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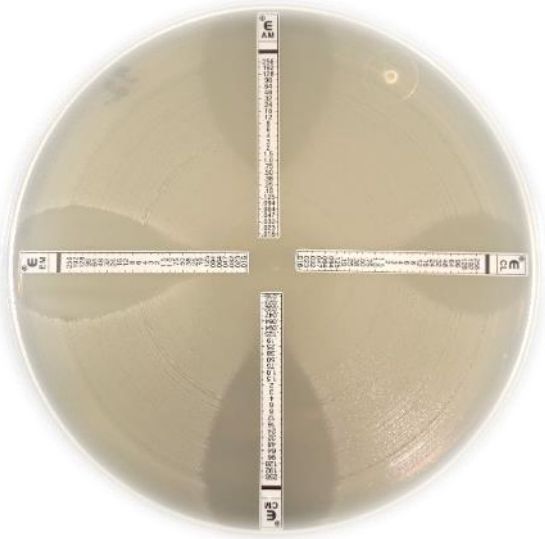
Antibiotic Susceptibility of Lactic Acid Bacteria



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Tiivistelmä/Referat – Abstract

Maitohappobakteereilla on pitkä käyttöhistoria elintarvikkeiden valmistuksessa johtuen niiden hyödyllisistä metabolisista ominaisuuksista sekä terveyttä edistävistä vaikutuksista. Pitkän käyttöhistoriansa vuoksi maitohappobakteereita pidetään turvallisina ja suurimmalla osalla niistä onkin FDA:n (U.S Food and Drug Administration) myöntämä GRAS (Generally Recognized As Safe) -status sekä EFSA:n (European Food Safety Authority) myöntämä QPS (Qualified Presumption of Safety) -status. Antimikrobiresistenssi on maailmanlaajuinen ongelma ja yhä useampi infektiosairaus on kasvavan resistenssin vuoksi vaikeampi hoitaa. Antimikrobiresistenssi on luonnollisesti suurin ongelma tautia aiheuttavissa mikrobeissa, mutta resistenssien lisääntyessä olisi hyvä ottaa huomioon myös ihmiselle hyödylliset mikrobit ja niiden mahdollinen kyky levittää geenejä ympäristöönsä ja myös patogeenisiin mikrobeihin. Fermentoidut elintarvikkeet luovat suotuisan ympäristön antimikrobisten geenien leviämiselle mm. sen vuoksi, että niissä voi olla korkeita määriä eläviä mikrobeja.

Tämän työn tarkoituksena oli selvittää *Lactobacillus rhamnosus*-, *Lactobacillus plantarum*-, *Leuconostoc* sp.- ja *Weissella* sp. -kantojen antibioottiherkkyyksiä ja etsiä kannoista mahdollisia antibioottiresistenssigeenejä. Kantojen fenotyyppistä antibioottiherkkyyttä tutkittiin E-testi -menetelmän avulla. Antibioottiherkkyyksiä testattiin kahdeksalle eri antibiootille, jotka olivat ampisilliini, kloramfenikoli, klindamysiini, erytromysiini, gentamisiini, kanamysiini, streptomysiini ja tetrasykliini. Kannoista etsittiin antibioottiresistenssigeenejä *blaZ*, *mecA*, *cat*, *lnuA*, *tetK* ja *tetM* spesifisillä PCR-menetelmillä. Antibioottiherkkyyksien selvittämisen lisäksi *Weissella* sp. -kannoille pyrittiin määrittämään raja-arvot (cut-off), joita sillä ei EFSA:n määrittämänä entuudestaan ole.

Fenotyyppisissä antibioottiherkkyysmäärityksissä suurimman osan testatuista kannoista huomattiin olevan resistenttejä kanamysiinille. *Leuconostoc* sp.- ja *L. rhamnosus* -kannoista huomattava osa osoittautui resistenteiksi myös kloramfenikolille. Tutkimuksessa yhden *L. rhamnosus* -kannan todettiin olevan resistentti sekä kloramfenikolille että klindamysiinille. Tämän lisäksi 48 %:lla testatuista *Leuconostoc* sp. -kannoista oli EFSA:n määrittämää raja-arvoa suurempi MIC-arvo streptomysiinille. Vaikka fenotyyppisissä määrityksissä havaittiinkin resistenssiä joillekin antibiooteille, etsittyjä resistenssigeenejä ei kuitenkaan löydetty yhdeltäkään testatuista maitohappobakteerikannoista. Tämä selittynee osittain sillä, että tutkimuksessa etsittiin vain pientä osaa tunnetuista antibioottiresistenssigeeneistä. EFSA:n asettamat raja-arvot ovat myös varsin tiukat, jolloin arvojen ylityksiä havaitaan helpommin. Tutkimuksessa saadut tulokset toivat paljon lisätietoa *L. rhamnosus*-, *L. plantarum*-, *Leuconostoc* sp.- ja *Weissella* sp. -kantojen antibioottiherkkyydestä ja niiden käyttöturvallisuudesta. Lisäksi tutkimuksessa pystyttiin määrittämään *Weissella* -kannoilta vielä puuttuvat cut-off-arvot.

Avainsanat – Nyckelord – Keywords

Antibioottiherkkyys, Maitohappobakteerit, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Leuconostoc*, *Weissella*

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Tiivistelmä/Referat – Abstract

Lactic acid bacteria have a long history of use in food industry due to their favorable metabolic properties and health benefits for human health. Therefore, they are generally recognized as safe (GRAS) by FDA (U.S Food and Drug Administration) and have QPS (Qualified Presumption of Safety) status granted by EFSA (European Food Safety Authority). Nowadays, antimicrobial resistance (AMR) is a serious global risk and due to the increasing AMRs, more and more microbial infections have become more difficult to treat with antibiotics. AMR has mainly been of concern in relation to pathogenic microbes. However, since fermented foods are favorable environments for AMR gene transfer it should also be considered in the context of beneficial bacteria and their potential to spread AMR genes into pathogenic microbes.

The aim of this study was to determine antibiotic susceptibilities of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Leuconostoc* sp. and *Weissella* sp. strains by E-test method and to detect selected specific antibiotic resistance genes by PCR. In addition, the goal was to define new cut-off values for *Weissella* strains since, so far, these have not been defined by EFSA. Antibiotic susceptibilities were determined against eight antibiotics: ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin and tetracycline. The detected AMR genes were *blaZ*, *mecA*, *cat*, *lnuA*, *tetK* and *tetM*.

Most of the determined strains were observed to exhibit a notable resistance to kanamycin. Several *Leuconostoc* sp. and *L. rhamnosus* strains showed also resistance to chloramphenicol. Interestingly, one *L. rhamnosus* strain was observed to exhibit multiresistance to chloramphenicol and clindamycin. Moreover, 48% Leuconostoc strains had higher MIC value for streptomycin than the cut-off value defined by EFSA. Any of the selected AMR genes were not detected even though a notable resistance during the phenotypic testing was observed. However, this might be explained by the small amount of detected AMR genes. The results obtained in the present study provided more information about the antibiotic susceptibility and the safety of *L. plantarum*, *L. rhamnosus*, *Leuconostoc* sp. and *Weissella* sp. strains. Moreover, new cut-off values were proposed for *Weissella* sp. strains.

Avainsanat – Nyckelord – Keywords
Antibiotic susceptibility, Lactic acid bacteria, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Leuconostoc*, *Weissella*

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Master's thesis was carried out and founded by VTT. It was supervised by Dr. Maria Saarela and Dr. Irina Tsitko.

1 Introduction

Antimicrobials including antibiotics, antifungals, antivirals and antiprotozoals, are substances that may kill or inhibit microbial growth [1]. Generally, antimicrobials are used for the treatment of microbial infections in humans and animals [1]. In agriculture, antimicrobials have also been used for the prevention of diseases in healthy animals but also for treatment and prevention of plant diseases [2, 3]. Moreover, some antimicrobials used against plant infections, such as tetracyclines and streptomycin, are also used to treat humans and animals [3]. In certain countries, including US, antimicrobials are also used as growth promoters although this has been banned in the EU since 2006 [2].

Antimicrobial resistance (AMR) is a serious global risk for human health [4]. Increased occurrence of resistant microbes is largely due to the misuse of antimicrobials in health care and agriculture [1, 3]. Fast action against AMR is essential since otherwise the treatment of increasing number of microbial infections will be much more challenging [1]. European Commission has accepted the EU one health action plan against antimicrobial resistance which forms the basis for wider action against AMR. The main aims of the action plan are to encourage the research and development of new antimicrobials and to provide novel knowledge and solutions for the treatment of microbial infections [1]. The goal is also to be a pioneer in preventing the spread of AMR and to form a worldwide plan against AMR [1].

Antimicrobial resistance can be either intrinsic or acquired. Intrinsic resistance occurs inherently in the strains of certain species and it is generally assumed to be caused by either active efflux pumps or reduced permeability of the bacterial outer membrane [5]. In the case of intrinsic resistance the risk of transferring AMR genes to other microbes is not considered to be as high as in acquired resistance [6]. Microbes that are inherently susceptible to a certain antibiotic can acquire resistance via a genetic mutation or gene transfer from one microbe to another [7, 8]. Gene transfer is not depended on species or genus lineages and therefore acquired resistance genes do not occur in all strains of a certain species [7, 9]. This is the hallmark of acquired resistance. Gene transfer over the species and genus borders is also known as horizontal gene transfer where the genes are typically located on mobile genetic elements such as plasmids and transposons [6, 8, 9]. Gene cassettes and integrons play an essential role in spreading of antimicrobial resistance genes due to the integrons ability to capture and express multiple gene cassettes with antimicrobial resistance genes [10,

11]. The principal mechanisms of microbial genetic material transfer are conjugation, transduction and transformation [8]. In transformation the lysis of donor cells releases free genetic material which can be incorporated into the resipient cell genome [12]. In transduction the genes are transmitted from one bacterial cell to another by bacteriophages while in conjugation the genetic material, mainly plasmids, is transferred from donor cell to resipient cell by cell-to-cell contact [12].

Acquired AMR resistance is a serious issue in pathogenic bacteria, but it should also be considered in the context of beneficial bacteria (starters, probiotics, protective cultures). Bacteria that are used in food/feed industry and agriculture (for example lactic acid bacteria, bifidobacteria, bacilli, enterococci) can also carry and spread acquired AMR genes and therefore determining their potential to carry acquired AMR genes is important [13]. The use of strains with acquired AMR genes should be avoided to prevent the further spread of the genes. Fermented foods are favorable environments for gene transfer due to the presence of large number of living bacterial cells and the presence of multiple stress factors such as low pH and antimicrobial compounds, such as lactic acid and other organic acids, antifungal peptides and bacteriocins [14].

Lactic acid bacteria (LAB) are gram-positive, nonsporulating, catalase- and oxidase-negative typically cocci- or rod-shaped bacteria that are generally defined by their ability to produce lactic acid as a major or sole fermentation end product [12]. The majority of lactic acid bacteria belong to the phylum *Firmicutes* [12]. This diverse group of bacteria consists of various phylogenetic branches including for instance the genera of *Lactobacillus*, *Lactococcus, Leuconostoc* and *Weissella* [15, 16].

Lactic acid bacteria have been widely used in the food industry for several decades and they are generally recognized as safe (GRAS) by the FDA (U.S Food and Drug Administration) [13]. In addition, many LAB species have QPS (Qualified Presumption of Safety) status defined by EFSA (European Food Safety Authority) [17]. QPS status is granted for microbes with sufficient evidence of their safe consumption and therefore microbes with QPS status do not need to go through the full safety assessment [17]. Lactic acid bacteria are important dairy starter cultures and play an essential role in the production of fermented food products [9]. In addition, these bacteria are often used as probiotics. World Health Organization (WHO) has defined probiotics as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” [18]. The interest in producing foods with health benefits is constantly increasing and therefore LAB, such as

Lactobacillus strains, are added in to the functional food products [12]. The reported health benefits of probiotics are usually connected to the enhancement of intestinal health that includes balancing of intestinal microbiota. A healthy intestinal microbiota have been described to be beneficial to the immune system [19]. In addition, the studies has reported that the consumption of probiotics may have beneficial effects for instance in the prevention of antibiotic associated diarrhea and allergic diseases [20, 21].

Lactic acid bacteria grow under anaerobic or micro-aerophilic conditions [22]. Most LAB species are aerotolerant meaning that they are not very sensitive to oxygen (O_2) [23]. Lactic acid bacteria generally obtain energy only via fermentation of sugars and therefore they usually grow in sugar-rich environments [12]. Lactic acid bacteria can utilize carbohydrates by either homolactic or heterolactic fermentation [22]. In homolactic fermentation, hexose is converted to lactic acid and hydrogen (H_2) [12]. This metabolic pathway is typical for *Lactococcus* and *Pediococcus* species and also for some *Lactobacillus* species [24, 25]. Heterolactic fermentation produces ethanol or/and acetic acid, carbon dioxide (CO_2) and hydrogen (H_2) besides lactic acid from a single hexose molecule [12, 26]. This type of fermentation is typical for instance in *Leuconostoc* species [27].

