Antibiotic susceptibility profiles of ropy slime-producing *Leuconostoc mesenteroides* isolated from cooked meat products

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Abstract

The transfer of antibiotic resistance *via* the food chain is a global concern. Nevertheless, more attention is required to non-pathogenic strains, such as spoilage bacteria, which could transmit genes to pathogens. Although Lactic Acid Bacteria are microorganisms generally recognized as safe, *Leuconostoc mesenteroides* may reach and maintain high concentration levels on the surface of cooked products and ready-to-eat products throughout the entire shelf life. It is therefore important to consider the possibility for this species to carry antibiotic-resistance genes. The present research deals with the antibiotic susceptibility profile of strains of *L. mesenteroides,* isolated from vacuum packaged cooked meat products. In this study, the antimicrobial susceptibility of *L.mesenteroides*, previously isolated from cooked ham, was investigated through disk diffusion assay according to CLSI standards. Isolated strains from ready-to-eat food show high levels of resistance to ampicillin and methicillin and, according to a settled panel of 21 antibiotics, the antibiotic resistance was demonstrated for the 50% of the tested molecules.

Introduction

Since 1945, the effects of antibiotics misuse were predicted by Alexander Fleming, who foresaw that the antimicrobial selective pressure would have led to the rise of antibiotic resistance in bacteria.[1-3] The rapid spread of antimicrobial resistance represents a threat to public health because of the slow progress in developing new antimicrobial, which can be used for therapy when resistance occurs.[4,5]

Nevertheless, most studies on the emergence and spread of antibiotic resistance are focused mainly on clinically relevant bacterial species. Infections caused by resistant strains, in fact, are more difficult to treat and require more time and money for their control.[6,7] In addition, genes encoding antibiotic resistance are also detected in bacteria isolated from uncontaminated and non-urbanized environments.[8]

Antibiotic resistance can be inherent in a bacterial species, which has been termed as *intrinsic* or *natural* resistance and/or *acquired* when a usually susceptible strain or susceptible species becomes resistant.[9] Intrinsic resistance is chromosomally regulated and is linked to the microorganism physiology.[10] Acquired resistance occurs through mutations in the genes encoding the antimicrobial target site or acquisition of resistance-encoding genetic material through mobile genetic elements such as plasmids, integrons, bacteriophages and transposons.[11,12] Horizontal gene transfer can happen through three independent mechanisms: conjugation, considered the most effective, transduction and transformation.[2]

Selective pressure imposed by the intensive use of antimicrobials has had an impact not only on pathogens but also on non-pathogenic and commensal strains.[13] Pathogens that carry resistance genes constitute a direct threat to humans and animal health while non-pathogenic and opportunistic bacteria represent an indirect threat, as their harbor resistance genes that can be transferred to pathogens through horizontal gene transfer.[14] Consequently, bacteria, act as reservoir of resistance genes and influence the dissemination of antimicrobial resistance-encoding genes in the microbial ecosystem. Scott stated that gene transfer occurs widely *in vivo* between gastrointestinal tract microbiota and pathogens.[15] The food chain can be considered one of the routes for antibiotic-resistance gene transfer between animals and humans.[6]

In the last decades, research has demonstrated the presence of antimicrobial resistance genes in *Lactobacillus* spp.,[16] and *Bifidobacterium* spp.[17] but information on the antimicrobial susceptibility profiles of *Leuconostoc* spp. are scarce.[18]

Leuconostoc spp. are heterofermentative lactic acid bacteria, which occur in a large variety of food as commensals. *Leuconostoc* belong to *Leuconostoc-Weissella* group. *Leuconostoc* are detected during manufacturing and ripening of several fermented foods and beverages. *Leuconostoc* have been used in dairy technology for technological and biological beneficial effects.[19] However, in meat products they could be responsible of undesirable modifications and alterations such as slime formation.[20] *Leuconostoc* spp. is considered a *generally regarded as safe* (GRAS) microorganism.[21] However in immunocompromised patients it can indirectly represent a threat by mediating antimicrobial resistance transfer.[16,22] *Leuconostoc* spp. grow at refrigeration temperatures (2-4°C).[23,24] At low temperature *Leuconostoc* spp. can survive and compete with other strains.[25] A typical characteristic of the species of the *Leuconostoc-Weissella* group have been shown to have intrinsic and non transferable resistance against glycopeptides, including vancomycin.[26] Resistance against vancomycin and other glycopeptides is determined by the presence of the dipeptide D-Alanine-D-Lactate as constituent of their peptidoglycan instead of the D-Alanine-D-Alanine dipeptide.[27] The aim of the this study was to investigate the antimicrobial susceptibility patterns of ropy slime-producers *L.mesenteroides* isolates, which were recovered from commercial cooked ham, through disk diffusion test. The aim is to monitor antimicrobial resistance and assess the risk posed by *Leuconostoc mesenteroides* isolates in the transfer of antimicrobial resistance.

