *Tn*916-*Tn*1545 family of conjugative transposons. Trends Microbiol. 3, 229–236

Clinical and Laboratory Standards Institute (CLSI). 2001. Performance standards for antimicrobial susceptibility testing; 21st informational supplement. CLSI M100–S21. no 1, vol. 31. Wayne, PA

Cocconcelli PS, Cattivelli D, Gazzola S. 2003. Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. Int J Food Microbiol. 88, 315–323

Cockerill FR. 1999. Genetic Methods for Assessing Antimicrobial Resistance. Antimicrob Agents Chemother. 43, 199–212

Comunian R, Daga E, Dupré I, Paba A, Devirgiliis C, Piccioni V, Perozzi G, Zonenschain D, Rebecchi A, Morelli L, De Lorentiis A, Giraffa G. 2009. Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. Int J Food Microbiol. 138, 151–156

Coppola R, Succi M, Tremonte P, Reale A, Salzano G, Sorrentino E. 2005. Antibiotic susceptibility of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. Le Lait. 85, 193–204

Courvalin P. 2006. Vancomycin resistance in Gram-positive cocci. Clin Infect Dis. 42, S25–S34

Crofts TS, Gasparrini AJ, Dantas G. 2017. Next-generation approaches to understand and combat the antibiotic resistome. Nat Rev. Microbiol. 15, 422–434

Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, McGee L, von Gottberg A, Song JH, Ko KS, Pichon B, Baker S, Parry CM, Lambertsen LM, Shahinas D, Pillai DR, Mitchell TJ, Dougan G, Tomasz A, Klugman KP, Parkhill J, Hanage WP, Bentley SD. 2011. Rapid pneumococcal evolution in response to clinical interventions. Science. 331, 430–434

D'Aimmo MR, Modesto M, Biavati B. 2007. Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. Int J Food Microbiol. 115, 35–42

Dale GE, Broger C, D'Arcy A, Hartman PG, DeHoogt R, Jolidon S, Kompis I, Labhardt AM, Langen H, Locher H, Page MG, Stüber D, Then RL, Wipf B, Oefner C. 1997. A single amino acid substitution in *Staphylococcus aureus* dihydrofolate reductase determines trimetoprim resistance. J Mol Bio. 266, 23–30

Dan T, Liu W, Sun Z, Lv Q, Xu H, Song Y, Zhang H. 2014. A novel multi-locus sequence typing (MLST) protocol for *Leuconostoc lactis* isolates from traditional dairy products in China and Mongolia. BMC Microbiol. 14, 1–9

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

Danielsen M, Andersen HS, Wind A. 2004. Use of folic acid casei medium reveals trimethoprim susceptibility of *Lactobacillus* species. Lett Appl Microbiol. 38, 206–210

Danilchanka O, Pavlenok M, Niederweis M. 2008. Role of porins for uptake of antibiotics by *Mycobacterium smegmatis*. Antimicrob Agents Chemother. 52, 3127–3134

Davies SC. 2013. Annual Report of the Chief Medical Officer, Volume Two, 2011, Infections and the rise of antimicrobial resistance. London: Department of Health. 1–154

Davies J and Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 74, 417–433

De Bruyne K, Schillinger U, Caroline L, Boehringer B, Cleenwerck I, Vancanneyt M, De Vuyst L, Franz CM, Vandamme P. 2007. *Leuconostoc holzapfelii* sp. nov., isolated from Ethiopian coffee fermentation and assessment of sequence analysis of housekeeping genes for delineation of *Leuconostoc* species. Int J Syst Evol Microbiol. 57, 2952–2959

de Paula AT, Jeronymo-Ceneviva AB, Silva LF, Todorov SD, Franco BD, Penna AL. 2015. *Leuconostoc mesenteroides* SJRP55: a potential probiotic strain isolated from Brazilian water buffalo mozzarella cheese. Annals of Microbiology. 65, 899–910

de Paula AT, Jeronymo-Ceneviva AB, Silva LF, Todorov SD, Franco BD, Choiset Y, Haertlé T, Chobert JM, Dousset X, Penna AL. 2014. *Leuconostoc mesenteroides* SJRP55: A Bacteriocinogenic Strain Isolated from Brazilian Water Buffalo Mozzarella Cheese. Probiotics Antimicrob Proteins. 6, 186–197

Dec M, Urban-Chmiel R, Stępień-Pyśniak D, Wernicki A. 2017. Assessment of antibiotic susceptibility in *Lactobacillus* isolates from chickens. Gut Pathog. 9, 1–16

Delgado S, Flórez AB, Mayo B. 2005. Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr Microbiol. 50, 202–207

Dellaglio F, Dicks LMT, Torriani S. 1995. The genus *Leuconostoc*. In: Wood B. J. B., Holzapfel W. H., editors. The genera of lactic acid bacteria. Blackie Academic & Professional, 235–278

Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. 2007. Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev. 20, 79–114

Devirgiliis C, Zinno P, Perozzi G. 2013. Update on antibiotic resistance in foodborne *Lactobacillus* and *Lactococcus* species. Front Microbiol. 4, 1–13

Devirgiliis C, Barile S, Perozzi G. 2011. Antibiotic resistance determinants in the interplay between food and gut microbiota. Genes Nutr. 6, 275–284

Devirgiliis C, Barile S, Caravelli A, Coppola D, Perozzi G. 2010. Identification of tetracycline- and erythromycin-resistant Gram-positive cocci within the fermenting microflora of an Italian dairy food product. J Appl Microbiol. 109, 313–323

Devirgiliis C, Coppola D, Barile S, Colonna B, Perozzi G. 2009. Characterization of the *Tn*916 conjugative transposon in a food-borne strain of *Lactobacillus paracasei*. Appl Environ Microbiol. 75, 3866–3871

Di Cagno R, Coda R, De Angelis M, Gobbetti M. 2013. Exploitation of vegetables and fruits through lactic acid fermentation. Food Microbiol. 33, 1–10

Djordjevic SP, Stokes HW, Roy Chowdhury P. 2013. Mobile elements, zoonotic pathogens and commensal bacteria: conduits for the delivery of resistance genes into humans, production animals and soil microbiota. Front Microbiol. 4, 1–12

European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10, 2740–2750

European Food Safety Authority (EFSA). 2008. Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J. 732, 1–15

European Food Safety Authority (EFSA). 2007. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 587, 1–16