Lactobacillus species are Gram-positive, non-spore forming and generally rod-shaped bacteria that utilize carbohydrates by either homolactic or heterolactic fermentation [26]. They are catalase-negative and usually non-motile [26]. *Lactobacillus* species have many nutritional requirements due to the need of additional energy and carbon sources, such as amino acids and vitamins, besides carbohydrates [26, 28]. Usually, the optimal growth temperature of *Lactobacillus* species varies between 30 and 40 °C and the optimal pH range is 5.5-6.2 [28, 29]. *Lactobacillus* species are aerotolerant but they typically grow in anaerobic environments [29]. The most common growing environments are dairy, grain and meat products as well as beer, wine and fruit juices [28]. In addition, some *Lactobacillus* species also belong to the resident microbiota of human and animal mouth and intestinal track [28]. *Lactobacillus* species have a long history of use in the food industry due to their efficacy in fermenting foods and potential to improve their structure, sensory properties, and storage stability. However, contaminating lactobacilli can sometimes turn into spoilage organisms especially if their metabolisms causes undesired changes in the sensory properties of the foods [28, 29].

Leuconostoc species are phylogenetically close to the genus *Lactobacillus* [30]. They are Gram-positive, non-motile and coccoid-shaped bacteria, which typically grow at the

temperatures between 20 and 30 °C in the pH range 6 to 7 [31]. *Leuconostoc* species grow in various environments for instance in dairy products, meat, and fermented vegetable products. [30, 31]. Besides their importance in the fermentation process, especially as acidity and flavor producers, *Leuconostoc* species may also cause food spoilage [30, 31].

Phylogenetically close to *Leuconostoc* genus is the genus *Weissella*, which also belongs to the family of *Leuconostocaceae* [32]. *Weissella* species are Gram-positive, catalase-negative, generally non-motile and non-spore-forming bacteria, which are shaped as rods or ovoid. Typically, their growth occurs between 15-45 °C depending on the strain [33]. Various species of *Weissella* grow in diverse habitats including environment (soil, sediments), plants, and fermented, mainly milk and plant based, foods. They can also be detected in e.g. saliva and faces of humans and animals [32, 34]. *Weissella* species are facultatively anaerobic and utilize glucose by heterolactic fermentation [32]. One of the main advantages in the metabolism of *Weissella* species is the production of exopolysaccharides, such as dextran, which have variable applications in food industry and especially in the fermentation of cereal-based products [34].

EFSA has a guidance for the safety assessment (including the presence of acquired AMR genes) of microbes used as feed additives or as production organisms in the EU [35]. According to this guidance the safety of the strain may be ensured by proper characterization including the strain identification, determination of antimicrobial susceptibilities and antimicrobial production and also the information about toxigenicity and pathogenicity, based on species specific requirements [35]. It can be assumed that in the future this guidance will most likely be applied also to microbes used in foods in the EU. EFSA has defined cut-off values to certain antimicrobials with the purpose of helping to identify the strains carrying acquired AMR genes [35]. The phenotypic antimicrobial susceptibility is determined by defining the MIC (Minimum Inhibitory Concentration) values for the antimicrobials listed in the guidance. The strains having higher MIC values than the defined cut-off values may carry acquired AMR genes requiring more investigation. The antimicrobials for which EFSA has defined cut-off values for lactic acid bacteria include ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin. The sites of inhibition and modes of action of these antibiotics are displayed in Table 1. In addition, the cut-off values defined by EFSA for *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Leuconostoc* spp. are presented in Table 2. For *Weissella* there are currently no EFSA's cut-off values.

Table 1 Antibiotic groups relevant in present study and the their modes of action [13, 36]

Site of action	Group	Antibiotic	Mode of action	
Cell wall synthesis	β-lactams	Ampicillin	Interaction with penicillin binding proteins (PBPs)	Disruption of peptidoglycan layer and cell lysis
	Glycopeptides	Vancomycin	Interaction with D-alanyl-D-alanine termini of peptidoglycan chain	Prevent the binding of D-alanyl subunit with the PBP
Protein synthesis	Aminoglycosides	Gentamicin	30S ribosomal subunit	Misreadings and premature termination of mRNA translation
		Kanamycin		
		Streptomycin		
	Chloramphenicols	Chloramphenicol	50S ribosomal subunit	Prevent binding of t-RNA to the A site
	Lincosamides	Clindamycin	50S ribosomal subunit	Affects the peptidyl transferase reactions resulting premature detachment of incomplete peptide chains
	Macrolides	Erythromycin	50S ribosomal subunit	Affects the peptidyl transferase reactions resulting premature detachment of incomplete peptide chains
	Tetracyclines	Tetracycline	30S ribosomal subunit	Prevent binding of t-RNA to the A site

Table 2. Microbiological cut-off values (μg mL^{-1}) for *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Leuconostoc* spp. defined by EFSA (EFSA 2018). n.r: not required

	Antibiotic (μg mL^{-1})							
LAB groups	**Ampicillin**	**Chloramphenicol**	**Clindamycin**	**Erythromycin**	**Gentamicin**	**Kanamycin**	**Streptomycin**	**Tetracycline**
Lactobacillus plantarum	2	8	4	1	16	64	n.r.	32
Lactobacillus rhamnosus	4	4	4	1	16	64	32	8
Leuconostoc spp.	2	4	1	1	16	16	64	8

The aim of this study was to characterize and evaluate the safety of *Lactobacillus*, *Leuconostoc* and *Weissella* strains by determining the MIC values of selected strains against eight antibiotics and by studying the presence of certain specific antibiotic resistance genes in the strains. In addition, the goal was also to try to set new cut-off values for *Weissella* and find out if the determined MIC values relate to the presence of antibiotic resistance genes in the strains.

2 Materials and methods

2.1 Bacterial strains and growth conditions

In total, 105 lactic acid bacterial strains representing the genera *Lactobacillus* (53), *Leuconostoc* (29) and *Weissella* (22) were selected for antibiotic susceptibility testing from the culture collection of VTT. The selected strains and their origins are shown in Table 3. The strains were cultivated on MRS medium (CM0361, Oxoid, UK) and incubated anaerobically (10 % (v/v) H_2, 5 % (v/v) CO_2 and 85 % (v/v) N_2) or in microaerophilic conditions (6 % (v/v) O_2) for 24 h at 25-37 °C depending on the strain. The favorable growing atmospheres were obtained by using Anoxomat (Mart Microbiology B.V., The Netherlands).

The correct identification of the strains was confirmed by MALDI Biotyper 4.1 system (Microflex LT/SH, Bruker Daltonics, Germany). For this, the bacteria were cultivated on MRS medium after which the identification was performed by direct colony method by following the manufacturer's instructions.

Table 3 Lactic acid bacteria strains and origins

VTT culture collection strain code	**Species**	**Origin/Source**	**Additional Info**
E-78076	*Lactobacillus plantarum*	Beer	
E-78079	*Lactobacillus plantarum*	Beer	
E-91468	*Lactobacillus plantarum*	Soft drink	
E-94566	*Lactobacillus plantarum*	Orange soft drink extract	
E-95618	*Lactobacillus plantarum*	Sour dough seed	
E-96608	*Lactobacillus plantarum*	Sour dough	
E-981065	*Lactobacillus plantarum*	Unknown	
E-981138	*Lactobacillus plantarum*	Brewery	
E-991158	*Lactobacillus plantarum*	Brewery	
E-991159	*Lactobacillus plantarum*	Brewery	
E-011800	*Lactobacillus plantarum*	Brewery	
E-032411	*Lactobacillus plantarum*	Beer	
E-062634	*Lactobacillus plantarum*	Brewery	
E-093106	*Lactobacillus plantarum*	Sour whole milk	
E-09683	*Lactobacillus plantarum*	Silage	DSM 2648
E-103137	*Lactobacillus plantarum*	Plant material	
E-133328	*Lactobacillus plantarum*	Cheese	

E-183579	*Lactobacillus plantarum*	Human saliva	
E-71034	*Lactobacillus plantarum*	Unknown	DSM 20205
E-093129^{T}	*Lactobacillus plantarum* subsp. *argentoratensis*	Fermented cassava roots (fufu)	DSM 16365
E-79098^{T}	*Lactobacillus plantarum* subsp. *plantarum*	Pickled cabbage	DSM 20174
E-78080*	*Lactobacillus rhamnosus*	Beer	
E-93444*	*Lactobacillus rhamnosus*	Unknown	ATCC 11443
E-96031^{T}	*Lactobacillus rhamnosus*	Unknown	DSM 20021
E-96666	*Lactobacillus rhamnosus*	Human faeces	ATCC 53103
E-97763	*Lactobacillus rhamnosus*	Ethanol fermentation	
E-97800*	*Lactobacillus rhamnosus*	Human faeces	
E-97948*	*Lactobacillus rhamnosus*	Human biopsy sample	
E-97951*	*Lactobacillus rhamnosus*	Human biopsy sample	
E-97959*	*Lactobacillus rhamnosus*	Human biopsy sample	
E 97960*	*Lactobacillus rhamnosus*	Human biopsy sample	
E 97962*	*Lactobacillus rhamnosus*	Human biopsy sample	
E-981000*	*Lactobacillus rhamnosus*	Human biopsy sample	
E-001125	*Lactobacillus rhamnosus*	Unknown	NCIMB 10463
E-052739	*Lactobacillus rhamnosus*	Infant faeces	
E-052740	*Lactobacillus rhamnosus*	Infant faeces	
E-052741	*Lactobacillus rhamnosus*	Infant faeces	
E-093103	*Lactobacillus rhamnosus*	Human faeces	
E-183563*	*Lactobacillus rhamnosus*	Human saliva	
E-183564*	*Lactobacillus rhamnosus*	Human faeces	
E-183565*	*Lactobacillus rhamnosus*	Human saliva	
E-183566*	*Lactobacillus rhamnosus*	Human saliva	
E-183567*	*Lactobacillus rhamnosus*	Human faeces	
E-183568*	*Lactobacillus rhamnosus*	Human saliva	
E-183569*	*Lactobacillus rhamnosus*	Human faeces	
E-183570*	*Lactobacillus rhamnosus*	Human faeces	
E-183571*	*Lactobacillus rhamnosus*	Human faeces	
E-183572*	*Lactobacillus rhamnosus*	Human saliva	
E-183573*	*Lactobacillus rhamnosus*	Human faeces	
E-183574*	*Lactobacillus rhamnosus*	Human faeces	
E-183575*	*Lactobacillus rhamnosus*	Human saliva	
E-183576*	*Lactobacillus rhamnosus*	Human	
E-183577*	*Lactobacillus rhamnosus*	Human	
E-183578	*Lactobacillus rhamnosus*	Human saliva	
E-90389	*Leuconostoc citreum*	Split kernel of barley	
E-90415	*Leuconostoc citreum*	Split kernel of barley	
E-91451	*Leuconostoc citreum*	Barley malt	
E-91452	*Leuconostoc citreum*	Barley malt	

E-93497	*Leuconostoc citreum*	Malting process	
E-93504^{T}	*Leuconostoc citreum*	Honey dew of rye ear	DSM 5577
E-981082	*Leuconostoc citreum*	Processed oat	
E-093125	*Leuconostoc citreum*	Sugar solutions and refineries	DSM 20188
E-143382	*Leuconostoc citreum*	Barley	
E-153421	*Leuconostoc gelidum*	Packaged broiler chicken cuts	
E-153484	*Leuconostoc kimchii*	Spontaneous faba bean fermentation	
E-98974^{T}	*Leuconostoc lactis*	Milk	DSM 20202
E-032298	*Leuconostoc lactis*	Syrup sample	
E-011779	*Leuconostoc mesenteroides*	Immobilized main beer fermentation	
E-093124	*Leuconostoc mesenteroides*	Sugar refineries	ATCC 11449
E-91461^{T}	*Leuconostoc mesenteroides* subsp. *mesenteroides*	Fermenting olives	DSM 20343
E-062512	*Leuconostoc mesenteroides* subsp. *mesenteroides*	Root beer	ATCC 10830A
E-143337	*Leuconostoc mesenteroides* subsp. *mesenteroides*	Organic carrot	
E-143338	*Leuconostoc mesenteroides* subsp. *mesenteroides*	Organic carrot	
E-143339	*Leuconostoc mesenteroides* subsp. *mesenteroides*	Organic carrot	
E-143340	*Leuconostoc mesenteroides* subsp. *mesenteroides*	Organic carrot	
E-98970^{T}	*Leuconostoc pseudomesenteroides*	Cane juice	DSM 284
E-981034	*Leuconostoc pseudomesenteroides*	Aromatic mineral water	
E-001383	*Leuconostoc* sp.	Beer	
E-143385	*Leuconostoc* sp.	Oat	
E-143386	*Leuconostoc* sp. *(citreum?)*	Oat	
E-143384	*Leuconostoc* sp. *(mesenteroides?)*	Oat	
E-143381	*Leuconostoc* sp. *(pseudomesenteroides)*	Barley	
E-143383	*Leuconostoc* sp. *(pseudomesenteroides)*	Barley	
E-072749	*Weissella cibaria*	Fermented wheat bran	
E-153485	*Weissella cibaria*	Faba bean fermentation	
E-163495	*Weissella cibaria*	Celery	
E-163497	*Weissella cibaria*	Faba bean flour	
E-082762^{T}	*Weissella cibaria*	Chili bo	DSM 15878
E-90392	*Weissella confusa*	Soured carrot mash	DSM 20194
E-143403	*Weissella confusa*	Protein-rich fraction of faba bean	
E-153454	*Weissella confusa*	Plums	
E-153455	*Weissella confusa*	Plums	
E-153457	*Weissella confusa*	Brussel sprouts	

E-153458	*Weissella confusa*	Brussel sprouts	
E-153459	*Weissella confusa*	Non peeled rye bran	
E-153460	*Weissella confusa*	Non peeled rye bran	
E-153461	*Weissella confusa*	Figs	
E-163496	*Weissella confusa*	Raw milk	
E-90393^{T}	*Weissella confusa*	Sugar cane	DSM 20196
E-072748	*Weissella* sp.	Fermented wheat bran	
E-072750	*Weissella* sp.	Fermented wheat bran	
E-083076	*Weissella* sp.	Fermented wheat bran	
E-153482	*Weissella* sp.	Faba bean fermentation	
E-072747	*Weissella viridescens*	Wheat bran	
E-98966^{T}	*Weissella viridescens*	Cured meat products	DSM 20410

T= Type strain, *= The MIC values for AM, CM, EM, GM, SM and TC were defined by Korhonen et al. (2010) . The values were used in the present study to obtain more comprehensive data sets.

