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Antimicrobial susceptibility of nisin resistant *Listeria monocytogenes* of dairy origin

Beatriz Martínez *, Ana Rodríguez

Instituto de Productos Lácteos de Asturias, IPLA-CSIC, Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain

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Abstract

The antibiotic susceptibility of wild *Listeria monocytogenes* strains and their corresponding nisin resistant variants was assessed. The resistant strains were more sensitive to most of the tested antibiotics than their wild-type counterparts. A slight increase in MIC was observed for a few antibiotics including the membrane disturbing polymixin B. Cross-resistance was detected with two synthetic antimicrobial peptides. A lower C15/C17 ratio in the membrane fatty acid composition of the nisin resistant strains was found, and one strain pair showed a significant difference in surface hydrophobicity. As judged by these results, no clear correlation could be established between resistance to nisin and to worldwide-used antibiotics.

Keywords: *Listeria monocytogenes*; Nisin resistance; Antibiotic cross-resistance; Synthetic antimicrobial peptides; Surface properties

1. Introduction

Food-borne illnesses and food intoxication are still issues of major concern. The increasing demand of minimally processed foods along with a consumer awareness of potential health risks associated to traditional preservatives has fueled researchers to examine new strategies for food preservation. In the last decades, a special effort has been focused in the use of naturally occurring antimicrobials, such as bacteriocins, as an additional hurdle to fight pathogen growth and prevent food spoilage [1,2].

Bacteriocins are ribosomally synthesised peptides which display antimicrobial activity. Bacteriocin production is a widespread trait among lactic acid bacteria (LAB), which are regarded as GRAS microorganisms due to their extended use as starters in the production of several fermented foods. Therefore, LAB bacteriocins have a great potential as food-grade antimicrobials, exemplified by the lantibiotic nisin, used as a food biopreservative in several countries. LAB bacteriocins are usually amphipathic positively charged peptides (2.5–10 kDa), heat stable, and have been classified into 3 main classes: (I) the lantibiotics, which are post-translationally modified and, thus, contain unusual amino acids such as lanthionine; (II) heat stable non-modified peptides, including class IIa or pediocin-like bacteriocins which are remarkably active against *Listeria* sp. and, (III) large heat labile proteins [3]. Most of LAB bacteriocins impair the overall integrity of the cytoplasmic membrane by pore formation [4]. Furthermore, nisin and related lantibiotics use the membrane-bound peptidoglycan precursor lipid II as a docking molecule for pore formation and, thus, cell wall synthesis is also inhibited [5,6].

* Corresponding author. Tel.: +34 985 89 33 59; fax: +34 985 89 22 33.

E-mail address: bmf1@ipla.csic.es (B. Martínez).

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Target microorganisms may become to some extent resistant to the pore-forming bacteriocins. The resistance may be transient or remain stable. Changes in the membrane composition and fluidity and polysaccharide production are examples of resistance mechanisms towards nisin [7,8]. Furthermore, an altered gene expression was detected in nisin resistant mutants in *Listeria monocytogenes* [9]. Resistance to class IIa bacteriocins has been related to the absence of one of the components of the mannose transport system [10,11].

Despite of the fact that bacteriocin resistance may hinder further applications, this issue has only been addressed in the last few years. Nonetheless, bacteriocin resistance raises concerns about the consequences of an intensive use of bacteriocins in food regarding potential cross-resistance in food pathogens towards clinically used antibiotics. In this context, it is worth to notice that nisin and vancomycin share the same target: the lipid II. Moreover, cationic antimicrobial peptides, structurally related to bacteriocins, are our first line of defence and non-specific cross resistance may be foreseen [12]. In fact, the EU has considered reviewing the use of nisin (E-234) in food, as one of the key actions within its strategy against antimicrobial resistance [13].

The aim of this work was to analyse the susceptibility of nisin resistant *L. monocytogenes*, derived from dairy isolates, towards several antibiotics as well as to synthetic cationic antimicrobial peptides which are being designed as novel anti-infective drugs. Surface properties such as membrane fatty acid composition and surface hydrophobicity, which could impair the interaction of the antimicrobial peptides with the cytoplasmic membrane, were compared between the wild types and the nisin resistant variants.