European Commission (SCAN). 2007. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J. 587, 1–16

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

Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, Grundman H, Hasman H, Holden MT, Hopkins KL, Iredell J, Kahlmeter G, Köser CU, MacGowan A, Mevius D, Mulvey M, Naas T, Peto T, Rolain JM, Samuelsen Ø, Woodford N. 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect. 23, 2–22

Fair RJ and Tor Y. 2014. Antibiotics and Bacterial Resistance in the 21st Century. Perspect Medicin Chem. 6, 25–64

Ferrando ML, de Greeff A, van Rooijen WJ, Stockhofe-Zurwieden N, Nielsen J, Wichgers Schreur PJ, Pannekoek Y, Heuvelink A, van der Ende A, Smith H, Schultsz C. 2015. Host-pathogen Interaction at the Intestinal Mucosa Correlates With Zoonotic Potential of *Streptococcus suis*. J Infect Dis. 212, 95–105

Flórez AB, Campedelli I, Delgado S, Alegría Á, Salvetti E, Felis GE, Mayo B, Torriani S. 2016. Antibiotic Susceptibility Profiles of Dairy *Leuconostoc*, Analysis of the Genetic Basis of Atypical Resistances and Transfer of Genes In Vitro and in a Food Matrix. PLoS One. 11, 1–20

Flórez AB, Alegría Á, Rossi F, Delgado S, Felis GE, Torriani S, Mayo B. 2014. Molecular Identification and Quantification of Tetracycline and Erythromycin Resistance Genes in Spanish and Italian Retail Cheeses. BioMed Research International. 2014, 1–10

Flórez AB, Delgado S, Mayo B. 2005. Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Can J Microbiol. 51, 51–58

Fluit AC, Visser MR, Schmitz FJ. 2001. Molecular Detection of Antimicrobial Resistance. Clin Microbiol Rev. 14, 836–871

Founou LL, Founou RC, Essack SY. 2016. Antibiotic resistance in the food chain: a developing country-perspective. Front Microbiol. 7, 1–19

Frantzen CA, Kot W, Pedersen TB, Ardö YM, Broadbent JR, Neve H, Hansen LH, Dal Bello F, Østlie HM, Kleppen HP, Vogensen FK, Holo H. 2017. Genomic Characterization of Dairy Associated *Leuconostoc* Species and Diversity of *Leuconostocs* in Undefined Mixed Mesophilic Starter Cultures. Front Microbiol. 8, 1–14

Gad G, Abdel-Hamid AM, Farag ZF. 2014. Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. Braz J Microbiol. 45, 25–33

Gao J, Yu FQ, Luo LP, He JZ, Hou RG, Zhang HQ, Li SM, Su JL, Han B. 2012. Antibiotic resistance of *Streptococcus agalactiae* from cows with mastitis. Vet J. 194, 423–424

Gazzola S, Fontana C, Bassi D, Cocconcelli PS. 2012. Assessment of tetracycline and erythromycin resistance transfer during sausage fermentation by culture-dependent and independent methods. Food Microbiol. 30, 348–354

Geenen PL, Koene MGJ, Blaak H, Havelaar AH, van de Giessen AW. 2010. Risk profile on antimicrobial resistance transmissible from food animals to humans. RIVM rapport 330334001. Bilhoven: National Institute for Public Health and the Environment (RIVM).

Gerritsen J, Smidt H, Rijkers GT, de Vos WM. 2011. Intestinal microbiota in human health and disease: the impact of probiotics. Genes Nutr. 6, 209–240

Gevers D, Masco L, Baert L, Huys G, Debevere J, Swings J. 2003a. Prevalence and diversity of tetracycline resistant lactic acid bacteria and their *tet* genes along the process line of fermented dry sausages. Syst Appl Microbiol. 26, 277–283

Gevers D, Huys G, Swings J. 2003b. In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiol Lett. 225, 125–130

Gevers D, Danielsen M, Huys G, Swings J. 2003c. Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl Environ Microbiol. 69, 1270–1275

Gibson MK, Forsberg KJ, Dantas G. 2015. Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. ISME J. 9, 207–2016

Giles-Gómez M, Sandoval García JG, Matus V, Campos Quintana I, Bolívar F, Escalante A. 2016. In vitro and in vivo probiotic assessment of *Leuconostoc mesenteroides* P45 isolated from pulque, a Mexican traditional alcoholic beverage. Springerplus. 5, 708

Gillings MR, Paulsen IT, Tetu SG. 2017. Genomics and the evolution of antibiotic resistance. Ann N Y Acad Sci. 1388, 92–107

Gilman J and Love J. 2016. Synthetic promoter design for new microbial chassis. Biochem Soc Trans. 44, 731–737

Goldstein E, Tyrrell KL, Citron DM. 2015. *Lactobacillus* species: taxonomic complexity and controversial susceptibilities. Clin Infect Dis. 2, S98–S107

Gordon NC, Price JR, Cole K, Everitt R, Morgan M, Finney J, Kearns AM, Pichon B, Young B, Wilson DJ, Llewelyn MJ, Paul J, Peto TEA, Crook D, Walker AS, Golubchik T. 2014. Prediction of *Staphylococcus aureus* antimicrobial resistance by whole-genome sequencing. J Clin Microbiol. 52, 1182–1191

Gu CT, Wang F, Li CY, Liu F, Hou GC. 2012. *Leuconostoc mesenteroides* subsp. *suionicum* subsp. nov. Int J Syst Evol Microbiol. 62, 1548–1551

Gueimonde M, Sánchez BG, de Los Reyes-Gavilán C, Margolles A. 2013. Antibiotic resistance in probiotic bacteria. Front Microbiol. 4, 1–6

Guo H, Pan L, Li L, Lu J, Kwok L, Menghe B, Zhang H, Zhang W. 2017. Characterization of Antibiotic Resistance Genes from *Lactobacillus* Isolated from Traditional Dairy Products. J Food Sci. 82, 724–730

Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain JM. 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother. 58, 212–220

Hampton T. 2013. Report reveals scope of US antibiotic resistance threat. JAMA. 310, 1661–1663

He LY, Ying GG, Liu YS, Su HC, Chen J, Liu SS, Zhao JL. 2016. Discharge of swine wastes risks water quality and food safety: Antibiotics and antibiotic resistance genes from swine sources to the receiving environments. Environ Int. 93, 210–219