2.2 Determination of MIC values

The MIC values of the eight antibiotics used in the EFSA's assessment were defined by Etest® method according to guidelines of the manufacturer [37]. The antibiotics used in this study were ampicillin (AM), chloramphenicol (CL), clindamycin (CM), erythromycin (EM), gentamicin (GM), kanamycin (KM), streptomycin (SM) and tetracycline (TC) (bioMérieux, France). The concentration range of the selected antibiotics was 0.16-256 μg mL^{-1} except for streptomycin it was 0.064-1024 μg mL^{-1}.

The colonies of selected strains were suspended in 5 mL of sterile 0.85 % (w/v) NaCl solution and the suspension corresponding to McFarland 1 was applied on LSM agar (90 % (v/v) Iso-sensitest broth, 10 % (v/v) MRS broth and 1.5 % (w/v) agar (Klare *et al*, 2005)). The plates containing the antibiotic strips were incubated anaerobically or in microaerophilic conditions for 48 h at 25-37 °C depending on the strain. After 48 h incubation the MIC values were determined as the concentration value were no growth was observed according to guidelines of the manufacturer given for each antibiotic.

For certain *L. rhamnosus* strains of VTT's culture collection the MIC values to AM, CM, EM, GM, SM and TC were already determined in the previous study [39]. These strains are shown in Table 3. The MIC values detected in Korhonen et al. (2010) study were used in the present study to obtain more comprehensive data set for *Lactobacillus rhamnosus* species.

The proposed cut-off values for *Weissella* sp. strains were defined by visual and statistical analysis. The statistical cut-off values were determined by using ECOFFinder (v. 2.0) program which estimates the cut-off values from the data set [40].

2.3 DNA extraction and detection of antibiotic resistance genes

Genomic DNA from the studied strains were extracted by using a commercial DNA extraction kit (NucleoSpin® Microbial DNA, Macherey-Nagel, Germany) and the concentrations of isolated DNA were then estimated by using NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Selected antibiotic resistance genes were detected by PCR (Polymerase Chain Reaction). Targeted AMR genes were selected based on the results of MIC determinations and phenotypic resistance patterns. The chosen AMR genes expressed resistance to ampicillin (*blaZ*: LS483313.1, *mecA:* KC243783*)*, chloramphenicol (*cat:* CP019573.1), clindamycin (*lnuA*: EU596446.1*)* and tetracycline (*tetM*: CP018888.1, *tetK:* NC_013452.1). These genes were selected based on the results of previous antibiotic susceptibility studies for lactic acid bacteria [41–43]. 16S rRNA gene amplification was performed to ensure the quality of the DNA extractions and the absence of PCR inhibitors. Positive controls for *blaZ*, *mecA*, *lnuA* and *tetM* genes were found by using CARD and MEGARes databases with Blastn 2.8.1+ search [44–46]. Two positive control strains (*Staphylococcus aureus* ssp. *aureus*, DSM 11822 and *Staphylococcus simulans*, DSM 20322) had other resistance genes as well since *cat* or *tetK* genes were detected from these strains. The presence of *cat* and *tetK* genes in the strains was confirmed by sequencing of the amplicons. Raw sequences were quality checked and consensus was built by using Geneious (version 6.1.8) software. The obtained sequences were compared to those included in to CARD database by using BLAST search.

The specific primers for AMR genes, product sizes, primer references, PCR programs, annealing temperatures and positive controls are shown in Table 4. PCR reactions was performed in 15 µl volumes containing 5.4 µl sterile H_2O, 7.5 µl 2x MyTaq^{TM} Red Mix (BIO-25043, Bioline, UK), 0.2 µM of each gene specific primers (Sigma-Aldrich, USA) and 1.5 µl of DNA template. PCR amplifications were done by using Mastercycler® Gradient thermal cycler (Eppendorf, Germany). PCR products were stained with Midori Green Advance (MG 04, Nippon Genetics, Japan) and analyzed by gel electrophoresis (120 V, 2 h) on 1 % agarose gel in 0.5 x TBE buffer (161-0770, Bio-rad, Germany). 1 Kb DNA Ladder

(15615-016, Invitrogen) and GeneRuler 100 bp Plus DNA Ladder (SM0321, Thermo Scientific) were used for the evaluation of the fragment sizes of the PCR products.

Table 4 Gene-specific primers, primer references, product sizes, annealing temperatures, used PCR programs and used positive controls for PCR detection

Antibiotic	**Gene**	**Primer sequence (5'-3')**	**Product size (bp)**	**Primer reference**	**Annealing temperature (°C)**	**PCR program**	**Positive control strains**
Ampicillin	*blaZ*	f-CAGTTCACATGCCAAAGAG r-TACACTCTTGGCGGTTTC	846	[47]	54	95 °C: 3 min, 95 °C: 30s, 54 °C: 30s, 72 °C: 1 min, 72 °C: 10 min, 30 cycles	*Staphylococcus simulans*, DSM 20322
	mecA	f-GGGATCATAGCGTCATTATTC r-AGTTCTGCAGTACCAGATTTGC	1429	[48]	58	95 °C: 3 min, 95 °C: 30s, 58 °C: 30s, 72 °C: 30 s, 72 °C: 10 min, 30 cycles	*Staphylococcus aureus* ssp. *aureus*, DSM 11822
Tetracycline	*tetM*	f-GAACTCGAACAAGAGGAAAGC r-ATGGAAGCCCAGAAAGGAT	740	[49]	60	95 °C: 3 min, 95 °C: 30s, 60 °C: 30s, 72 °C: 1 min, 72 °C: 10 min, 30 cycles	*Lactobacillus amylophilus*, DSM 20533
	tetK	f-TTATGGTGGTTGTAGCTAGAAA r-AAAGGGTTAGAAACTCTTGAAA	348	[50]	55	95 °C: 3 min, 95 °C: 30s, 55 °C: 30s, 72 °C: 1 min, 72 °C: 10 min, 30 cycles	*Staphylococcus simulans*, DSM 20322
Clindamycin	*lnuA (=linA)*	f-GGTGGCTGGGGGGTAGATGTATTAACTGG r-GCTTCTTTTGAAATACATGGTATTTTTCGATC	323	[51]	57	94 °C: 3 min, 94 °C: 30s, 57 °C: 30s, 72 °C: 30 s, 72 °C: 10 min, 30 cycles	*Lactobacillus reuteri*, ATCC 55730
Chloramphenicol	*cat*	f-TTAGGTTATTGGGATAAGTTA r-GCATGRTAACCATCACAWAC	300	[52]	54	95 °C: 3 min, 95 °C: 30s, 54 °C: 30s, 72 °C: 45 s, 72 °C: 10 min, 30 cycles	*Staphylococcus aureus* ssp. *aureus*, DSM 11822

3 Results

3.1 Identification of lactic acid bacterial strains

Since the strains were selected from the culture collection and some strains had been identified to the species level more than 10 years ago by partial 16S rRNA gene sequencing, MALDI TOF based identification of the strains was performed. This was also done in order to check the purity of the strains.

In general, the species-level confirmation was detected in the genera *Lactobacillus* and *Leuconostoc*. For *Weissella* strains mainly genus-level identification were obtained due to the small number of reference strains in the MALDI Biotyper database.

3.2 MIC determinations

The MIC values were determined at the concentration value where no growth was observed. In Figure 1 the inhibition zones of *Leuconostoc citreum* E-93497 strain are displayed to illustrate the E-test method.

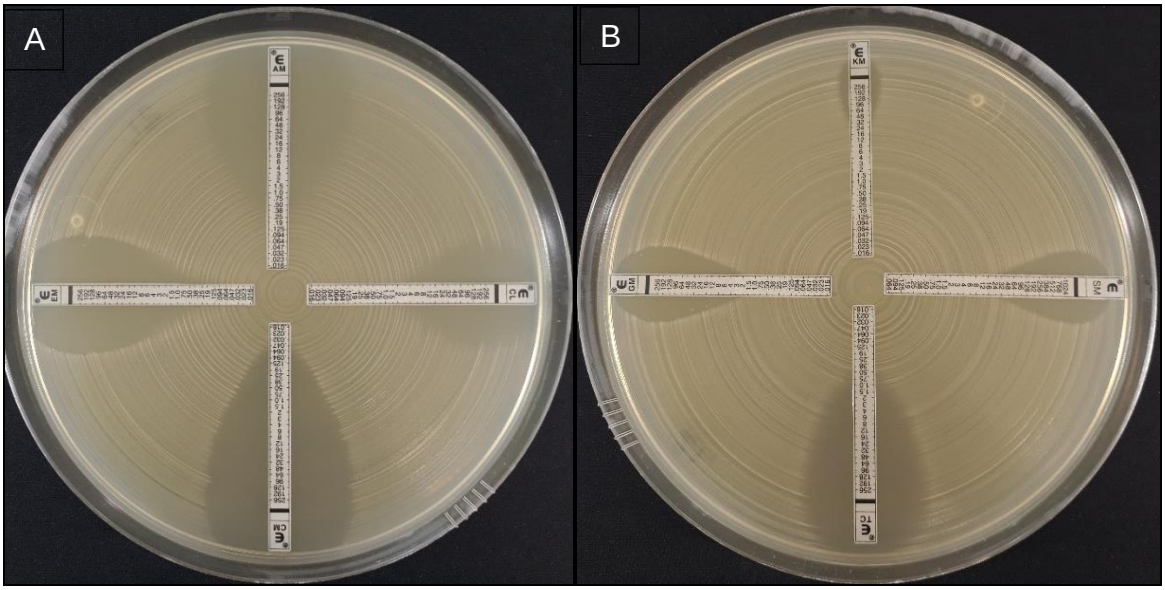

Figure 1 Inhibition zones of *Leuconostoc citreum* E-93497 strain for A: ampicillin, chloramphenicol, clindamycin, erythromycin and B: gentamicin, kanamycin, streptomycin and tetracycline

MIC distributions of ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin and tetracycline for *L. plantarum*, *L. plantarum*, *Leuconostoc* sp. and *Weissella* sp. strains are displayed as histograms in Figures 2-9. The determined values are also summarized in Annex 1. The strain was considered as resistant if the determined MIC value was much higher than the cut-off value defined by EFSA. In addition, significantly different MIC values in the end of the concentration range are generally assumed to indicate acquired resistance.

MIC distributions of ampicillin are shown in Figure 2. Generally, the distributions are following the normal distribution and no clearly resistant strains were detected. However, an additional peak at the MIC value of 2 µg mL^{-1} in the *Leuconostoc* sp. histogram was observed. The strains with MIC value of 2 µg mL^{-1} or higher represented *L. mesenteroides*. In addition, MIC value (2 µg mL^{-1}) of one *Weissella* strain (*Weissella cibaria* E-153485) was slightly higher than the other determined MIC values.

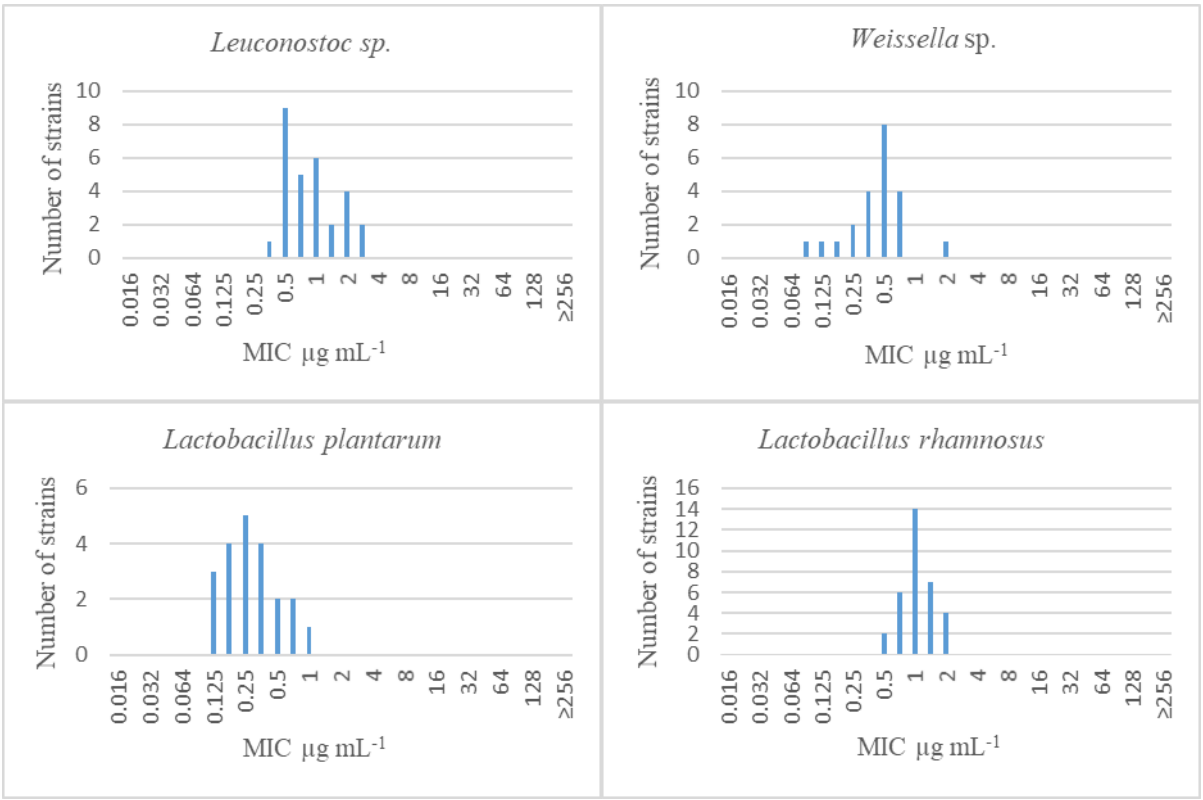

Figure 2. Ampicillin MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

MIC distributions of chloramphenicol seemed to conform to a normal distribution and no big variations in the MIC values were observed with the exception of one *L. rhamnosus* strain (E-001125) which exhibited clear resistance to chloramphenicol (Figure 3).