2. Material and methods

2.1. Bacterial strains and growth conditions

The wild *L. monocytogenes* strains Lm4 y Lm41 were previously isolated from short ripened cheeses manufactured in Asturias (northern Spain) [14]. They belong to the serotypes 1/2b and 4b, respectively. *L. monocytogenes* ScottA was used as a reference strain. Spontaneous nisin resistant derivatives (ScottAR, Lm4R and Lm41R) were isolated on TSA plates containing nisin at 1.5 μM that is, approximately, twofold the original nisin MIC values. Other *L. monocytogenes* strains and their nisin resistant derivatives are listed in Table 2 and were kindly supplied by Dr. A. Gravesen (Royal Veterinary and Agricultural University, Denmark). All the strains were routinely grown at 37 °C in Trypticase Soy broth (TSB) (Scharlau, Barcelona, Spain). For the nisin resistant strains, nisin (0.75 μM) was added (TSB-Nisin), unless indicated. All the strains were kept at −80 °C in TSB containing glycerol 10% (wt v^{-1}).

2.2. Etest antimicrobial susceptibility test

The Etest (AB Biodisk, Solna, Sweden) was carried out following the manufacturer instructions with some modifications. Exponentially growing cultures in TSB were diluted in 30 ml soft-TSB (agar 0.7%) to give a final concentration ca. 10^5 cfu/ml and further spread on TSA (agar 2%) 150-mm plates. Etest strips were laid on the agar surface and plates were incubated at 37 °C. MIC readings were carried out at 24 and 48 h. The tested antibiotics were: (i) protein synthesis inhibitors: chloramphenicol, clindamycin, kanamycin, streptomycin, azithromycin, clarithromycin, dirithromycin, erythromycin, doxycycline, minocycline, tetracycline; all of them in a range 0.016–256 μg/ml) and quinupristin/dalfopristin (0.002–32 μg/ml); (ii) RNA synthesis inhibitor: rifampicin (0.002–32 μg/ml); and (iii) cell wall synthesis inhibitor: vancomycin (0.016–256 μg/ml).

2.3. Disk diffusion susceptibility test

Inoculated plates were prepared and incubated as described above for the Etest. The disks (Fluka, Buchs SG, Switzerland) contained the following cell wall synthesis inhibitors: ampicillin (10 μg), amoxicillin plus clavulanic acid (20 + 10 μg), oxacillin (5 μg), methicillin (10 μg) and cefalexin (30 μg). Fosfomycin disks (50 μg) were purchased from BioMérieux (Marcy-L'Ëtoile, France). The inhibition halos were measured after 24 h incubation at 37 °C.

2.4. Broth microdilution MIC determination

It was performed to calculate the MIC values of nisin (Applin & Barrett Ltd., Dorset, UK), penicillin G (Sigma, Madrid, Spain), polimyxin B (Sigma) and the synthetic antimicrobial peptides (AMPs) P19/G7 and P19(6/E) [15], pilosulin2 [16] and amphipathic-3D (A-3D) [17]. The AMPs were kindly supplied by A. Tossi (University of Trieste, Italy) and H.G. Sahl (University of Bonn, Germany). Serial twofold dilutions (100 μl) were made in a microtiter plate (Nunc GmbH & Co., KG, Germany). Each well was further inoculated with 100 μl of an exponentially growing culture in TSB with an $OD_{600\ nm}$ of 1.0 diluted 1:10000 in TSB. Plates were incubated at 37 °C for 24 h. The MIC was taken as the lowest concentration that completely inhibited bacterial growth.

2.5. Cellular fatty acid composition

The fatty acid composition was determined by gas chromatography at the DSMZ external service

(Braunschweig, Germany) using the Microbial Identification System (MIDI, Newark, DE, USA). The cell extracts were obtained from exponentially growing cultures in TSB or TSB-Nisin of the wild type and nisin resistant strains, respectively. The cells were collected by centrifugation, washed twice with sterile deionised water and freeze dried. The fatty acids were converted to methyl esters and extracted in a four-step procedure according to the manufacturer instructions (http://www.midi-inc.com/media/pdfs/TechNote_101.pdf).