Hemme D and Foucaud-Scheunemann C. 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int Dairy J. 14, 467–494

Herve-Jimenez L, Guillouard I, Guedon E, Boudebbouze S, Hols P, Monnet V, Maguin E, Rul F. 2009. Postgenomic analysis of streptococcus thermophilus cocultivated in milk with *Lactobacillus delbrueckii* subsp. *bulgaricus*: involvement of nitrogen, purine, and iron metabolism. Appl Environ Microbiol. 75, 2062–2073

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 11, 506–514

Hill CM, Krause KM, Lewis SR, Blais J, Benton BM, Mammen M, Humphrey PP, Kinana A, Janc JW. 2010. Specificity of induction of the *van*A and *van*B operons in vancomycin-resistant enterococci by telavancin. Antimicrob Agents Chemother. 54, 2814–2818

Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin PJ, Piddock LJ. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 387, 176–187

Howard SJ, Hopwood S, Davies SC. 2014. Antimicrobial resistance: a global challenge. Sci Transl Med. 6, 236ed10

Huddleston JR. 2014. Horizontal gene transfer in the human gastrointestinal tract: potential spread of antibiotic resistance genes. Appl Environ Microbiol. 73, 730–739

Hummel A, Holzapfel WH, Franz CM. 2007a. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Syst Appl Microbiol. 30, 1–7

Hummel AS, Hertel C, Holzapfel WH, Franz CMAP. 2007b. Antibiotic Resistances of Starter and Probiotic Strains of Lactic Acid Bacteria. Drug Resist. 7, 167–176

Huovinen P. 2001. Resistance to trimetoprim-sulfamethoxazole. Clin Infect Dis. 32, 1608–1614

Huys G, D'Haene K, Cnockaert M, Tosi L, Danielsen M, Flórez AB, Mättö J, Axelsson L, Korhonen J, Mayrhofer S, Egervärn M, Giacomini M, Vandamme P. 2010. Intra- and

interlaboratory performances of two commercial antimicrobial susceptibility testing methods for bifidobacteria and nonenterococcal lactic acid bacteria. Antimicrob Agents Chemother. 54, 2567–2574

Iulietto MF, Sechi P, Borgogni E, Goga BTC. 2016. Antibiotic susceptibility profiles of ropy slime-producing *Leuconostoc mesenteroides* isolated from cooked meat products. Microbiol Res. 7

Jeon HH, Kim KH, Chun BH, Ryu BH, Han NS, Jeon CO. 2017. A proposal of *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* subsp. nov. and reclassification of *Leuconostoc mesenteroides* subsp. *suionicum* (Gu *et al*., 2012) as *Leuconostoc suionicum* sp. nov. based on complete genome sequences. Int J Syst Evol Microbiol. 67, 2225–2230

Jeon CO, Jeon HH, Kim HR, Kim KH. Pan-genome Analysis of *Leuconostoc mesenterides* reveals its Metabolic Capabilities and Diversities during Fermentation. (Unpublished)

Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson TA, Brinkman FS, Wright GD, McArthur AG. 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res. 45, D566–D573

Johansson P, Paulin L, Säde E, Salovuori N, Alatalo ER, Björkroth KJ, Auvinen P. 2011. Genome sequence of a food spoilage lactic acid bacterium, *Leuconostoc gasicomitatum* LMG 18811T, in association with specific spoilage reactions. Appl Environ Microbiol. 77, 4344–4351

Jorgensen JH and Ferraro MJ. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis. 49, 1749–1755

Jorgensen JH and Ferraro MJ. 1998. Antimicrobial susceptibility testing: general principles and contemporary practices. Clin Infect Dis. 26, 973–980

Jung JY, Lee SH, Jeon CO. 2012a. Complete genome sequence of *Leuconostoc carnosum* strain JB16, isolated from Kimchi. J Bacteriol. 194, 6672–6673

Jung JY, Lee SH, Lee HJ, Seo HY, Park WS, Jeon CO. 2012b. Effects of *Leuconostoc mesenteroides* starter cultures on microbial communities and metabolites during kimchi fermentation. Int J Food Microbiol. 153, 378–387

Kastner S, Perreten V, Bleuler H, Hugenschmidt G, Lacroix C, Meile L. 2006. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. Syst Appl Microbiol. 29, 145–155

Katla AK, Kruse H, Johnsen G, Herikstad H. 2001. Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. Int J Food Microbiol. 67, 147–152

Kaur J, Lee S, Park YS, Sharma A. 2017. RAPD analysis of *Leuconostoc mesenteroides* strains associated with vegetables and food products from Korea. Food Sci Technol. 77, 383–388

Kechagia M, Basoulis D, Konstantopoulou S, Dimitriadi D, Gyftopoulou K, Skarmoutsou N, Fakiri EM. 2013. Health Benefits of Probiotics: A Review. ISRN Nutrition. 2013, 1–7

Keessen EC, Gaastra W, Lipman LJ. 2011. *Clostridium difficile* infection in humans and animals, differences and similarities. Vet Microbiol. 153, 205–217

Kim DW, Choi SH, Kang A, Nam SH, Kim RN, Kim A, Park HS. Draft Genome Sequence of *Leuconostoc lactis* KCTC 3528. (Unpublished)

Kim DS, Choi SH, Kim DW, Kim RN, Nam SH, Kang A, Kim A, Park HS. 2011a. Genome Sequence of *Leuconostoc gelidum* KCTC 3527, Isolated from Kimchi. J Bacteriol. 193, 799–800

Kim DS, Choi SH, Kim DW, Kim RN, Nam SH, Kang A, Kim A, Park HS. 2011b. Genome sequence of *Leuconostoc inhae* KCTC 3774, isolated from Kimchi. J Bacteriol. 193, 1278–1279

Kim DW, Choi SH, Kang A, Nam SH, Kim RN, Kim A, Kim DS, Park HS. 2011c. Genome sequence of *Leuconostoc pseudomesenteroides* KCTC 3652. J Bacteriol. 193, 4299

Kim JF, Jeong H, Lee JS, Choi SH, Ha M, Hur CG, Kim JS, Lee S, Park HS, Park YH, Oh TK. 2008. Complete genome sequence of *Leuconostoc citreum* KM20. J Bacteriol. 190, 3093–3094