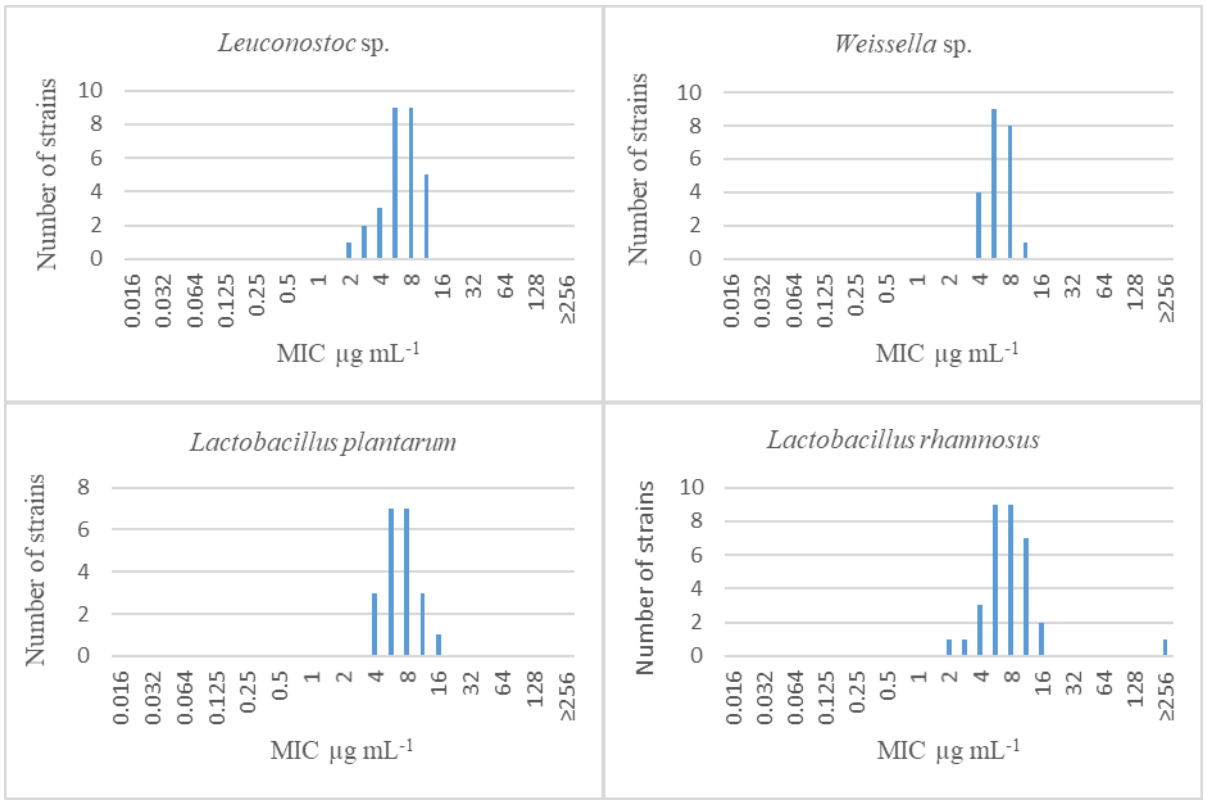

Figure 3. Chloramphenicol MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

Clindamycin MIC distributions are displayed in Figure 4. Generally, *Leuconostoc* sp. and *Lactobacillus rhamnosus* strains were susceptible to clindamycin and the determined MIC values followed a normal distribution. However, six *Leuconostoc* strains (*Leuconostoc lactis* E-032298, *Leuconostoc citreum* E-91451, *Leuconostoc* sp. (pseudomesenteroides) E-143383, *Leuconostoc pseudomesenteroides* E-98970T, *Leuconostoc* sp. (pseudomesenteroides) E-143381, *Leuconostoc* sp. E-143385) had higher MIC values (16, 32, and ≥256 µg mL^{-1}). In addition, one *L. rhamnosus* strain (E-001125) showed clear resistance to clindamycin with the MIC value of ≥256 µg ml^{-1}.

Clindamycin MIC distributions of *Weissella* sp. and *L. plantarum* strains were more challenging to analyze due to the larger variation in the MIC values. As a result, the obtained MIC distributions were not following the normal distribution very well. In the case

of *L. plantarum*, six strains were more susceptible to clindamycin (0.023-0.047 µg mL^{-1}) and two strains (*L. plantarum* E-96608, *L. plantarum* E-981065) were more resistant with MIC value of 24 µg mL^{-1}. A similar phenomenon was detected in the MIC distribution of *Weissella* sp. strains as the MIC values varied between 0.032 and 4 µg mL^{-1}.

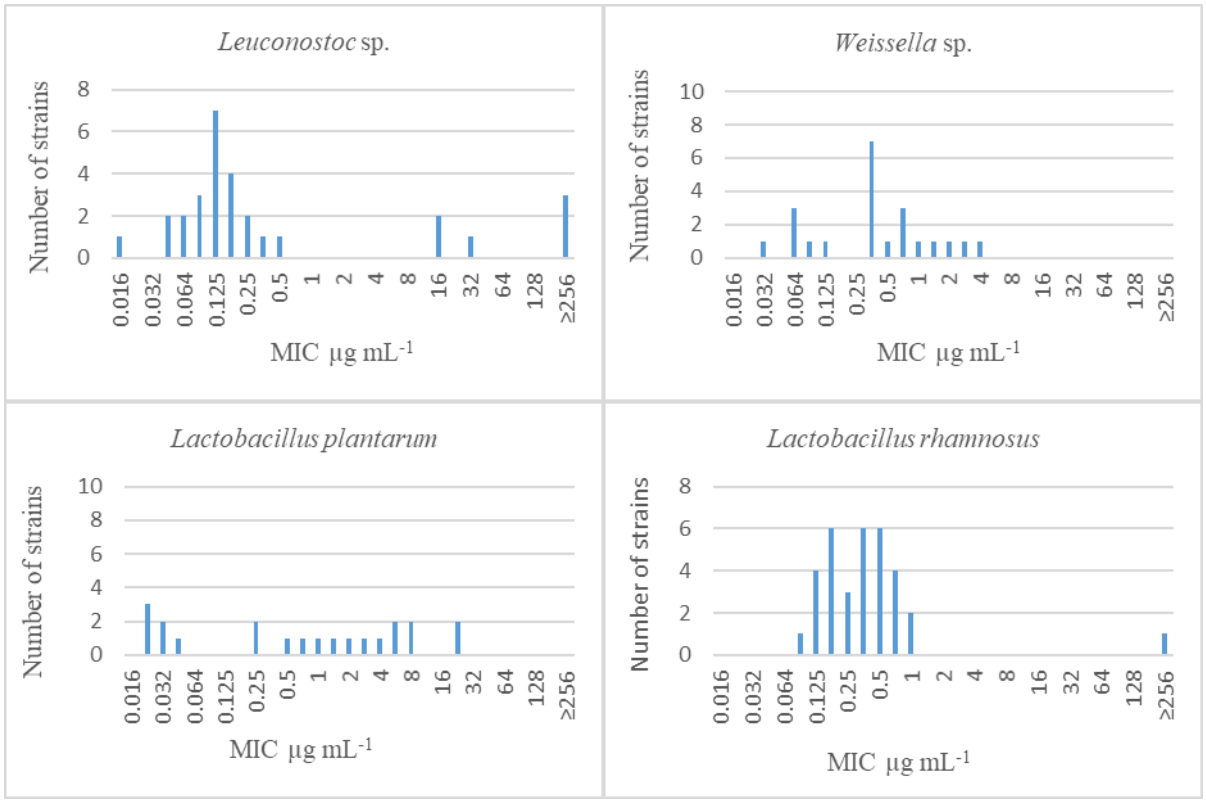

Figure 4. Clindamycin MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

Concerning erythromycin, the distributions of *Leuconostoc* sp., *Weissella* sp. and *L. plantarum* strains appeared to conform the normal distribution and no significant resistances could be observed (Figure 5). A bimodal distribution, although separated by a single dilution, was detected in the distribution of *L. rhamnosus* strains.

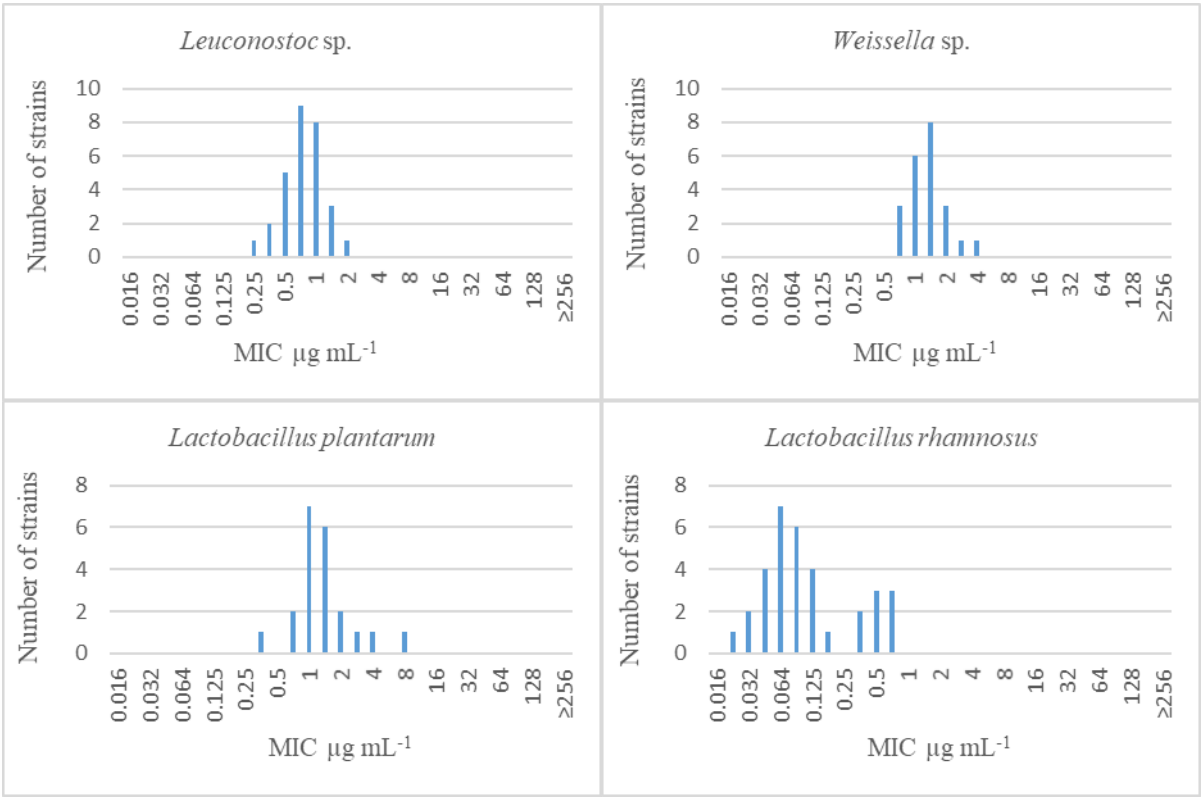

Figure 5. Erythromycin MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

Gentamicin MIC distributions are displayed in Figure 6. Generally, the distributions appeared to follow a normal distribution. Eight *Leuconostoc* sp. strains, which were representing several species, formed an additional high peak (MIC value of 8 µg mL^{-1}) at the end of the distribution. In addition, one *Weissella* sp. strain (*W. cibaria* E-163495) had slightly higher MIC value (MIC value of 16 µg mL^{-1}) than the other strains.