2.6. Cell surface hydrophobicity

Overnight cultures of *L. monocytogenes* in TSB were washed twice with sodium phosphate buffer 50 mM, pH 6.5 and adjusted to an $OD_{600\ nm}$ of 1.0. Cells (3 ml) were vigorously vortexed with 0.6 ml of hexadecane (Sigma–Aldrich, Madrid, Spain) and allowed to stand for 10 min at room temperature. The aqueous phase was carefully extracted and the $OD_{600\ nm}$ was checked. The percentage of the cells (measured as OD) that migrated into the organic phase was taken as a measure of the cell surface hydrophobicity. All the measurements were done in triplicate.

2.7. Statistical analysis

For each pair of strains, wild type and the nisin resistant counterpart, the cell surface hydrophobicity values were compared using the one-way ANOVA analysis with the SPSS 11.0 software for windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Antibiotic susceptibility

An undesirable consequence of an extended use of natural antimicrobials such as nisin in food might be cross-resistance to clinically used antibiotics in foodborne pathogens such as *L. monocytogenes*. However, only few studies have comprehensively addressed this issue [8,18].

In this work, we have analysed the antibiotic susceptibility of two *L. monocytogenes* dairy isolates (Lm4 and Lm41) and the clinical isolate ScottA alongside their nisin resistant counterparts Lm4R, Lm41R and ScottAR, respectively (Table 1). These nisin resistant strains have an enhanced MIC for nisin of two to fourfold (shown in Table 2). The antibiotic susceptibility profile of these *Listeria* strains was in agreement with previous reports [19,20]. Our wild type strains were susceptible to most of the antibiotics and resistant to cephalosporins and fosfomycin, towards them *Listeria* is intrinsically resistant (Table 1 and data not shown). Hence, a particular antibiotic resistant phenotype was not displayed by these strains.

The nisin resistant strains were more susceptible to the antibiotics than their wild type counterparts (Table 1). This could be related to the fitness cost commonly associated to the development of the nisin resistant phenotype [21]. There was only cross-resistance to the aminoglicosides kanamycin and streptomycin and to the membrane disturbing polymixin B (Table 1). Only in the latter case, though, the cross-resistance was consistently displayed by the 3 strain pairs. This result seems reasonable as nisin shares the same primary target

Table 1
Susceptibility of *Listeria monocytogenes* strains and their nisin resistant counterparts against several antibiotics

Antibiotic	Test	Target strains					
		ScottA	ScottAR	Lm4	Lm4R	Lm41	Lm41R
Chloramphenicol	E	8	4	4	4	8	8
Clindamycin	E	1.5	1.5	2	1	1.5	1.5
Kanamycin	E	24	24	12	24	8	16
Streptomycin	E	96	96	48	48	12	24
Clarithromycin	E	0.38	0.25	0.38	0.19	0.25	0.25
Erythromycin	E	0.75	0.75	0.75	0.38	0.38	0.38
Doxycycline	E	0.094	0.094	0.19	0.094	0.25	0.25
Minocycline	E	0.047	0.032	0.094	0.047	0.125	0.125
Rifampicin	E	0.19	0.094	0.125	0.125	0.047	0.064
Ampicillin	D	32	35	29	31	32	36
Methicillin	D	29	30	23	27	17	25
Amoxicillin plus clavulanic acid	D	35	37	33	39	38	39
Penicillin G	B	0.125	0.063	0.125	0.125	0.250	0.125
Polymixin B	B	100	200	50	200	100	200

Susceptibility was tested by Etest (E), disks (D) or by the broth microdilution method (B). The results are the MIC in μg/ml for the Etest and broth microdilution method and the diameter of the inhibition halo (mm) for the disk susceptibility test.
Only those antibiotics whose MIC values were at least twofold higher or lower than those of the wild type strains are displayed. A full list of the tested antibiotics is described in Section 2.