Kim B, Lee J, Jang J, Kim J, Han H. 2003. *Leuconostoc inhae* sp. nov., a lactic acid bacterium isolated from kimchi. Int J Syst Evol Microbiol. 53, 1123–1126

Kim J, Chun J, Han HU. 2000. *Leuconostoc kimchii* sp. nov., a new species from kimchi. Int J Syst Evol Microbiol. 5, 1915–1919

Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Müller-Bertling S, Witte W, Goossens H. 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. J Antimicrob Chemother. 59, 900–912

Klare I, Konstabel C, Müller-Bertling S, Reissbrodt R, Huys G, Vancanneyt M, Swings J, Goossens H, Witte W. 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl. Environ. Microbiol. 71, 8982–8986

Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, Bron PA. 2010. The extracellular biology of the lactobacilli. FEMS Microbiol Rev. 34, 199–230

Kline L and Sugihara TF. 1971. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Appl Microbiol. 21, 459–465

Klose V, Bayer K, Kern C, Goelß F, Fibi S, Wegl G. 2014. Antibiotic resistances of intestinal lactobacilli isolated from wild boars. Vet Microbiol. 168, 240–244

Kobierecka PA, Wyszyńska AK, Aleksandrzak-Piekarczyk T, Kuczkowski M, Tuzimek A, Piotrowska W, Górecki A, Adamska I, Wieliczko A, Bardowski J, Jagusztyn-Krynicka EK. 2017. In vitro characteristics of *Lactobacillus* spp. strains isolated from the chicken digestive tract and their role in the inhibition of *Campylobacter* colonization. Microbiologyopen. 6, 1–15

Köser CU, Ellington MJ, Peacock SJ. 2014. Whole-genome sequencing to control antimicrobial resistance. Trends Genet. 30, 401–407

Kothari D and Goyal A. 2015. Gentio-oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase as prebiotics and as a supplement for functional foods with anti-cancer properties. Food Funct. 6, 604–611

Lampkowska J, Feld L, Monaghan A, Toomey N, Schjørring S, Jacobsen B, van der Voet H, Andersen SR, Bolton D, Aarts H, Krogfelt KA, Wilcks A, Bardowski J. 2008. A standardized conjugation protocol to asses antibiotic resistance transfer between lactococcal species. Int J Food Microbiol. 127, 172–175

Lavanya B, Sowmiya S, Balaji S, Muthuvelan B. 2011. Screening and Characterization of Lactic Acid Bacteria from Fermented Milk. British Journal of Dairy Sciences. 2, 5–10

Laverde Gomez JA, van Schaik W, Freitas AR, Coque TM, Weaver KE, Francia MV, Witte W, Werner G. 2011. A multiresistance megaplasmid pLG1 bearing a hylEfm genomic island in hospital *Enterococcus faecium* isolates. Int J Med Microbiol. 301, 165–175

Lee Ventola CMS. 2015. The Antibiotic Resistance Crisis. Part 1: Causes and Threats. P T. 40, 277–283

Levy SB and Marshall B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med. 10, 122–129

Li Y, Canchaya C, Fang F, Raftis E, Ryan KA, van Pijkeren JP, van Sinderen D, O'Toole PW. 2009. Distribution of megaplasmids in *Lactobacillus salivarius* and other lactobacilli. J Bacteriol. 189, 6128–6139

Liu L, Zhu W, Cao Z, Xu B, Wang G, Luo M. 2015. High correlation between genotypes and phenotypes of environmental bacteria *Comamonas testosteroni* strains. BMC genomics. 16, 1–14

Liu S, Han Y, Zhou Z. 2011. Lactic acid bacteria in traditional fermented Chinese foods. Food Research International. 44, 643–651

Liu B and Pop M. 2009. ARDB–Antibiotic Resistance Genes Database. Nucleic Acids Res. 37, D443–D447

Liu C, Zhang ZY, Dong K, Yuan JP, Guo XK. 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomed Environ Sci. 22, 401–412

Liu LC, Tsai JC, Hsueh PR, Tseng SP, Hung WC, Chen HJ, Teng LJ. 2008. Identification of *tet*(S) gene area in tetracycline-resistant *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates. J Antimicrob Chemother. 61, 453–435

Loman NJ and Pallen MJ. 2015. Twenty years of bacterial genome sequencing. Nat Rev Microbiol. 13, 787–794

Luh KT, Hsueh PR, Teng LJ, Pan HJ, Chen YC, Lu JJ, Wu JJ, Ho SW. 2000. Quinupristin-dalfopristin resistance among gram-positive bacteria in Taiwan. Antimicrob Agents Chemother. 44, 3374–3380

Lyhs U, Snauwaert I, Pihlajaviita S, De Vuyst L, Vandamme P. 2015. *Leuconostoc rapi* sp. nov., isolated from sous-vide-cooked rutabaga. Int J Syst Evol Microbiol. 65, 2586–2590

Ma Q, Fu Y, Sun H, Huang Y, Li L, Yu Q, Dinnyes A, Sun Q. 2017. Antimicrobial resistance of *Lactobacillus* spp. from fermented foods and human gut. LWT - Food Science and Technology. 86, 201–208

Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S,

Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee JH, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O'Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D. 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci USA. 103, 15611–15616

Marcobal AM, Sela DA, Wolf YI, Makarova KS, Mills DA. 2008. Role of hypermutability in the evolution of the genus *Oenococcus*. J Bacteriol. 190, 564–570

Martínez JL, Coque TM, Baquero F. 2015. What is a resistance gene? Ranking risk in resistomes. Nat Rev Microbiol. 13, 116–123

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

Mayrhofer S, Mair C, Kneifel W, Domig KJ. 2011. Susceptibility of bifidobacteria of animal origin to selected antimicrobial agents. Chemother Res Pract. 2011, 1–6

Mayrhofer S, van Hoek AH, Mair C, Huys G, Aarts HJ, Kneifel W, Domig KJ. 2010. Antibiotic susceptibility of members of the *Lactobacillus acidophilus* group using broth microdilution and molecular identification of their resistance determinants. Int J Food Microbiol. 144, 81–87

McArthur AG and Wright GD. 2015. Bioinformatics of antimicrobial resistance in the age of molecular epidemiology. Curr Opin Microbiol. 27, 45–50

McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ, Bhullar K, Canova MJ, De Pascale G, Ejim L, Kalan L, King AM, Koteva K, Morar M, Mulvey MR, O'Brien JS, Pawlowski AC, Piddock LJ, Spanogiannopoulos P, Sutherland AD, Tang I, Taylor PL, Thaker M, Wang W, Yan M, Yu T, Wright GD. 2013. The comprehensive antibiotic resistance database. Antimicrob Agents Chemother. 57, 3348–3357

McDermott PF, Tyson GH, Kabera C, Chen Y, Li C, Folster JP, Ayers SL, Lam C, Tate HP, Zhao S. 2016. Whole-Genome Sequencing for Detecting Antimicrobial Resistance in Nontyphoidal *Salmonella*. Antimicrob Agents Chemother. 60, 5515–5520

McSweeney PLH and Suosa MJ. 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review. Lait. 80, 293–324

Miller AW, Kohl KD, Dearing MD. 2014. The Gastrointestinal Tract of the White-Throated Woodrat (*Neotoma albigula*) Harbors Distinct Consortia of Oxalate-Degrading Bacteria. Appl Environ Microbiol. 80, 1595–1601

Million M, Angelakis E, Paul M, Armougom F, Leibovici L, Raoult D. 2012. Comparative meta-analysis of the effect of *Lactobacillus* species on weight gain in humans and animals. Microb Pathog. 53, 100–108

Mo H, Zhu Y, Chen Z. 2008. Microbial fermented tea – a potential source of natural food preservatives. Trends in Food Science & Technology. 19, 124–130

Morandi S, Cremonesi P, Silvetti T, Brasca M. 2013. Technological characterisation, antibiotic susceptibility and antimicrobial activity of wild-type *Leuconostoc* strains isolated from north Italian traditional cheeses. J Dairy Res. 80, 457–466

Morelli L, Vogensen FK, von Wright A. 2004. Genetics of lactic acid bacteria. In: Salminen S, von Wright A, Ouwehand A (eds) Lactic acid Bacteria. Microbiology and Functional aspects. Marcel Dekker, Inc., New York

Moreno-Arribas MV, Polo MC, Jorganes F, Munoz R. 2003. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. Int J Food Microbiol. 84, 117–123

Munita JM and Arias CA. 2016. Mechanisms of Antibiotic Resistance. Microbiol Spectr. 4, 1–37

Nam SH, Kim A, Choi SH, Kang A, Kim DW, Kim RN, Kim DS, Park HS. 2011a. Genome sequence of *Leuconostoc carnosum* KCTC 3525. J Bacteriol. 193, 6100–6101

Nam SH, Choi SH, Kang A, Kim DW, Kim DS, Kim RN, Kim A, Park HS. 2011b. Genome sequence of *Leuconostoc fallax* KCTC 3537. J Bacteriol. 193, 588–589

Naser SM, Vancanneyt M, Snauwaert C, Vrancken G, Hoste B, De Vuyst L, Swings J. 2006. Reclassification of *Lactobacillus amylophilus* LMG 11400 and NRRL B-4435 as *Lactobacillus amylotrophicus* sp. nov. Int J Syst Evol Microbiol. 56, 2523–2527

Nawaz M, Wang J, Zhou A, Ma C, Wu X, Moore JE, Millar BC, Xu J. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol. 62, 1081–1089

Nesmelova IV and Hackett PB. 2010. DDE transposases: Structural similarity and diversity. Adv Drug Deliv Rev. 62, 1187–1195

Nicoloff H and Bringel F. 2003. ISL*pl1* is a functional IS*30*-related insertion element in *Lactobacillus plantarum* that is also found in other lactic acid bacteria. Appl Environ Microbiol. 69, 6032–6040

Nieto-Arribas P, Seseña S, Poveda JM, Palop L, Cabezas L. 2010. Genotypic and technological characterization of *Leuconostoc* isolates to be used as adjunct starters in Manchego cheese manufacture. Food Microbiol. 27, 85–93

Nionelli L, Curri N, Curiel JA, Di Cagno R, Pontonio E, Cavoski I, Gobbetti M, Rizzello CG. 2014. Exploitation of Albanian wheat cultivars: characterization of the flours and lactic acid bacteria microbiota, and selection of starters for sourdough fermentation. Food Microbiol. 44, 96–107

O'Sullivan DJ and Klaenhammer TR. 1993. Rapid mini-prep isolation of high quality DNA from *Lactococcus* and *Lactobacillus* species. Appl Environ Microbiol. 59, 2730–2733

Ogier JC, Casalta E, Farrokh C, Saïhi A. 2008. Safety assessment of dairy microorganisms: the *Leuconostoc* genus. Int J Food Microbiol. 126, 286–290

Oh HM, Cho YJ, Kim BK, Roe JH, Kang SO, Nahm BH, Jeong G, Han HU, Chun J. 2010. Complete genome sequence analysis of *Leuconostoc kimchii* IMSNU 11154. J Bacteriol. 192, 3844–3845

Oki K, Rai AK, Sato S, Watanabe K, Tamang JP. 2011. Lactic acid bacteria isolated from ethnic preserved meat products of the Western Himalayas. Food Microbiol. 28, 1308–1315

Ouoba LI, Lei V, Jensen LB. 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. Int J Food Microbiol. 121, 217–224

Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, de Crécy-Lagard V, Diaz N, Disz T, Edwards R, Fonstein M, Frank ED, Gerdes S, Glass EM, Goesmann A, Hanson A, Iwata-Reuyl D, Jensen R, Jamshidi N, Krause L, Kubal M, Larsen N, Linke B, McHardy AC, Meyer F, Neuweger H, Olsen G, Olson R, Osterman A, Portnoy V, Pusch GD, Rodionov DA, Rückert C, Steiner J, Stevens R, Thiele I, Vassieva O, Ye Y, Zagnitko O, Vonstein V. 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res. 33, 5691–5702

Pagès JM, James CE, Winterhalter M. 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol. 6, 893–903

Palmieri C, Princivalli MS, Brenciani A, Varaldo P, Facinelli B. 2011. Different Genetic Elements Carrying the *tet*(W) Gene in Two Human Clinical Isolates of *Streptococcus suis*. Antimicrob Agents Chemother. 55, 631–636

Pan L, Hu X, Wang. 2011. Assessment of antibiotic resistance of lactic acid bacteria in Chinese fermented foods. Food Control. 22, 1316–1321

