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Purification and characterization of a new bacteriocin active against *Campylobacter* produced by *Lactobacillus salivarius* SMXD51

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ABSTRACT

Strain SMXD51, isolated from chicken ceca and identified as *Lactobacillus salivarius*, produced a component that inhibits the growth of Gram-positive and Gram-negative bacteria and especially *Campylobacter jejuni*. The active peptide from the cell-free supernatant of *Lb. salivarius* SMXD51 was purified in three steps: (i) precipitation with 80% saturated ammonium sulfate, (ii) elution on a reversed phase SPE UPTICLEAN cartridge using different concentrations of acetonitrile, (iii) final purification by reversed phase HPLC on a C_{18} column. The mode of action of this peptide of 5383.2 Da was identified as bactericidal, and its amino acid composition was established. This new bacteriocin SMXD51 appears potentially very useful to reduce *Campylobacter* in poultry prior to processing.

1. Introduction

Human campylobacteriosis is a zoonosis and the leading cause of recognized bacterial gastroenteritis in industrialized countries. It is characterized by watery or bloody diarrhea, abdominal cramps and nausea. This pathogenic organism is also associated with Guillain–Barré syndrome (Olson et al., 2008) *Campylobacter* has become increasingly resistant to clinical antibiotics, causing a serious threat to public health (Allos, 2001). The majority of human infections (>80%) are due to *Campylobacter jejuni* with the remainder being predominantly caused by *Campylobacter coli* (EFSA, 2009).

Poultry flocks are frequently colonized by *C. jejuni* without any apparent symptoms (Shane, 2000) and risk assessment analyses have identified the handling and consumption of poultry meat as one of the most important sources of human campylobacteriosis (Potter et al., 2003). Several studies have demonstrated that horizontal transmission is the major route of *Campylobacter* colonization of poultry as the same strain has been shown to colonize multiple flocks on a farm (Newell and Fearnley, 2003). Raw poultry meat is considered an important vector of *Campylobacter* (Samuel et al., 2004).

Campylobacter colonization sites are the cecum, small intestine, large intestine, and cloaca. It is generally accepted that *Campylobacter* has evolved to colonize the avian gut rapidly and efficiently (Beery et al., 1988). Elimination of *Campylobacter* in the poultry reservoir is a crucial step in the control of this food safety problem.

Several farm-based interventions have been proposed to control campylobacteria in poultry such as the use of antimicrobial peptides (Svetoch and Stern, 2010). At present, there is an urgent need for control measures that can be used in the field and that are acceptable to consumers.

Lactic acid bacteria (LAB) are important components of the healthy intestinal microbiota of chicken. The LAB population levels in the chicken digestive tract are higher than 10^8–10^9viable cells per g (Dumonceaux et al., 2006). The dominance of LAB species can vary depending on the anatomical site (Zhu and Joerger, 2003). They are capable of producing a wide range of ribosomally synthesized proteins and peptides, which have antimicrobial activity to compete with other bacteria of the same species (narrow spectrum) or to counteract bacteria of other genera (broad-spectrum) (Drider et al., 2006). Most of the bacteriocins produced

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by LAB are only active against other Gram-positive bacteria. A cursory search for the pertinent literature reveals well over a thousand research articles published in the past 30 years describing the isolation of bacteriocins. Of these, only a small percentage displays antibacterial activity toward more phylogenetically distant Gram-positive bacteria and, under certain conditions, Gram-negative bacteria.