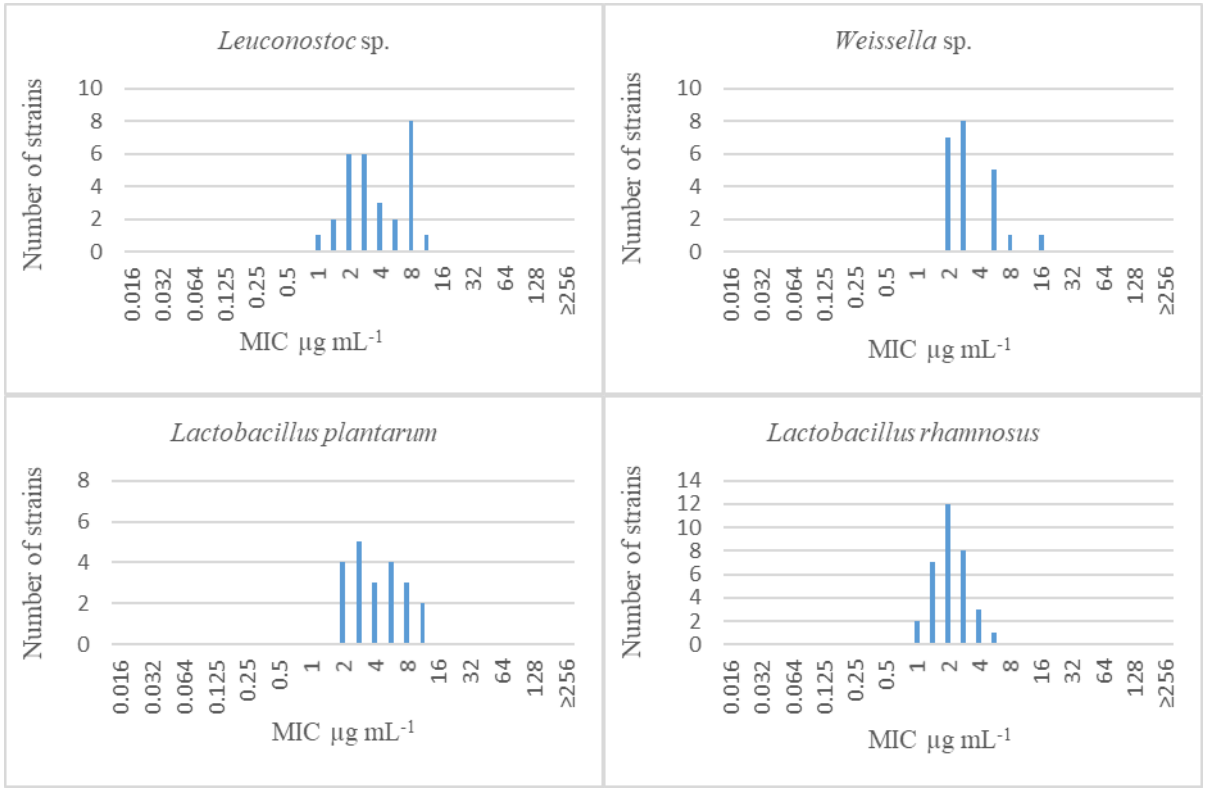

Figure 6. Gentamicin MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

Most of the determined strains exhibited resistance to kanamycin to a certain extent (Figure 7). MIC distributions of *Leuconostoc* sp. and *Weissella* sp. strains conformed to a normal distribution, although several strains (12 *Leuconostoc* sp. and four *Weissella* sp. strains) showed clear resistance to kanamycin (MIC ≥ 256 µg mL^{-1}). In addition, four *Weissella* strains (two *W. cibaria*: E-082762T, E-153485 and two *W. confusa*: E-153459, E-153460) with a MIC value of 64 µg mL^{-1} formed an additional high peak at the end of the distribution. In contrast, only four *L. plantarum* strains were somewhat sensitive to kanamycin while the rest of the strains showed clear resistance to kanamycin. Similar results were also obtained with *L. rhamnosus* strains as the MIC values of 64 % (21/33) strains were above 96 µg mL^{-1}.

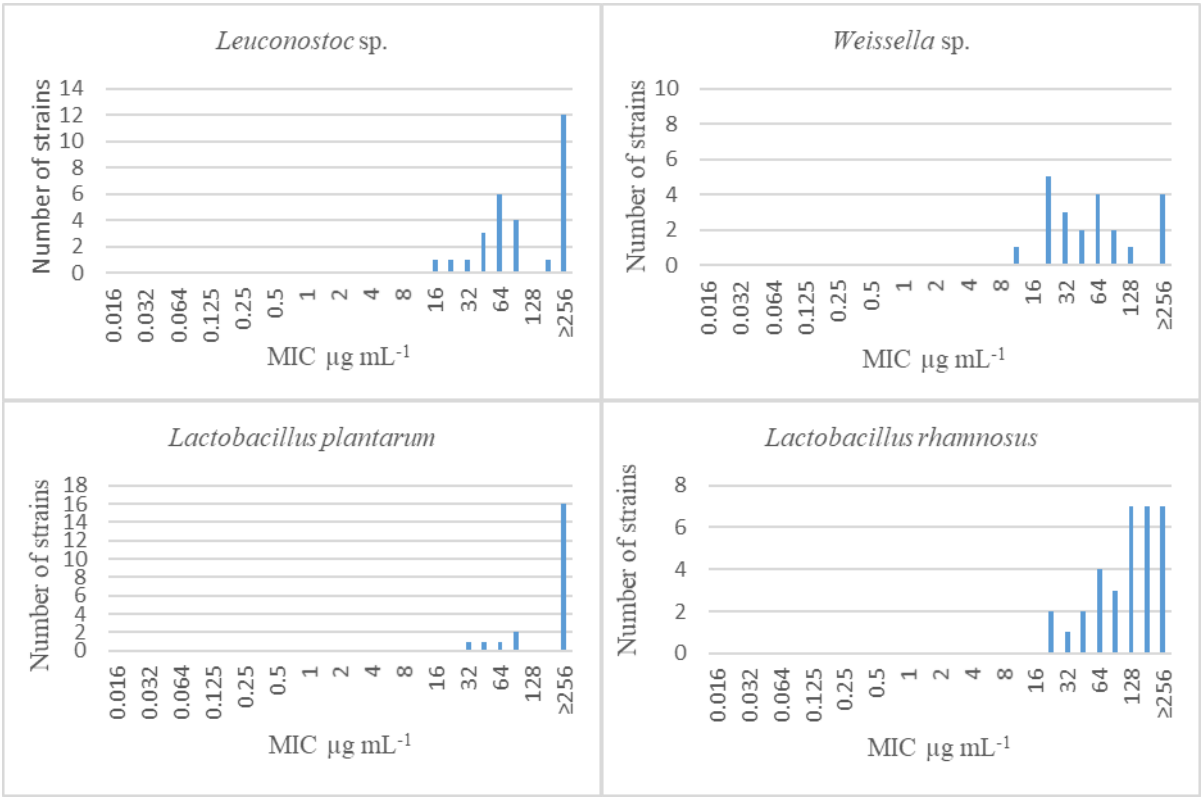

Figure 7 Kanamycin MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

Generally, streptomycin MIC distributions followed a normal distribution with an exception of *L. rhamnosus* strains, where a bimodal distribution was observed (Figure 8). *L. plantarum* strains were uniformly susceptible to streptomycin. In contrast, one *Leuconostoc* sp. strain (*L. pseudomesenteroides* E-98970T) exhibited notable resistance with an unusually high MIC value (512 µg mL^{-1}). The MIC distribution of *Weissella* sp. strains was more challenging to analyze since the determined MIC values were quite evenly distributed within different concentration levels. As a result, the distribution was not following a normal distribution pattern. Moreover, four *Weissella* sp. strains (were *Weissella cibaria* E-082762T, *Weissella confusa* E-143403, *Weissella cibaria* E-153485, *Weissella cibaria* E-163495) were more resistant to streptomycin than the others with MIC values of 256 and 384 µg mL^{-1}.

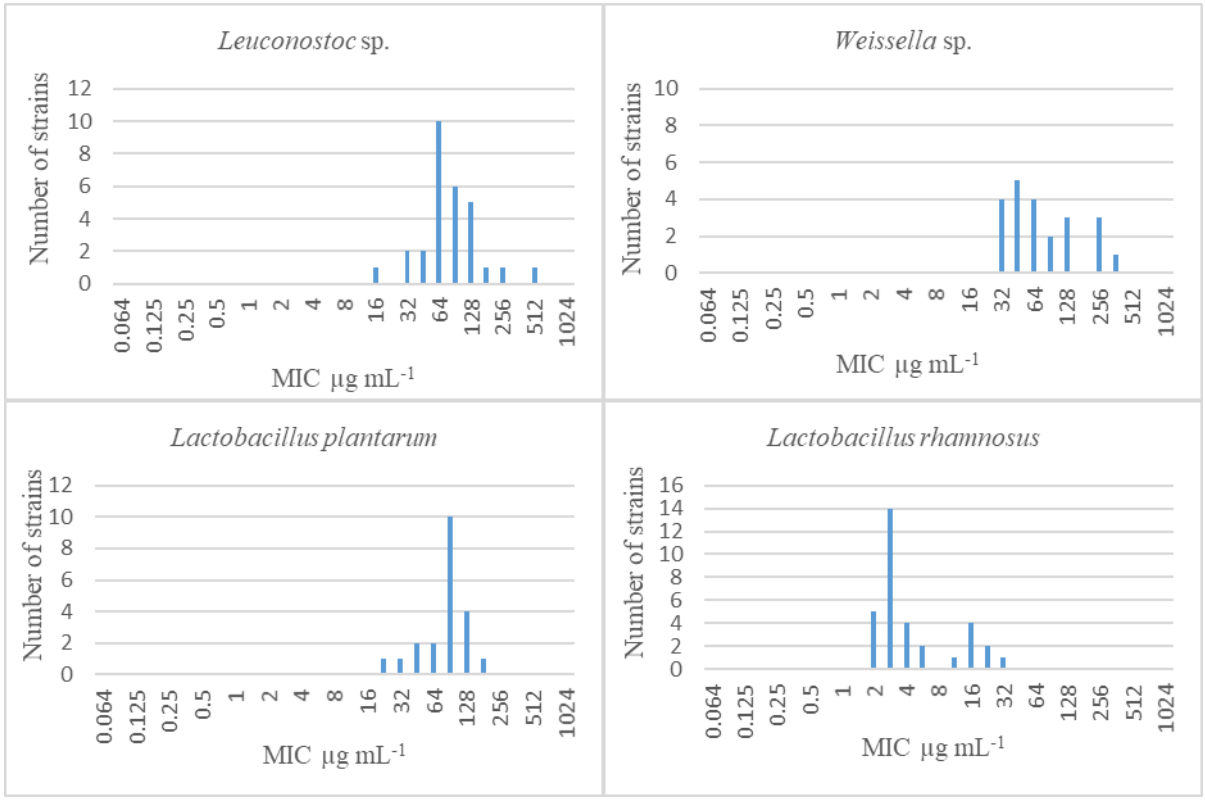

Figure 8. Streptomycin MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

Tetracycline MIC distributions of *Leuconostoc*, *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains were generally following a normal distribution (Figure 9). No clear deviation were detected in the distribution of *Leuconostoc* sp. and *L. rhamnosus* strains. By contrast, two *Weissella* sp. strains (*Weissella confusa* E-153461, *Weissella viridescens* E-98966T) exhibited slightly higher resistance to tetracycline with MIC values of 12 and 16 µg mL^{-1}. Furthermore, one *L. plantarum* strain (E-183579) was clearly resistant (MIC ≥ 256 µg mL^{-1}).

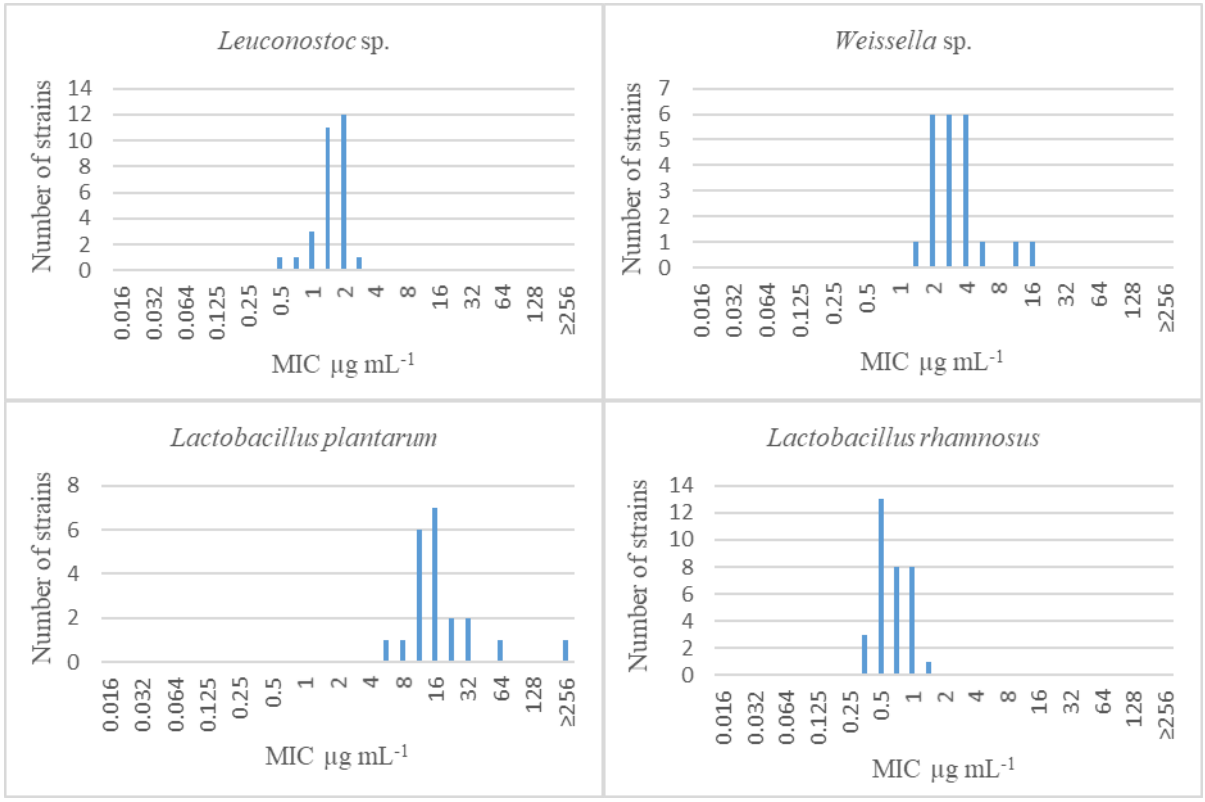

Figure 9. Tetracycline MIC distributions of *Leuconostoc* sp., *Weissella* sp., *L. plantarum* and *L. rhamnosus* strains. On the X-axis is the concentration of antibiotic (µg mL^{-1}) and on the Y-axis the number of strains.