Papizadeh M, Rohani M, Nahrevanian H, Javadi A, Pourshafie MR. 2017. Probiotic characters of *Bifidobacterium* and *Lactobacillus* are a result of the ongoing gene acquisition and genome minimization evolutionary trends. Microb Pathog. 111, 118–131

Park GS and Shin JH. Complete genome sequence of *Leuconostoc mesenteroides* subsp. *dextranicum* DSM 20484. (Unpublished)

Park IS and Walsh CT. 1997. D-Alanyl-D-Lactate and D-Alanyl-D-Alanine synthesis by D-Alanyl-D-Alanine ligase from vancomycin-resistant *Leuconostoc mesenteroides*. Effects of a phenylalanine 261 to tyrosine mutation. J Biol Chem. 272, 9210–9214

Pedersen TB, Kot WP, Hansen LH, Sørensen SJ, Broadbent JR, Vogensen FK, Ardö Y. 2014. Genome Sequences of Two *Leuconostoc pseudomesenteroides* Strains Isolated from Danish Dairy Starter Cultures. Genome Announc. 2, 1–2

Pedersen TB, Ristagno D, McSweeney PLHF, Vogensen K, Ardö Y. 2013. Potential impact on cheese flavour of heterofermentative bacteria from starter cultures. Int Dairy J. 33, 112–119

Pérez Pulido R, Ben Omar N, Abriouel H, Lucas López R, Martínez Cañamero M, Gálvez A. 2005. Microbiological study of lactic acid fermentation of Caper berries by molecular and culture-dependent methods. Appl Environ Microbiol. 71, 7872–7879

Puopolo KM, Klinzing DC, Lin MP, Yesucevitz DL, Cieslewicz MJ. 2007. A composite transposon associated with erythromycin and clindamycin resistance in group B *Streptococcus*. J Med Microbiol. 56, 947–955

Qin S, Wang Y, Zhang Q, Chen X, Shen Z, Deng F, Wu C, Shen J. 2012. Identification of a novel genomic island conferring resistance to multiple aminoglycoside antibiotics in *Campylobacter coli*. Antimicrob Agents Chemother. 56, 5332–5339

Rahkila R, De Bruyne K, Johansson P, Vandamme P, Björkroth J. 2014. Reclassification of *Leuconostoc gasicomitatum* as *Leuconostoc gelidum* subsp. *gasicomitatum* comb. nov., description of *Leuconostoc gelidum* subsp. *aenigmaticum* subsp. nov., designation of *Leuconostoc gelidum* subsp. *gelidum* subsp. nov. and emended description of *Leuconostoc gelidum*. Int J System Evol Microbiol. 64, 1290–1295

Ramirez MS and Tolmasky ME. 2010. Aminoglycoside Modifying Enzymes. Drug Resist Updat. 13, 151–171

Reichmann P, Nuhn M, Denapaite D, Brückner R, Henrich B, Maurer P, Rieger M, Klages S, Reinhard R, Hakenbeck R. 2011. Genome of *Streptococcus oralis* strain Uo5. J Bacteriol. 193, 2888–2889

Reygaert WC. 2014. The antimicrobial possibilities of green tea. Front Microbiol. 5, 434

Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Girones R, Koutsoumanis K, Lindqvist R, Nørrung B, Robertson L, Ru G, Fernandez Escamez PS, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlström H, Cocconcelli PS, Peixe L, Maradona MP, Querol A, Suarez JE, Sundh I, Vlak J, Correia S and Herman L. 2017. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 6: suitability of taxonomic units notified to EFSA until March 2017. EFSA J. 15, 4884–4916

Riveros-Mckay F, Campos I, Giles-Gómez M, Bolívar F, Escalante A. 2014. Draft genome sequence of *Leuconostoc mesenteroides* P45 isolated from Pulque, a traditional mexican alcoholic fermented beverage. Genome Announc. 2, e01130–14

Rizzotti L, La Gioia F, Dellaglio F, Torriani S. 2009a. Molecular diversity and transferability of the tetracycline resistance gene *tet*(M), carried on *Tn*916-1545 family transposons, in enterococci from a total food chain. Antonie van Leeuwenhoek. 96, 43–52

Rizzotti L, La Gioia F, Dellaglio F, Torriani S. 2009b. Characterization of tetracycline-resistant *Streptococcus thermophilus* isolates from Italian soft cheeses. Appl Environ Microbiol. 75, 4224–4229

Rizzotti L, Simeoni D, Cocconcelli PS, Gazzola S, Dellaglio F, Torriani S. 2005. Contribution of enterococci to the spread of antibiotic resistance in the production chain of swine meat commodities. J Food Protect. 68, 955–965

Roberts MC and Schwarz S. 2009. Tetracycline and Chloramphenicol Resistance Mechanisms. Antimicrobial Drug Resistance. 183–193

Roberts MC. 2008. Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. FEMS Microbiol Lett. 282, 147–159

Roberts MC. 2002. Resistance to tetracycline, macrolide-lincosamide-streptogramin, trimethoprim, and sulfonamide drug classes. Mol Biotechnol. 20, 261–283

Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H. 1999. Nomenclature for macrolide and macrolide–lincosamide–streptogramin B resistance determinants. Antimicrob Agents Chemother. 43, 2823–2830

Rodríguez-Alonso P, Fernández-Otero C, Centeno JA, Garabal JI. 2009. Antibiotic resistance in lactic acid bacteria and *Micrococcaceae*/*Staphylococcaceae* isolates from artisanal raw milk cheeses, and potential implications on cheese making. J Food Sci. 74, M284–M293

Rojo-Bezares B, Sáenz Y, Poeta P, Zarazaga M, Ruiz-Larrea F, Torres C. 2006. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. Int J Food Microbiol. 111, 234–240

Rolain JM. 2013. Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. Front Microbiol. 4, 1–10

Rosander A, Connolly E, Roos S. 2008. Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. Appl Environ Microbiol. 74, 6032–6040

Rossi F, Rizzotti L, Felis GE, Torriani S. 2014. Horizontal gene transfer among microorganisms in food: Current knowledge and future perspectives. Food Microbiol. 42, 232–243

Salvetti E and O'Toole PW. 2017. When regulation challenges innovation: The case of the genus *Lactobacillus*. Trends in Food Science & Technology. 66, 187–194