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Materials and Methods

Commercial cooked ham marketed in Italy and affected by ropy slime was sampled and examined for the occurrence of slime-producing bacteria. The detection of ropy slime-producing bacteria was conducted through biochemical and biomolecular analysis (PCR and sequencing). According to the phenotypic and genotypic findings, two strains of ropy slime-producing *Leuconostoc mesenteroides* were isolated, identified and classified as 649 and 650 (Laboratory collection ID). Briefly, isolates

were plated on selective agar, *i.e.* MRS agar (Oxoid, CM0361) added with vancomycin (20 g/mL) at 30°C for 48 hours, in microaerophilic conditions. Then, *Leuconostoc mesenteroides* was detected according to PCR conditions indicated by Robert and colleagues,[28] with primers which target the *16S* rRNA gene: L.mesF (5'-AACTTAGTGTCGCATGAC-3') and L.mesR (5'-AGTCGAGTTACAGACTACAA-3'). According to Yost and Nattress,[29] universal primers Y1 (5'-TGGCTCAGAACGAACGCTGGCCCG-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3') for bacterial 16S rRNA gene were used as positive control to ensure that template DNA was readily amplifed; primers Lu1r (5'-CCACAGCGAAAGGTGCTTGCAC-3') and Lu2 (5'-GATCCATCTCTAGGTGACGCCG-3') were used to specifically amplify a fragment of approximately 175 bp from *Leuconostoc* spp. Amplification products were sent for sequencing (Table 1).

Disk diffusion susceptibility test

Antimicrobial resistance patterns were determined against a panel of 21 antibiotics, using the disk diffusion test (Kirby-Bauer) with antimicrobial susceptibility disks (Thermo Scientific™, Oxoid, Waltham, MA, USA) (Table 2). The antibiotics were selected and disk diffusion patterns were evaluated according to the microbiological breakpoints for selected lactic acid bacteria as defined by EFSA (2012).[16] The antibiotics used for this study were cell wall synthesis, nucleic acid synthesis, folate synthesis and protein synthesis inhibitors. The agar disk diffusion method was performed on Mueller Hinton agar (Oxoid, CM0337), according to Clinical and Laboratory Standards Institute guidelines.[30]

The inoculum was prepared from colonies on a primary culture plate. The strains (n=2) of the Laboratory Collection, previously isolated, were inoculated into MRS broth, incubated at 30°C for 24 h. The cell density of the cultures was adjusted to approximately $1*10^8$ cfu mL^{-1}, equivalent to an absorbance at 600 nm of 1668 OD. The broth was transferred on diluent solution until 0.5 McFarland Standard. Mueller-Hinton agar plates were inoculated by dipping a sterile swab into the solution, removing the excess against the side of the tube; followed by streaking the swab all over the surface of the medium three times, by rotating the plate through an angle of 60° after each application. Within 15 minutes of swabbing, four antibiotic disks were placed aseptically on the agar surface. Agar plates with antibiotic disks were then incubated for 24 h at 30°C, under anaerobic conditions. After overnight incubation, the diameters of the inhibition zones were measured using a ruler under a colony counter apparatus and the results were recorded in mm. The result was interpreted according to CLSI standard criteria.[30]

Results

Antimicrobial susceptibility was evaluated using zone diameter interpretive criteria after an average of 2 readings. Isolates were expressed as sensitive (S), intermediate (I) and resistant (R) according to CLSI published breakpoint interpretations based on pharmacokinetic and pharmacodynamic data.[30] Results are summarised in Table 2.

Susceptibility to inhibitors of cell wall synthesis

The isolates showed resistance towards all β-lactams including ampicillin, and methicillin except for ticarcillin. Resistance was also observed against cephalosporins tested *i.e.* cefotaxime, ceftriaxone, cephalotin. Assays for β-lactamase inhibitors revealed resistance to amoxicillin-clavulanic acid. All isolates were resistant to vancomycin.