Table 2
Susceptibility of *Listeria monocytogenes* and the nisin resistant variants (strains R and N) to synthetic cationic antimicrobial peptides and nisin

Target strain	Cationic antimicrobial peptides MIC (μM)				
	P19(G/7)	P19(6/E)	A-3D	Pilosulin2	Nisin
ScottA	>4	2	>4	4	1.86
ScottAR	>4	4	>4	>4	7.46
Lm4	>4	2	4	2	1.86
Lm4R	>4	2	>4	4	7.46
Lm41	>4	2	4	4	1.86
Lm41R	>4	2	4	>4	3.73
Lm322	>4	2	>4	2	1.86
Lm322N	>4	2	>4	4	7.46
Lm409	>4	2	4	4	1.86
Lm409N	>4	4	>4	>4	7.46
Lm412	>4	2	>4	4	1.86
Lm412N	>4	2	>4	4	3.73

Susceptibility was tested by the broth microdilution method.

with polymixin B: the cytoplasmic membrane. Nisin resistance in *L. monocytogenes* has been partially correlated with changes in the membrane composition which potentially interfere with the pore forming ability of nisin in the cytoplasmic membrane [8,22]. Moreover, changes in the cell envelope such as a thickener cell wall, polysaccharide production or a higher degree of D-alanine substitution in the teichoic acids were also described as resistance strategies to avoid killing by cationic antimicrobial peptides [23–25]. Basically, these mechanisms lower the net negative surface charge and restrict the accessibility of nisin and, hence, of other cationic drugs such as polymixin B and aminoglycosides, to their targets.

As judged by these results, the development of nisin resistance in *L. monocytogenes* would not hinder the current antibiotic therapy towards listeriosis based on β-lactam treatment to which the nisin resistant strains were more susceptible than the wild types. A higher sensitivity to β-lactams has been frequently correlated to enhanced nisin resistance in *L. monocytogenes* [8,18,26].

3.2. Cross-resistance to synthetic antimicrobial peptides

Ribosomally synthesised cationic antimicrobial peptides (AMPs) are found as components of the innate defence system against infection in virtually all forms of life [27]. Most of them act as membrane disturbing molecules, although additional internal targets are not discarded [17,28], and display a broad inhibitory spectrum including multidrug resistance pathogens. Besides, due to their amenability to perform structure/activity studies, these AMPs are thought to be potential leading structures for developing new antibiotics [15]. As nisin can be considered a component of the wide AMPs family, we have also analysed the potential cross-resistance of nisin resistant strains with synthetic AMPs which were rationally designed to improve their antimicrobial activity (Table 2). The strains Lm322, Lm409 and Lm412 and their nisin resistant variants were also included. The molecular basis of the nisin resistance phenotype of these strains has been partially characterized. Both Lm322N and Lm412N overexpressed a gene encoding a penicillin binding protein (PBP) while Lm409 did not [9,18]. This PBP could shield lipid II and hamper nisin binding to lipid II.

The peptides A-3D and P19(G/7) were hardly active against *L. monocytogenes* and their MIC values were over the 4 μM cut-off established for proper antibiotic candidates. These AMPs were followed by pilosulin2 and P19(6/E) which displayed MICs in the same range as nisin (1.86–2 μM) towards the wild type strains. These values are also similar to those previously described for other natural antimicrobial peptides [29]. A clear correlation was found between nisin resistance and a lower susceptibility to pilosulin2 as observed with five out of the six strains tested (Table 2). Cross resistance between nisin and P19(6/E) was also obvious at prolonged incubations (data not shown), except for the strains whose resistant phenotype involves a higher expression of the PBP coding gene, i.e., Lm322N and Lm412N. These observations support the hypothesis that nisin resistance in these strains relies essentially on the higher PBP synthesis and not in changes in the overall composition of the cytoplasmic membrane [9].

The different AMPs susceptibility profile found within the strains reflects the complexity of the nisin resistant phenotypes which appears to be strain/isolate dependent and multifaceted. On the other hand, AMPs have shown a relative large variability in their ways of killing, albeit they share in common the ability to disrupt microbial membranes. Therefore, isolate-specific responses to AMPs are also expected [15,17,30].

3.3. Cell surface properties of nisin resistant strains

In an attempt to clarify the possible mechanisms underlying nisin and AMP resistance in our dairy

isolates, surface properties such as cellular fatty acid composition and the hydrophobicity were analysed and compared between the wild types and the nisin resistant strains.