Sanders ME, Akkermans LMA, Haller D, Hammerman C, Heimbach J, Hörmannsperger G, Huys G, Levy DD, Lutgendorff F, Mack D, Phothirath P, Solano-Aguilar G, Vaughan E. 2010. Safety assessment of probiotics for human use. Gut Microbes. 3, 164–185

Sasirekha B, Usha MS, Amruta JA, Ankit S, Brinda N, Divya R. 2014. Incidence of constitutive and inducible clindamycin resistance among hospital-associated *Staphylococcus aureus*. Biotech. 4, 85–89

Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev. 32, 234–258

Schleifer KH and Kilpper-Bälz R. 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int J Syst Bacteriol. 34, 31–34

Schmittgen TD and Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 3, 1101–1108

Schürch AC and van Schaik W. 2017. Challenges and opportunities for whole-genome sequencing-based surveillance of antibiotic resistance. Ann N Y Acad Sci. 1388, 108–120

Schwarz S, Loeffler A, Kadlec K. 2017. Bacterial resistance to antimicrobial agents and its impact on veterinary and human medicine. Vet Dermatol. 28, 82–95

Sharma P, Tomar SK, Sangwan V, Goswami P, Singh R. 2016. Antibiotic Resistance of *Lactobacillus* sp. Isolated from Commercial Probiotic Preparations. J Food Saf. 36, 38–51

Sharma P, Tomar SK, Goswami P, Sangwan V, Singh R. 2014. Antibiotic resistance among commercially available probiotics. Food Res Int. 57, 176–195

Shaw BG and Harding CD. 1989. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Int J Syst Evol Microbiol. 39, 217–223

Shaw JH and Clewell DB. 1985. Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon *Tn*917 in *Streptococcus faecalis*. J Bacteriol. 164, 782–796

Silva LF, Casella T, Gomes ES, Nogueira MC, De Dea Lindner J, Penna AL. 2015. Diversity of lactic acid bacteria isolated from Brazilian water buffalo mozzarella cheese. J Food Sci. 80, 411–417

Simeoni D, Rizzotti L, Cocconcelli P, Gazzola S, Dellaglio F, Torriani S. 2008. Antibiotic resistance genes and identification of staphylococci collected from the production chain of swine meat commodities. Food Microbiol. 25, 196–201

Singh KV, Weinstock GM, Murray BE. 2002. An *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob Agents Chemother. 46, 1845–1850

Sköld O. 2001. Resistance to trimetoprim and sulfonamides. Vet Res. 32, 261–273

Smith JL, Goldberg JM, Grossman AD. 2014. Complete Genome Sequences of *Bacillus subtilis* subsp. subtilis Laboratory Strains JH642 (AG174) and AG1839. Genome Announc. 2, e00663-14

Sornplang P, Leelavatcharamas V, Sukon P, Yowarach S. 2001. Antibiotic Resistance of Lactic Acid Bacteria Isolated from a Fermented Fish Product, Pla-chom. Research Journal of Microbiology. 6, 898–903

Soucy SM, Huang J, Gogarten JP. 2015. Horizontal gene transfer: building the web of life. Nat Rev Genet. 16, 472–482

Spigaglia P, Barbanti F, Mastrantonio P. 2011. Multidrug resistance in European *Clostridium difficile* clinical isolates. J Antimicrob Chemother. 66, 2227–2234

Srinivasan V, Metcalf BJ, Knipe KM, Ouattara M, McGee L, Shewmaker PL, Glennen A, Nichols M, Harris C, Brimmage M, Ostrowsky B, Park CJ, Schrag SJ, Frace MA, Sammons SA, Beall B. 2014. *van*G element insertions within a conserved chromosomal site conferring vancomycin resistance to *Streptococcus agalactiae* and *Streptococcus anginosus*. MBio. 5, e01386-14

Stanhope MJ, Lefébure T, Walsh SL, Becker JA, Lang P, Bitar PDP, Miller LA, Italia MJ, Amrine-Madsen H. 2008. Positive selection in penicillin-binding proteins 1a, 2b, and 2x from *Streptococcus pneumoniae* and its correlation with amoxicillin resistance development. Infect Genet Evol. 8, 331–339

Stogios PJ, Kuhn ML, Evdokimova E, Courvalin P, Anderson WF, Savchenko A. 2014. Potential for reduction of streptogramin A resistance revealed by structural analysis of the VatA acetyltransferase. Antimicrob Agents Chemother. 58, 7083–7092

Sun Z, Harris HM, McCann A, Guo C, Argimón S, Zhang W, Yang X, Jeffery IB, Cooney JC, Kagawa TF, Liu W, Song Y, Salvetti E, Wrobel A, Rasinkangas P, Parkhill J, Rea MC, O'Sullivan O, Ritari J, Douillard FP, Paul Ross R, Yang R, Briner AE, Felis GE, de Vos WM, Barrangou R, Klaenhammer TR, Caufield PW, Cui Y, Zhang H, O'Toole PW. 2015.

Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. Nat Commun. 6, 1–13

Sun J, Deng Z, Yan A. 2014. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem Biophys Res Commun. 453, 254–267

Syvanen M. 2012. Evolutionary implications of horizontal gene transfer. Annu Rev Genet. 46, 341–358

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30, 2725–2729

Tanasupawat S, Pakdeeto A, Thawai C, Yukphan P, Okada S. 2007. Identification of lactic acid bacteria from fermented tea leaves (miang) in Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov. J Gen Appl Microbiol. 53, 7–15

Terzic-Vidojevic A, Mihajlovic S, Uzelac G, Veljovic K, Tolinacki M, Nikolic M, Topisirovic L, Kojic M. 2014. Characterization of lactic acid bacteria isolated from artisanal Travnik young cheeses, sweet creams and sweet kajmaks over four seasons. Food Microbiol. 39, 27–38

Thaker M, Spanogiannopoulos P, Wright GD. 2010. The tetracycline resistome. Cell Mol Life Sci. 67, 419–431

Thumu SC and Halami PM. 2012a. Acquired Resistance to Macrolide–Lincosamide–Streptogramin Antibiotics in Lactic Acid Bacteria of Food Origin. Indian J Microbiol. 52, 530–537

Thumu SC and Halami PM. 2012b. Presence of erythromycin and tetracycline resistance genes in lactic acid bacteria from fermented foods of Indian origin. Antonie Van Leeuwenhoek. 102, 541–551