The determined MIC values exceeded the cut-off values defined by EFSA for certain antibiotics (Table 5). The main overruns were detected in the case of chloramphenicol and kanamycin. 85 % (28/33) of *L. rhamnosus* and 79 % (23/29) of *Leuconostoc* sp. strains had higher MIC values than the EFSA's cut-off value for chloramphenicol (4 µg mL^{-1}). A similar phenomenon was observed in the case of kanamycin as 86 % (18/21) *L. plantarum*, 73 % (24/33) *L. rhamnosus* and 97 % (28/29) *Leuconostoc* sp. strains had higher MIC value than the defined cut-off value (64 and 16 µg mL^{-1}, respectively). In addition, 52 % (11/21) of the MIC values for erythromycin in *L. plantarum* strains and 48 % (14/29) streptomycin MIC values of *Leuconostoc* sp. strains were detected to have higher MIC values than the defined cut-off values (1 and 64 µg mL^{-1}, respectively)

The proposed cut-off values for *Weissella* sp. were compared to the results obtained in statistical analysis (Table 6). The results of visual analysis were corresponding well with the results obtained in statistical analysis with a few exceptions (Table 6). The

statistical analysis defined higher cut-off values for chloramphenicol, clindamycin and streptomycin distributions than obtained in visual analysis.

Table 5. Number of the strains with higher MIC value than the cut-off value (μg mL^{-1}) defined by EFSA

Antibiotic	***L. plantarum***	**EFSA's cut-off**	***L. rhamnosus***	**EFSA's cut-off**	***Leuconostoc* sp.**	**EFSA's cut-off**
Ampicillin	0 % (0/21)	2	0 % (0/33)	4	7 % (2/29)	2
Chloramphenicol	19 % (4/21)	8	85 % (28/33)	4	79 % (23/29)	4
Clindamycin	29 % (6/21)	4	3 % (1/33)	4	21 % (6/29)	1
Erythromycin	52 % (11/21)	1	0 % (0/33)	1	14 % (6/29)	1
Gentamicin	0 % (0/21)	16	0 % (0/33)	16	0 % (0/29)	16
Kanamycin	86 % (18/21)	64	73 % (24/33)	64	97 % (28/29)	16
Streptomycin	-	-	0 % (0/33)	32	48 % (14/29)	64
Tetracycline	10 % (2/21)	32	0 % (0/33)	8	0 % (0/29)	8

Table 6. Defined cut-off values for *Weissella* sp. strains by visual and statistical analysis (μg mL^{-1})

Method	**Ampicillin**	**Chloramphenicol**	**Clindamycin**	**Erythromycin**	**Gentamicin**	**Kanamycin**	**Streptomycin**	**Tetracycline**
Visual analysis	2	12	4	4	16	128	128	8
Statistical analysis	2	16	8	4	16	128	256	8

3.3 PCR detection of AMR genes

The positive controls for selected AMR genes were used to ensure the validity of PCR reactions. The presence of the genes in the positive controls were verified by PCR (Figure 10).

Additionally, to check the quality of isolated DNA a PCR with primers specific for 16S rRNA gene was performed (data not shown). Positive controls and 16S rRNA reactions gave strong amplicons indicating that there were no technical problems in the PCR.

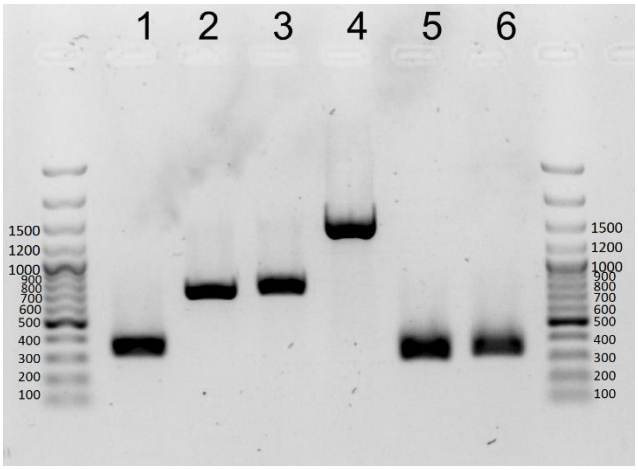

Figure 10 Amplicons of the positive controls. 1: *tetK* (348 bp) 2: *tetM* (740 bp) 3: *blaZ* (846 bp) 4: *mecA* (1429 bp) 5: *lnuA* (323 bp) 6: *cat* (300 bp) Molecular marker: GeneRuler 100 bp Plus DNA Ladder

Ampicillin, chloramphenicol, clindamycin and tetracycline resistance genes *blaZ*, *mecA*, *cat*, *lnuA*, *tetM* and *tetK* were not detected by PCR in any of the strains even though several strains were found to exhibit resistance in the phenotypic testing.

4 Discussion

Lactic acid bacteria play an essential role in the food industry as starters and probiotics [9]. As the antimicrobial resistance is getting more widespread and the risk of spreading AMR genes is increasing, it is important to consider beneficial bacteria as a potential pool of transmissible AMR genes. Especially as fermented foods contain large numbers of live bacteria and are thus favorable habitats for gene transfer [14].

Antibiotic susceptibility of lactic acid bacteria has been studied by using several methods including broth microdilution, disk diffusion and E-test method. E-test method was selected for the present study due to its simple technological requirements and suitability for screening multiple strains with several antibiotics at the same time. However, E-test method has also some inherent challenges. If pinpoint colonies and hazes inside the inhibition zones in addition to double zones of inhibition are detected the defining of exact MIC values can be challenging. These phenomena were detected with clindamycin, gentamicin, streptomycin and kanamycin. These observations may be explained by spontaneous mutations and decreased antibacterial activity during incubation period [53, 54]. Moreover, the E-test method is based on a visual evaluation of the MIC values and therefore the results may depend on the person performing the analysis. Therefore, E-test is best suited for screening of clear antimicrobial resistances in a bacterial population and not for defining exact MIC values.

Previous studies have reported that *L. rhamnosus*, *L. plantarum*, *Leuconostoc* sp. and *Weissella* sp. strains are generally sensitive to ampicillin as the determined MIC values have typically remained under 2 μg mL^{-1} [8, 14, 55–58]. The same results were observed in the present study since majority of the determined MIC values (except two MIC values of *Leuconostoc* sp. strains) remained under EFSA's cut-off values. In addition, the genes expressing ampicillin resistance, *blaZ* and *mecA*, were not detected in any of the studied strains. Moreover, the additional high peak at the end of the distribution of *Leuconostoc* sp. strains seemed to be species specific, which might indicate that the susceptibility determinations should be performed in species-level instead of genus also for *Leuconostoc*s.

Chloramphenicol MIC values for *L. rhamnosus* and *Leuconostoc* sp. strains were generally somewhat above the cut-off value (4 μg mL^{-1}) defined by EFSA. In addition, one *L. rhamnosus* strain exhibited clear resistance to chloramphenicol. However, *cat* gene

were not observed in any of the strains. Several studies have obtained similar results and the detected MIC values have mainly been above 4 μg mL^{-1} [8, 56, 59]. On the other hand, few studies have also determined MIC values under 4 μg mL^{-1} correlating well with the defined cut-off values [14, 43, 55, 57, 60]. Chloramphenicol MIC values of *L. plantarum* strains have generally reported to be under 4 μg mL^{-1} [43, 55, 58]. The results obtained in this study were slightly higher although only four *L. plantarum* strains had a MIC value above the EFSA's cut-off value (8 μg mL^{-1}). As for *Weissella* sp. strains, the suggested cut-off value of chloramphenicol is 12 μg mL^{-1} in the visual analysis and 16 μg mL^{-1} in statistical analysis. The results of previous studies are correlating well with the observations of this study since the chloramphenicol MIC values of *Weissella* sp. strains are reported not to exceed 16 μg mL^{-1} [43, 56, 61].

Generally, *Leuconostoc* sp. and *L. rhamnosus* strains were susceptible to clindamycin having MIC values below the EFSA's cut-off value (1 and 4 μg mL^{-1}, respectively). Similar results have also been reported in earlier studies [8, 57]. However, several strains exhibited clear resistance to clindamycin as significantly higher MIC values than the defined cut-off values were detected. Interestingly, *L. rhamnosus* strain (E-001125), which showed clear resistance to chloramphenicol, was also resistant to clindamycin. These observations might indicate acquired resistance even though the detected clindamycin resistance gene, *lnuA,* was not detected in this or any other of the examined strains. Previously, chloramphenicol and clindamycin have been reported to have partly overlapping inhibition sites, which could explain the cross-resistance for these antibiotics [62]. About one third of *L. plantarum* strains had clindamycin MIC value above the defined cut-off value (4 μg mL^{-1}). However, a wide clindamycin MIC range was detected in *L. plantarum* and *Weissella* sp. strains. Similar phenomenon was observed in previous antibiotic susceptibility studies of *L. plantarum* [6, 57, 63, 64]. Earlier studies of clindamycin susceptibilities in *Weissella* species are limited and the reported MIC values have varied between 0.064 and 0.5 μg mL^{-1} [65, 66]. There is no obvious explanation for the wide MIC ranges but one theory is that the phenomenon could be caused by mutations [67]. The mode of action of clindamycin is based on the inhibition of protein synthesis in the 50S ribosomal subunit by affecting the peptidyl transferase reactions [68]. It has been suggested that clindamycin's ability to inhibit protein synthesis might decrease if the synthesized peptide has already reached the critical length [68]. This observation might also explain the wide range of determined MIC values.

Several *Leuconostoc* sp. and *L. plantarum* strains had MIC values above the defined cut-off value (1 μg mL^{-1}) for erythromycin. Cross-resistance between clindamycin and erythromycin, also known as MLS_B (macrolide-lincosamide-streptogramin B) resistance, is considered to be to the overlapping inhibition sites of these antibiotics [6, 69]. However, the strains with high clindamycin MIC values in the present study did not have high erythromycin MIC values and therefore no cross-resistance was observed. Generally, MIC values below 1 μg mL^{-1} for erythromycin have been detected for *Weissella* sp. strains although more resistance strains have also been observed (MIC 16 μg mL^{-1}) [43, 56, 65]. However, since MIC distributions in *Weissella* have been reported only to a limited extent and therefore it is difficult to compare the results. All the detected *L. rhamnosus* strains were susceptible to erythromycin even though a bimodal distribution was observed for the MIC values. Similar phenomenon was detected in the streptomycin MIC distribution of *L. rhamnosus* strains. Several studies have reported the occurrences of bimodal distributions in the context of streptomycin and erythromycin [14, 70, 71]. In closer analysis, Korhonen et al. (2010) detected systematically somewhat lower MIC values for *L. rhamnosus* strains with both antibiotics, which can be observed as bimodal distribution. Interestingly, this phenomenon was not observed in the MIC distributions of other tested antibiotics. These differences might be explained by different growth conditions and techniques applied in the MIC determination.

About half of the studied *Leuconostoc* sp. strains had higher MIC value for streptomycin than the defined cut-off value (64 μg mL^{-1}). A wide variety of streptomycin MIC values (2-2048 μg mL^{-1}) has been reported for *Leuconostoc* strains in previous studies [8, 14, 56]. One potential explanation for the wide streptomycin MIC range may be the common occurrence of pinpoint colonies at the inhibition zone during the susceptibility testing which hampers the correct identification of the MIC values [39, 54]. High streptomycin MIC values were also detected in *L. plantarum* and *Weissella* sp. strains. Similar results were obtained in previous studies as well [6, 56, 57, 65].

Relatively high MIC values for aminoglycosides including streptomycin, gentamicin and kanamycin is typical among many lactic acid bacteria and is probably associated to the lack of cytochrome-mediated electron transport that affects uptake of the drug [52]. This correlates well also with the kanamycin MIC distributions as 86 % *L. plantarum*, 73 % *L. rhamnosus* and 97 % *Leuconostoc* sp. strains had higher MIC value than the defined cut-off values (64, 64, and 16 μg $mL^{-1,}$ respectively). On the other hand,

susceptible strains were also detected. Re-evaluation of the cut-off values is thus warranted and can be done when more data on the MIC distribution has accumulated.

Interestingly, all the examined strains were susceptible to gentamicin even though they exhibited resistance to streptomycin and kanamycin. Similar results have been observed in previous studies [57, 71, 72]. Susceptibility to gentamicin might be explained by its ability to pass through the membrane more effectively than other aminoglycosides due to the differences in structures and interactions with the membrane [73].

Generally, all the examined *L. rhamnosus, L. plantarum, Leuconostoc* sp. and *Weissella* sp. strains were susceptible to tetracycline with an exception of two *L. plantarum* strains which had MIC values above the defined cut-off value (32 μg mL^{-1}). However, none of the studied strains carried the detected tetracycline resistance genes (*tetK* and *tetM*). Similar tetracycline MIC ranges have also been reported in several studies [6, 39, 56, 57, 64, 66].