Susceptibility to inhibitors of nucleic acid synthesis

Strains were resistant to sulphamethoxazole-trimethoprim and sulphonamide. Concerning quinolones, the isolates were resistant to nalidixic acid, but susceptible to enrofloxacin and cyprofloxacin

Susceptibility to protein synthesis inhibitors

All strains were susceptible to tetracycline, to chloramphenicol and to erythromycin. The

Table 1. *Leuconostoc mesenteroides* Genbank accession number and nucleotide sequences of the *16S* rRNA gene.

Strains	ID	GenBank accession number	Nucleotide sequences of the *16S* rRNA gene
Leuconostoc mesenteroides	649	KC568533.1	CWWTKGAGYMTGCGMACTAAGTTTTATTCGGTATTAGCATC TGTTTCCAAATGTTATCCCCAGCCTTGAGGCAGGTTGTCCAC GTGTTACTCACCCGTTCGCCACTCACTTGAAAGGTGCAAGCA CCTTTCGCTGTGGA
Leuconostoc mesenteroides	650	gb|M23035.1|	CTWATTTGKGYMTGCGAMACTAAGTTTTATTCGGTATTAGC ATCTGTTTCCAAATGTTATCCCCAGCCTTGAGGCAGGTTGTC CACGTGTTACTCACCCGTTCGCCACTCACTTGAAAGGTGCAA GCACCTTTCGCTGTGGA

Table 2. Antimicrobials disks used in susceptibility assay and susceptibility profiles of strains of *Leuconostoc mesenteroides*.

Antimicrobials	Concentration, µg/disk	*Leuconostoc mesenteroides*
Cell wall synthesis inhibitors		
Amoxicillin/clavulanic acid	20-10	R
Ampicillin	10	R
Cefotaxime	30	R
Ceftriaxone	30	R
Cephalothin	30	R
Methicillin	5	R
Ticarcillin	75	S
Vancomycin	30	R
Nucleic acid synthesis inhibitors		
Cyprofloxacin	5	S
Enrofloxacin	5	S
Nalidixic acid	30	R
Chloramphenicol	30	S
Folate synthesis inhibitors		
Compound sulphonamides	300	R
Sulphamethoxazole/trimethoprim	25	R
Protein synthesis inhibitors		
Erythromycin	15	S
Tetracycline	30	S
Amikacyn	30	S
Gentamicin	10	S
Kanamycin	30	S
Neomycin	30	S
Streptomycin	10	I

R, resistant; I, marginally susceptible; S, susceptible.

isolates were also susceptible to the following aminoglycosides: gentamicin, neomicyn, amikacyn and kanamycin. Finally, susceptibility was observed for all the inhibitors of protein synthesis tested with the exception of partial resistance to streptomycin.

Discussion and Conclusions

Comparing to data reported in previous studies, our results showed that ropy slime-producing *L.mesenteroides* strains were resistant to β-lactams and susceptible to chloramphenicol, kanamycin and partially resistant to streptomycin. Concerning resistance to glycopeptides, nalidixic acid, gentamicin, kanamycin, streptomycin and sulphamethoxazhole/thrimetroprim and susceptibility to chloramphenicol, erythromycin and tetracycline our results are in accordance with previous studies.[16,27,31]

The effects of antibiotic resistance represent a major health concern even if it is a common characteristic in bacteria; further assessment in the ecology of the phenomenon is required.[32] The transmission of antibiotic resistance genes to other organisms is one the most important safety issues because the food chain is considered as one of the paths for the diffusion antibiotic resistance-encoding genes.[6] When considering microbiota interaction, genetic material is shifted from one strain to another, and also genes coding for resistance to a certain antibiotic may be transferred to other species.[18]

Nowadays, increased attention is given to food safety, taking into account all the bacteria present in food products. Lactic acid bacteria naturally occur in many environments, including vegetables, meat, gastrointestinal tract, and strains with multi-drug resistance-encoding genes constitute a potential threat for the wellbeing of humans or animals.[6] Naturally present or intentionally added lactic acid bacteria represent a source of antibiotic resistance determinants for potentially pathogenic strains through horizontal gene transfer.[5,7] Ropy slime producing bacteria are responsible of spoilage of cooked meat products and, in association with the abundant secretion of exopolysaccharides, they maintain a stationary phase which persist for the entire shelf life of the product. The attention to these strains is not only due to the economical impact of the spoilage they provokes but also for investigating the potential risk of transmission of antibiotic-resistance genes to pathogens, considering their multi-drug resistance. In fact, when the phenomenon of ropy slime is not evident and the product reaches the final consumer, the risk is enhanced.

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