Tomich PK, An FY, Clewell DB. 1980. Properties of erythromycin-inducible *Tn*917 in *Streptococcus faecalis*. J Bacteriol. 141, 1366–1374

Tomich PK, An FY, Clewell DB. 1979. A transposon (*Tn*917) in *Streptococcus faecalis* that exhibits enhanced transposition during induction of drug resistance. Cold Spring Harbor Quant Biol. 43, 1217–1221

Toomey N, Bolton D, Fanning S. 2010. Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs. Res Microbiol. 161, 127–135

Toomey N, Monaghan A, Fanning S, Bolton D. 2009a. Transfer of antibiotic resistance marker genes between lactic acid bacteria in model rumen and plant environments. Appl Environ Microbiol. 75, 3146–3152

Toomey N, Monaghan A, Fanning S, Bolton DJ. 2009b. Assessment of horizontal gene transfer in Lactic acid bacteria - a comparison of mating techniques with a view to optimising conjugation conditions. J Microbiol Methods. 77, 23–28

Tyson GH, McDermott PF, Li C, Chen Y, Tadesse DA, Mukherjee S, Bodeis-Jones S, Kabera C, Gaines SA, Loneragan GH, Edrington TS, Torrence M, Harhay DM, Zhao S. 2015. WGS accurately predicts antimicrobial resistance in *Escherichia coli*. J Antimicrob Chemother. 70, 2763–2769

van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. 2014. Ten years of next-generation sequencing technology. Trends Genet. 30, 418–426

van Hoek AH, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJ. 2011. Acquired antibiotic resistance genes: an overview. Front Microbiol. 2, 1–27

van Hoek A, Margolles A, Domig KJ, Korhonen JM, Zycka-Krzesinska J, Bardowski JK, Danielsen M, Huys G, Morelli L, Aarts H. 2008. Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance. Int J Probiotics Prebiotics. 3, 271–280

van Reenen CA and Dicks LM. 2011. Horizontal gene transfer amongst probiotic lactic acid bacteria and other intestinal microbiota: what are the possibilities? A review. Arch Microbiol. 193, 157–168

Vannuffel P and Cocito C. 1996. Mechanism of action of streptogramins and macrolides. Drugs. 51, 20–30

Venema K and Meijerink M. 2015. Lactobacilli as Probiotics: Discovering New Functional Aspects and Target Sites. in: Probiotics and Prebiotics: Current Research and Future Trends. 29–42

Vera-Pingitore E, Jimenez ME, Dallagnol A, Belfiore C, Fontana C, Fontana P, von Wright A, Vignolo G, Plumed-Ferrer C. 2016. Screening and characterization of potential probiotic and starter bacteria for plant fermentations. Food Sci Technol. 71, 288–294

Verraes C, Van Boxstael S, Van Meervenne E, Van Coillie E, Butaye P, Catry B, de Schaetzen MA, Van Huffel X, Imberechts H, Dierick K, Daube G, Saegerman C, De Block J, Dewulf J, Herman L. 2013. Antimicrobial resistance in the food chain: a review. Int J Environ Res Public Health. 10, 2643–2669

von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, Savelkoul PHM, Wolffs PFG. 2016. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol. 7, 1–10

Wang N, Hang X, Zhang M, Liu X, Yang H. 2017. Analysis of newly detected tetracycline resistance genes and their flanking sequences in human intestinal bifidobacteria. Sci Rep. 7, 6267

Wang HH, Manuzon M, Lehman M, Wan K, Luo H, Wittum TE, Yousef A, Bakaletz LO. 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. FEMS Microbiol Lett. 254, 226–231

Wegener HC. 2003. Antibiotics in animal feed and their role in resistance development. Curr Opin Microbiol. 6, 439–445

Werner T. 2010. Next generation sequencing in functional genomics. Brief Bioinform. 11, 499–511

WHO – World Health Organization. 2011. Tackling antibiotic resistance from a food safety perspective in Europe. WHO Regional Office for Europe. 1–65

Wiegand I, Hilpert K, Hancock RE. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 3, 163–175

Wong A, Ngu DYS, Dan LA, Ooi A, Lim RLH. 2015. Detection of antibiotic resistance in probiotics of dietary supplements. Nutrition Journal. 14, 1–6

Wright LD and Grossman AD. 2016. Autonomous replication of the conjugative transposon *Tn*916. Journal of Bacteriology. 195, 2255–2266

Xavier BB, Das AJ, Cochrane G, De Ganck S, Kumar-Singh S, Aarestrup FM, Goossens H, Malhotra-Kumar S. 2016. Consolidating and Exploring Antibiotic Resistance Gene Data Resources. J Clin Microbiol. 54, 851–859

Yan M, Han J, Xu X, Liu L, Gao C, Zheng H, Chen Y, Tao Y, Zhou H, Li Y, Wu Z. 2016. Gsy, a novel glucansucrase from *Leuconostoc mesenteroides*, mediates the formation of cell aggregates in response to oxidative stress. Sci Rep. 6, 1–13

Yu L, Yan X, Wang L, Chu J, Zhuang Y, Zhang S, Guo M. 2012. Molecular cloning and functional characterization of an ATP-binding cassette transporter OtrC from *Streptomyces rimosus*. BMC Biotechnol. 12, 52

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 67, 2640–2644

Zapun A, Morlot C, Taha MK. 2016. Resistance to β-lactams in *Neisseria ssp* due to chromosomally encoded penicillin-binding proteins. Antibiotics. 5, 35

Zhao S, Tyson GH, Chen Y, Li C, Mukherjee S, Young S, Lam C, Folster JP, Whichard JM, McDermott PF. 2016. Whole-Genome Sequencing Analysis Accurately Predicts Antimicrobial Resistance Phenotypes in *Campylobacter* spp. Appl Environ Microbiol. 82, 459–466

Zheng J, Ruan L, Sun M, Gänzle M. 2015. A genomic view of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. Appl Environ Microbiol. 81, 7233–7243

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

Zonenschain D, Rebecchi A, Morelli L. 2009. Erythromycin- and tetracycline-resistant lactobacilli in Italian fermented dry sausages. J Appl Microbiol. 107, 1559–1568

Zuo FL, Feng XJ, Chen LL, Chen SW. 2014. Identification and partial characterization of lactic acid bacteria isolated from traditional dairy products produced by herders in the western Tianshan Mountains of China. Lett Appl Microbiol. 59, 549–556

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