The problem with many beneficial bacteria is that there is much less information about MIC values than for pathogenic bacteria. Improving the current cut-off values can only happen with the accumulation of new MIC distribution data for these species/genera. Differences in the MIC values detected in different studies are probably partially explained by the different sources of the studied strains and variations in the methods used. Generally, no major disagreements in the results of various antibiotic susceptibility test methods, such as E-test, disk diffusion and microdilution, have been observed. The most notable differences between agar-based methods and broth micro dilution methods are reported with clindamycin, erythromycin and tetracycline [39, 54]. These observations are explained by the changes in the action of antimicrobial agents in different mediums and by the observations of pinpoint colonies and double zones of inhibition in agar-based methods [39, 54].

So far, EFSA has not defined any cut-off values for *Weissella* species because no sufficient evidence of their safe consumption has been reported and therefore QPS status for *Weissella* sp. strains has not been granted [74]. The cut-off values of genus *Leuconostoc* were compared to the proposed cut-off values of *Weissella* strains since these two genera are phylogenetically close. Generally, the MIC values of both genera were correlating well and only small differences were detected. The cut-off values were the same for ampicillin, gentamicin and tetracycline. The proposed cut-off values of clindamycin and erythromycin were slightly higher in *Weissella* sp. than in *Leuconostoc* sp.. The differences of clindamycin

cut-off values might be explained with the wider MIC distribution of *Weissella* sp. strains. For chloramphenicol, kanamycin and streptomycin, the proposed cut-off values were significantly higher in *Weissella* sp. than in *Leuconostoc* sp. (12/16, 128, 128/256 μg mL^{-1} versus 4, 16, 64 μg mL^{-1}, respectively). However, for these antibiotics, most of the determined *Leuconostoc* sp. strains had higher MIC values than the defined cut-off values as well indicating that EFSA's cut-off values may require re-evaluation. These results demonstrate that *Weissella* sp. strains do not cause greater safety risk than *Leuconostoc* species while considering their antibiotic resistance. However, further antibiotic susceptibility studies are needed for defining better cut-off values since only small number of *Weissella* strains were examined in present study.

Interestingly, none of the screened antibiotic resistance genes (*blaZ*, *mecA*, *cat*, *lnuA*, *tetM* and *tetK*) was detected in the studied strains even though a clear phenotypic resistance was observed in several strains. Since no positive PCR signals were obtained in any of the strains it is obvious that none of the strains had inactive AMR genes which would lead into phenotypical susceptibility [52, 75]. These results might be explained by the small number of the studied genes, as based on MEGARes database, multiple genes encode resistance to ampicillin, chloramphenicol, clindamycin and tetracycline. The genes studied in present study were selected based on the observations of previous studies which indicate that these genes, particularly resistance genes to chloramphenicol and tetracycline, can be found among lactic acid bacteria [41–43, 71]. Different methods have been used for the detection of AMR genes including PCR and DNA microarray methods in addition to whole genome sequencing [14, 64, 71]. For further studies, DNA microarray method might be more suitable method for screening of the resistance genes since multiple genes can be detected at the same time [64]. After the screening, the positive results can be ensured with more specific methods [64]. Whole genome sequencing (WGS) is a suitable option if not too many strains have to be studied. WGS enables the identification of new potential AMR resistance mechanisms and genetic elements [76]. In addition to DNA, RNA can be used as a starting material for sequence analysis enabling the detection of expressed genes [76]. However, this method is too laborious and complex to be used for the studies on several strains.

The results obtained in the present study provided more information about the antibiotic susceptibility profiles and the safety of *L. plantarum*, *L. rhamnosus*, *Leuconostoc* sp. and *Weissella* sp. strains. In addition, new, tentative cut-off values for *Weissella* sp. strains were proposed. In the future, even more strains of different origins should be studied to produce

robust MIC distributions, preferably in species-specific manner. Antibiotic susceptibility determinations for lactic acid bacteria should be performed using standardized methods as the incubation conditions might have an effect on the obtained results. With standardized methods, more reliable comparison of the results of various studies can be performed.

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6 References

1. **European Commission**. One Health Action Plan against Antimicrobial Resistance. European Commission 2017;24.

2. **O'Neil, J.** Antimicrobials in agriculture and the environment: reducing unnecessary use and waste. *Review on Antimicrobial Resistance* 2015. https://ec.europa.eu/health/amr/sites/amr/files/amr_studies_2015_am-in-agri-and-env.pdf, (accessed 13 December 2018).

3. **FAO**. Antimicrobial Resistance and Foods of Plant Origin. Summary Report of an FAO Meeting of Experts FAO Antimicrobial Resistance Working Group. Food and Agriculture Organization of the United Nations 2018. http://www.fao.org/3/BU657en/bu657en.pdf (accessed 28 January 2019).

4. **WHO**. Antibiotic resistance. World Health Organization 2018. https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (accessed 13 December 2018).

5. **Cox G, Wright GD**. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology* 2013;303:287–292. DOI: 10.1016/J.IJMM.2013.02.009

6. **Korhonen JM, Danielsen M, Mayo B, Egervärn M, Axelsson L, Huys G, Wright A.V.** Antimicrobial Susceptibility and Proposed Microbiological Cut-off Values of Lactobacilli by Phenotypic Determination. *International Journal of Probiotics and Prebiotics* 2008;3:257–268.

7. **Rosander A, Connolly E, Roos S**. Removal of antibiotic resistance gene-carrying plasmids from Lactobacillus reuteri ATCC 55730 and characterization of the resulting daughter strain, L. reuteri DSM 17938. *Applied and Environmental Microbiology* 2008;74:6032–6040. DOI: 10.1128/AEM.00991-08

8. **Flórez AB, Campedelli I, Delgado S, Alegría Á, Salvetti E, Felis G, Mayo B, Torriani S.** 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* 2016;11:1–20. DOI: 10.1371/journal.pone.0145203

9. **Teuber M, Meile L, Schwarz F**. Acquired antibiotic resistance in lactic acid bacteria from food. In: Konings W.N., Kuipers O.P., In 't Veld J.H.J.H. (eds) *Lactic Acid Bacteria: Genetics, Metabolism and Applications*. Dordrecht: Springer;1999. pp. 115–137.

10. **Partridge SR, Tsafnat G, Coiera E, Iredell JR**. Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiology Reviews* 2009;33:757–784. DOI: 10.1111/j.1574-6976.2009.00175.x

11. **Partridge SR, Kwong SM, Firth N, Jensen SO**. Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews* 2018;31:e00088-17. DOI: 10.1128/CMR.00088-17

12. **Madigan MT, Bender KS, Buckley DH, Daniel H, Sattley WM, Stahl DA**. Brock biology of microorganisms, Global Edition [Internet]. NY, NY: Pearson; 2019. pp. 342-368: 530-566. https://helka.finna.fi/Record/helka.3103818 (accessed 1 October 2018).

13. **Korhonen JM**. Antibiotic Resistance of Lactic Acid Bacteria. *Publications of the University of Eastern Finland, Dissertations in Forestry and Natural Sciences*. e-publication. 2010. http://epublications.uef.fi/pub/urn_isbn_ 978-952-61-0097-5/urn_isbn_978-952-61-0097-5.pdf (accessed 21 January 2019). ISBN: 978-952-61-0097-5 (PDF)

14. **Casado Muñoz M del C, Benomar N, Lerma LL, Gálvez A, Abriouel H**. Antibiotic resistance of Lactobacillus pentosus and Leuconostoc pseudomesenteroides isolated from naturally-fermented Aloreña table olives throughout fermentation process. *International Journal of Food Microbiology* 2014;172:110–118.

15. **Schleifer KH, Ludwig W**. Phylogenetic relationships of lactic acid bacteria. In: Wood B.J.B., Holzapfel W.H. (eds) *The Genera of Lactic Acid Bacteria. The Lactic Acid Bacteria*, vol 2. Boston: Springer; 1995. pp. 7–18.

16. **Axelsson L**. Lactic Acid Bacteria: Classification and Physiology. In Salminen S, Wright A.V, Ouwehand A. (eds) *Lactic Acid Bacteria Microbiological and Functional Aspects*, 3rd edition. NY: Marcel Dekker; 2004. pp. 1–66.

17. **EFSA**. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. *EFSA Journal* 2007;5:587.DOI: 10.2903/j.efsa.2007.587

18. **FAO, WHO**. Probiotics in food : health and nutritional properties and guidelines for evaluation. Food and Agriculture Organization of the United Nations, Whorld Health Organization 2006. https://catalogue.nla.gov.au/Record/3788914 (accessed 21 January 2019).

19. **Havenaar R, Huis In't Veld JHJ**. Probiotics: A General View. In Wood B.J.B. (eds) *The Lactic Acid Bacteria*, Volume 1. Boston, MA: Springer; 1992. pp. 151–170.

20. **Dunne C, Murphy L, Flynn S, O'Mahony L, O'Halloran S, Feeney M, Morrissey D, Thornton G, Fitzgerald G, Daly C, Kiely B, Quigley E M, O'Sullivan G C, Shanahan F, Collins J K.** Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie Van Leeuwenhoek* 1999;76:279–92.

21. **Salminen S, Gueimonde M**. Human Studies on Probiotics: What Is Scientifically Proven. *Journal of Food Science* 2006;69:M137–M140. DOI: 10.1111/j.1365-2621.2004.tb10723.x

22. **Holzapfel WH, Wood BJB**. Lactic acid bacteria in contemporary perspective. In Wood B.J.B., Holzapfel W.H. (eds) *The Genera of Lactic Acid Bacteria. The Lactic Acid Bacteria*, vol 2. Boston: Springer; 1995. pp. 1–6.

23. **Holzapfel WH, Haberer P, Geisen R, Björkroth J, Schillinger U**. Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition* 2001;73:365s–373s. DOI: 10.1093/ajcn/73.2.365s

24. **König H, Fröhlich J**. Lactic Acid Bacteria. In König H, Unden G, Fröhlich J. (eds) *Biology of Microorganisms on Grapes, in Must and in Wine*. Cham: Springer International Publishing. 2017. pp. 3–41.

25. **Mayo B, Aleksandrzak - Piekarczyk T, Fernández M, Kowalczyk M, Álvarez - Martín P, Bardowski J.** Updates in the Metabolism of Lactic Acid Bacteria. In Mozzi F, Raya RR, Vignolo GM. (eds) *Biotechnology of Lactic Acid Bacteria Novel Applications.* Iowa: Blackwell Publishing. 2010. pp. 3-33

26. **Hammes WP, Weiss N, Holzapfel WH**. The genera *Lactobacillus* and *Carnobacterium*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) *The Prokaryotes 2nd ed. Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications Vol. 2*. New York, NY: Springer Verlag. 1992 pp. 1535–1594.

27. **Dellaglio F, Dicks LMT, Torriani S**. The Genus *Leuconostoc*. In Wood B.J.B, Holzapfel W.H. (eds) *The Genera of Lactic Acid Bacteria*. *The Lactic Acid Bacteria*, vol 2. Boston, MA: Springer US. 1995 pp. 235–278.

28. **Hammes WP, Hertel C**. *Lactobacillus*. In Whitman WP, Rainey F, Kämpfer P, Trujillo M, Chun J, DeVos P, Hedlund B, Dedysh S. (eds) *Bergey's Manual of Systematics of Archaea and Bacteria*. Chichester, UK: John Wiley & Sons, Ltd. 2015 pp. 1–76. DOI:10.1002/9781118960608.gbm00604

29. **Pot B, Felis GE, Bruyne K De, Tsakalidou E, Papadimitriou K, Leisner J, and Vandamme P.** The genus *Lactobacillus*. In Wood B.J.B, Holzapfel W.H. (eds) *Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd. 2014 pp. 249–353.

30. **Holzapfel WH, Schillinger U**. The genus *Leuconostoc*. In Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) *The Prokaryotes*, 2nd ed. New York, NY: Springer New York. 1992 pp. 1508-1534.

31. **Björkroth J, Dicks LMT, Endo A, H.Holzapfel W**. The genus *Leuconostoc*. In Wood BJB, Holzapfel WH. (eds) *Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd. 2014 pp. 391–404.

32. **Björkroth J, Dicks LMT, Endo A**. The genus *Weissella*. In Wood BJB, Holzapfel WH. (eds) *Lactic Acid Bacteria*. Chichester, UK: John Wiley & Sons, Ltd. 2014 pp. 417–428.

33. **Björkroth J, Dicks LMT, Holzapfel WH**. *Weissella*. In Whitman WP, Rainey F, Kämpfer P, Trujillo M, Chun J, DeVos P, Hedlund B, Dedysh S. (eds) *Bergey's Manual of Systematics of Archaea and Bacteria*. Chichester, UK: John Wiley & Sons, Ltd. 2015 pp. 1–15. DOI: 10.1002/9781118960608.gbm00609

34. **Fusco V, Quero GM, Cho G-S, Kabisch J, Meske D, Neve H, Bockelmann W, Franz C.** The genus *Weissella*: taxonomy, ecology and biotechnological potential. *Frontiers in microbiology* 2015;6:155. DOI: 10.3389/fmicb.2015.00155

35. **Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos M de L, *et al.*** Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal* 2018;16:1–24. DOI: 10.2903/j.efsa.2018.5206

36. **Kapoor G, Saigal S, Elongavan A**. Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of anaesthesiology, clinical pharmacology* 2017;33:300–305. DOI: 10.4103/joacp.JOACP_349_15

37. **bioMérieux**. Etest Antimicrobial Susceptibility Testing. bioMérieux. 2012. www.biomerieux.com/techlib (accessed 22 November 2018).