The membrane of the *L. monocytogenes* strains were composed principally of C14, C15, C16 and C17 fatty acids, accounting for ca. 2.1%, 53.2%, 5.3%, and 35.8% of the total fatty acids, respectively (data not shown). They were mainly synthesized as the iso/anteiso branched fatty acids. Minor amounts of 2- and 3-hydroxy fatty acids were also detected. A similar overall composition has been previously described in this species [31]. The comparison of the most abundant fatty acids C15 and C17 among the wild type strains and their variants is shown in Fig. 1. There were hardly differences in their C15 content between the strain pairs. In contrast, the C17 content was persistently higher in the nisin resistant strains. This led to a lower ratio C15/C17, which has been directly related to a more rigid membrane and frequently associated, but not always, to the nisin resistance phenotype as well as to resistance to other pore-forming bacteriocins [22,31,32].

According to the hydrophobicity measurements (Fig. 2), no significant differences ($p > 0.05$) were observed between Lm4 and ScottA and their nisin resistant counterparts, respectively. On the contrary, Lm41R was more hydrophobic ($p < 0.05$) than the corresponding wild type (Fig. 2). This implies a substantial change in the surface architecture of this nisin resistant mutant which might involve a different protein display at the surface. Further in-depth characterization of this nisin resistant mutant should be carried out to verify this hypothesis.

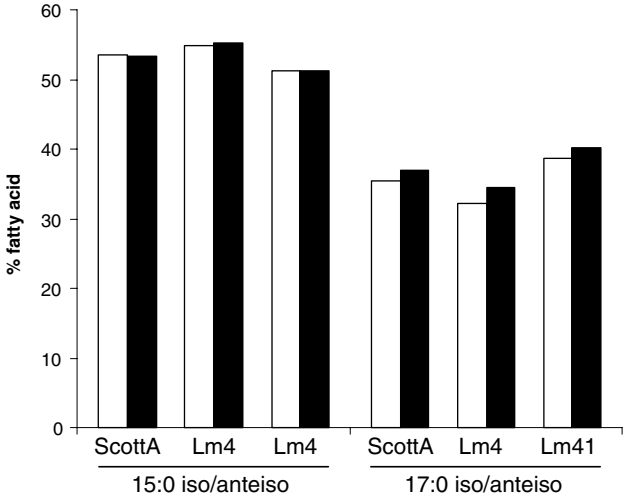

Fig. 1. Fatty acid composition of the *Listeria monocytogenes* strains ScottA, Lm4 and Lm41 (open bars) and their nisin resistant counterparts ScottAR, Lm4R and Lm41R, respectively (solid bars). Only the most abundant fatty acids C15 and C17 are displayed.

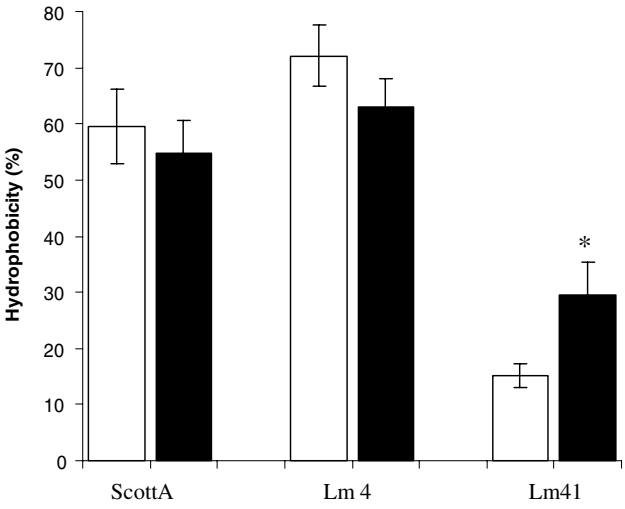

Fig. 2. Surface hydrophobicity of the *Listeria monocytogenes* strains ScottA, Lm4 and Lm41 (open bars) and their nisin resistant counterparts ScottAR, Lm4R and Lm41R, respectively (solid bars). $^{*}p < 0.05$.

3.4. Concluding remarks

According to these results, the development of nisin resistance in dairy *L. monocytogenes* would not jeopardize the antibiotic therapy since no cross-resistance to the most common clinically used antibiotics was observed. However, it might hinder the future use of synthetic antimicrobial peptides whose mechanism of action is mainly based on membrane permeabilization. On the other hand, several nisin resistant phenotypes can be envisaged depending on the strains. Therefore, it would be advisable to extend the survey to a larger number of *L. monocytogenes* isolates from several sources, mainly from foods relying on nisin preservation and susceptible to *L. monocytogenes* contamination.

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