38. **Klare I, Konstabel C, Müller-Bertling S, Reissbrodt R, Huys G, Vancanneyt M, Swings J, Goossens H, Witte W.** Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. *Applied and environmental microbiology* 2005;71:8982–6. DOI: 10.1128/AEM.71.12.8982-8986.2005

39. **Korhonen JM, Van Hoek AHAM, Saarela M, Huys G, Tosi L, Mayrhofer S, Von Wright, A.** Antimicrobial susceptibility of *Lactobacillus rhamnosus*. *Beneficial Microbes* 2010;1:75–80. DOI: 10.3920/BM2009.0002

40. **Turnidge J, Kahlmeter G, Kronvall G**. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clinical Microbiology and Infection* 2006;12:418–425. DOI: 10.1111/j.1469-0691.2006.01377.x

41. **Dec M, Urban-Chmiel R, Stępień-Pyśniak D, Wernicki A**. Assessment of antibiotic susceptibility in *Lactobacillus* isolates from chickens. *Gut Pathogens* 2017;9:54. DOI: 10.1186/s13099-017-0203-z

42. **Aquilanti L, Garofalo C, Osimani A, Silvestri G, Vignaroli C, Clementi, F.** Isolation and molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine meat products. *Journal of Food Protection 2007;03:557-565.*

43. **Basbülbül G, Özteber M, Biyik HH**. Antibiotic resistance in lactic acid bacteria isolated from fermented dairy products and boza. *Journal of Microbiology, Biotechnology and Food Science* 2015;04:513–517. DOI: 10.15414/jmbfs.2015.4.6.513-51

44. **Lakin SM, Dean C, Noyes NR, Dettenwanger A, Ross AS, Doster E, Rovira P, Abdo Z, Jones KL, Ruiz J, Belk KE, Morley PS, Boucher C..** MEGARes: an antimicrobial resistance database for high throughput sequencing. *Nucleic Acids Research* 2017;45:D574–D580. DOI: 10.1093/nar/gkw1009

45. **Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang K, Lago B, Dave B, Pereira S, Sharma A, Doshi S, Courtot M, Lo R, Williams L, Frye J, Elsayegh T, Sardar D, Westman E, Pawlowski A, Johnson T, Brinkman Fiona, Wright G, McArthur A.** CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Research* 2017;45:D566–D573. DOI: 10.1093/nar/gkw1004

46. **Zhang Z, Schwartz S, Wagner L, Miller W**. A Greedy algorithm for aligning DNA Sequences. *Journal of Computational Biology* 2000;7:203–214. DOI: 10.1089/10665270050081478

47. **Perreten V, Vorlet-Fawer L, Slickers P, Ehricht R, Kuhnert P, Frey J.** Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. *Journal of Clinical Microbiology* 2005;43:2291–302. DOI: 10.1128/JCM.43.5.2291-2302.2005

48. **Garofalo C, Vignaroli C, Zandri G, Aquilanti L, Bordoni D, Osimani A, Clementi F, Biavasco F.** Direct detection of antibiotic resistance genes in specimens of chicken and pork meat. *International Journal of Food Microbiology* 2007;113:75–83. DOI: 10.1016/J.IJFOODMICRO.2006.07.015

49. **Olsvik B, Olsen I, Tenover FC**. Detection of *tet(M)* and *tet(Q)* using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. *Oral Microbiology and Immunology* 1995;10:87–92. DOI: 10.1111/j.1399-302X.1995.tb00124.x

50. **Gevers D, Danielsen M, Huys G, Swings J**. Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Applied and Environmental Microbiology* 2003;69:1270–1275. DOI: 10.1128/AEM.69.2.1270-1275.2003

51. **Lina G, Quaglia A, Reverdy ME, Leclercq R, Vandenesch F, Etienne J.** Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. *Antimicrobial Agents Chemotherapy* 1999;43:1062–6.

52. **Hummel AS, Hertel C, Holzapfel WH, Franz CMAP**. Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Applied and environmental microbiology* 2007;73:730–9. DOI: 10.1128/AEM.02105-06

53. **Curragh HJ, Collins MA**. High levels of spontaneous drug resistance in *Lactobacillus*. *Journal of Applied Bacteriology* 1992;73:31–36. DOI: 10.1111/j.1365-2672.1992.tb04965.x

54. **Mayrhofer S, Domig KJ, Mair C, Zitz U, Huys G, Kneifel W.** Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of *Lactobacillus acidophilus* group members. *Applied and Environmental Microbiology* 2008;74:3745–3748. DOI: 10.1128/AEM.02849-07

55. **Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou E, Tassou C.** Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiology* 2013;33:282–291. DOI: 10.1016/j.fm.2012.10.005

56. **Jeong DW, Lee JH**. Antibiotic resistance, hemolysis and biogenic amine production assessments of *Leuconostoc* and *Weissella* isolates for kimchi starter development. *LWT - Food Science and Technology* 2015;64:1078–1084. DOI: 10.1016/j.lwt.2015.07.031

57. **Danielsen M, Wind A**. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology* 2003;82:1–11. DOI: 10.1016/S0168-1605(02)00254-4

58. **Guo H, Pan L, Li L, Lu J, Kwok L, Menghe B, Zhang H, Zhang W.** Characterization of antibiotic resistance genes from *Lactobacillus* isolated from traditional dairy products. *Journal of Food Science* 2017;82:724–730. DOI: 10.1111/1750-3841.13645

59. **Klein G**. Antibiotic resistance and molecular characterization of probiotic and clinical *Lactobacillus* strains in relation to safety aspects of probiotics. *Foodborne Pathogens and Disease* 2011;8:267–281. DOI: 10.1089/fpd.2010.0672

60. **Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Müller-Bertling S, Witte W, Goossens H.** Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *Journal of Antimicrobial Chemotherapy* 2007;59:900–912. DOI: 10.1093/jac/dkm035

61. **Muñoz-Atienza E, Gómez-Sala B, Araújo C, Campanero C, Del Campo R, Hernández P, Herranz C, Cintas L.** Antimicrobial activity, antibiotic susceptibility and virulence factors of Lactic Acid Bacteria of aquatic origin intended for use as probiotics in aquaculture. *BMC Microbiology*; 2013;13. DOI: 10.1186/1471-2180-13-15.

62. **Kehrenberg C, Schwarz S, Jacobsen L, Hansen LH, Vester B**. A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: Methylation of 23S ribosomal RNA at A2503. *Molecular Microbiology* 2005;57:1064–1073. DOI: 10.1111/j.1365-2958.2005.04754.x

63. **Flórez AB, Egervärn M, Danielsen M, Tosi L, Morelli L, Lindgren S, Mayo B.** Susceptibility of *Lactobacillus plantarum* strains to six antibiotics and definition of new susceptibility-resistance cutoff values. *Microbial drug resistance* 2006;12;4

64. **Ammor MS, Flórez AB, Van Hoek AHAM, De Los Reyes-Gavilán CG, Aarts HJM, Margolles A, Mayo B.** Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *Journal of Molecular Microbiology and Biotechnology* 2007;14:6–15. DOI: 10.1159/000106077

65. **Adimpong DB, Nielsen DS, Sørensen KI, Derkx PM, Jespersen L**. Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products. *BMC Microbiology* 2012;1:75. DOI: 10.1186/1471-2180-12-75.

66. **Ayeni FA, Sánchez B, Adeniyi BA, De Los Reyes-Gavilán CG, Margolles A, Ruas-Madiedo P.** Evaluation of the functional potential of *Weissella* and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow's intestine. *International Journal of Food Microbiology* 2011;147:97–104. DOI: 10.1016/j.ijfoodmicro.2011.03.014

67. **Mayrhofer S, van Hoek AHAM, Mair C, Huys G, Aarts HJM, Kneifel W, Domig K.** Antibiotic susceptibility of members of the *Lactobacillus acidophilus* group using broth microdilution and molecular identification of their resistance determinants. *International Journal of Food Microbiology* 2010;144:81–87. DOI: 10.1016/J.IJFOODMICRO.2010.08.024

68. **Tenson T, Lovmar M, Ehrenberg M**. The Mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome. *Journal of Molecular Biology* 2003;330:1005–1014. DOI: 10.1016/S0022-2836(03)00662-4

69. **Weisblum B**. Erythromycin resistance by ribosome modification. *Antimicrobial Agents and Chemotherapy* 1995;39:577–585. DOI: 10.1128/AAC.39.3.577

70. **Duranti S, Lugli GA, Mancabelli L, Turroni F, Milani C, Mangifesta M, Ferrario C, Anzalone R, Viappiani A, van Sinderen D, Ventura M.** Prevalence of antibiotic resistance genes among human gut-derived bifidobacteria. *Applied and Environmental Microbiology* 2017;83:1–14. DOI: 10.1128/AEM.02894-16

71. **Campedelli I, Mathur H, Salvetti E, Clarke S, Rea MC, Torriani S, Ross RP, Hill C, O'Toole PW.** Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. *Applied and Environmental Microbiology 2018*;85. DOI: 10.1128/AEM.01738-18.

72. **Georgieva R, Yocheva L, Tserovska L, Zhelezova G, Stefanova N, Atanasova A, Danguleva A, Ivanova G, Karapetkov N, Rumyan N, Karaivanova E.** Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures. *Biotechnology and Biotechnological Equipment* 2015;29:84–91. DOI: 10.1080/13102818.2014.987450

73. **Elkins CA, Mullis LB**. Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. *Applied and Environmental Microbiology* 2004;70:7200–7209. DOI: 10.1128/AEM.70.12.7200-7209.2004

74. **Koutsoumanis K, Allende A, Álvarez-Ordóñez A, Bolton D, Bover-Cid S, Chemaly M, Davies R, Hilbert F, Lindqvist R, Nauta M, Peixe L, Ru G, Simmons M, Skandamis P, Suffredini E, Cocconcelli PS, Fernández Escámez, PS, Maradona MP, Querol A, Suarez JE, Sundh I, Vlak J, Barizzone F, Correia S, Herman L.** Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 9: suitability of taxonomic units notified to EFSA until September 2018. *EFSA Journal* 2019;17:1–46. DOI: 10.2903/j.efsa.2019.5555

75. **Mättö J, Maukonen J, Alakomi H-L, Suihko M-L, Saarela M**. Influence of oral doxycycline therapy on the diversity and antibiotic susceptibility of human intestinal bifidobacterial population. *Journal of Applied Microbiology* 2008;105:279–289. DOI: 10.1111/j.1365-2672.2008.03792.x

76. **Chan K-G**. Whole-genome sequencing in the prediction of antimicrobial resistance. *Expert Review of Anti-infective Therapy* 2016;7:617-619. DOI: 10.1080/14787210.2016.1193005.

Annex 1 MIC distributions of ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, tetracycline and streptomycin for *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Leuconostoc* sp. and *Weissella* sp. strains.

MIC values (µg mL^{-1})																														
		N	**0.016**	**0.023**	**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.19**	**0.25**	**0.38**	**0.5**	**0.75**	**1**	**1.5**	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**48**	**64**	**96**	**128**	**192**	**≥256**
Ampicillin	*Leuconostoc* sp.	29										1	9	5	6	2	4	2													
	Weissella sp.	22						1	1	1	2	4	8	4			1														
	L. plantarum	21							3	4	5	4	2	2	1																
	L. rhamnosus	33											2	6	14	7	4														
Chloramphenicol	*Leuconostoc* sp.	29															1	2	3	9	9	5									
	Weissella sp.	22																	4	9	8	1									
	L. plantarum	21																	3	7	7	3	1								
	L. rhamnosus	33															1	1	3	9	9	7	2								1
Clindamycin	*Leuconostoc* sp.	29	1			2	2	3	7	4	2	1	1										2		2						2
	Weissella sp.	22			1		3	1	1			7	1	3	1	1	1	1	1												
	L. plantarum	21		3	2	1					2		1	1	1	1	1	1	1	2	2			2							
	L. rhamnosus	33						1	4	6	3	6	6	4	2																1
Erythromycin	*Leuconostoc* sp.	29									1	2	5	9	8	3	1														
	Weissella sp.	22												3	6	8	3	1	1												
	L. plantarum	21										1		2	7	6	2	1	1		1										
	L. rhamnosus	33		1	2	4	7	6	4	1		2	3	3																	

		N	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32	48	64	96	128	192	≥256
Gentamicin	*Leuconostoc* sp.	29													1	2	6	6	3	2	8	1								
	Weissella sp.	22															7	8		5	1		1							
	L. plantarum	21															4	5	3	4	3	2								
	L. rhamnosus	33													2	7	12	8	3	1										
Kanamycin	*Leuconostoc* sp.	29																					1	1	1	3	6	4		1	12
	Weissella sp.	22																				1		5	3	2	4	2	1		4
	L. plantarum	21																							1	1	1	2			16
	L. rhamnosus	33																						2	1	2	4	3	7	7	7
Tetracycline	*Leuconostoc* sp.	29											1	1	3	11	12	1													
	Weissella sp.	22														1	6	6	6	1		1	1								
	L. plantarum	21																		1	1	6	7	2	2		1				1
	L. rhamnosus	33										3	13	8	8	1															

		N	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32	48	64	96	128	192	256	384	512	768	1024
Streptomycin	*Leuconostoc* sp.	29																	1		2	2	10	6	5	1	1		1		
	Weissella sp.	22																			4	5	4	2	3		3	1			
	L. plantarum	21																		1	1	2	2	10	4	1					
	L. rhamnosus	33											5	14	4	2		1	4	2	1										