Anti-*Campylobacter* bacteriocin treatment could be an effective strategy to reduce the *C. jejuni* load in chickens. Commensal bacteria showing an inhibitory effect towards *Campylobacter* have been isolated from the intestinal tract of chicken but only a few bacteriocins have been purified and characterized, including SRCAM 602 from *Paenibacillus polymyxa* (Stern et al., 2005), OR-7 from *Lactobacillus salivarius* (Stern et al., 2006), and E-760 and E 50-52 from *Enterococcus* spp. (Line et al., 2008; Svetoch et al., 2008). In a recent study, LAB have been isolated from chicken ceca (Messaoudi et al., 2011) and tested for their antimicrobial activity. The aim of the present study was to purify and characterize the 5383.2 Da bacteriocin produced by the strain *Lb. salivarius* SMXD51 isolated from chicken ceca with a broad-spectrum activity against both Gram-positive and Gram-negative bacteria, and to identify whether its mechanism of action against *C. jejuni* is bacteriostatic or bactericidal.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Lb. salivarius SMXD51, identified by whole 16S rRNA gene sequencing, was isolated from Tunisian chicken ceca (Messaoudi et al., 2011). This strain showed an antimicrobial activity against *C. jejuni*. *Lb. salivarius* NRRL B-30514 (Stern et al., 2006) was used as the positive control and was grown aerobically at 37 °C in de Man–Rogosa–Sharpe (MRS) medium (AES, Bruz, France) (de Man et al., 1960) for 18–24 h. Indicator strains used to determine the antimicrobial activity of partially purified bacteriocin from *Lb. salivarius* SMXD51 are shown in Table 1.

Table 1
Antimicrobial activity spectrum of partially purified bacteriocin of *Lactobacillus salivarius* SMXD51.

Strains	Sources	Conditions of growth	Antimicrobial activity
Campylobacter jejuni	NCTC 11168	Brucella, 18–24 h at 41 °C	++
Campylobacter jejuni	81176	Brucella, 18–24 h at 41 °C	+
Campylobacter coli	CIP 702	Brucella, 18–24 h at 41 °C	+
Campylobacter coli	CIP 7081	Brucella, 18–24 h at 41 °C	+
Listeria monocytogenes	EGDe 107776	Elliker, 18–24 h, 37 °C	++
Listeria innocua F	ONIRIS, Nantes	Elliker, 18–24 h, 37 °C	–
Salmonella enterica	CIP 81.3	BHI, 18–24 h, 37 °C	+
Bacillus cereus	CIP 78.3	BHI, 18–24 h, 37 °C	++
Staphylococcus aureus	CIP 76.25	BHI, 18–24 h, 37 °C	++
Pseudomonas fluorescens	CIP 6.913T	BHI, 18–24 h, 37 °C	–
Lactobacillus brevis	F1.114	MRS, 18–24 h, 37 °C	–
Lactobacillus sakei	ATCC 15531	MRS, 18–24 h, 30 °C	–
Lactobacillus plantarum	ATCC 14917	MRS, 18–24 h, 30 °C	–
Lactobacillus salivarius	101 NRRL B-30514	MRS, 18–24 h, 37 °C	–
Enterococcus faecium S34	ONIRIS, Nantes	MRS, 18–24 h, 37 °C	–
Enterococcus faecalis	CIP105042	BHI, 18–24 h, 30 °C	–
Fungi			
Penicillium roqueforti	LUBEM, Brest	Potato Dextrose, 48 h, 30 °C	–
Geotrichum candidum	LUBEM, Brest	Potato Dextrose, 48 h, 30 °C	–
Mucor plumbeus	LUBEM, Brest	Potato Dextrose, 48 h, 30 °C	–
Fusarium sp.	CBS 1385	Potato Dextrose, 48 h, 30 °C	–
Yeasts			
Debaryomyces hansenii	LUBEM, Brest	Potato Dextrose, 48 h, 30 °C	–
Kluyveromyces lactis	LUBEM, Brest	Potato Dextrose, 48 h, 30 °C	–

(–) no inhibition; (+) diameter of inhibition < 3 mm; (++) diameter of inhibition of 3–6 mm.

2.2. Bacteriocin purification

Lb. salivarius SMXD51 was evaluated for bacteriocin production following the procedures of (Batdorj et al., 2006). The strain was grown in 1 L of MRS medium at 37 °C for 18 h in aerobic conditions ($OD_{600} = \pm 1$). The culture was then centrifuged at 12,000 g for 10 min to remove the cells and the supernatant was adjusted to pH 6.8 by adding 1 N NaOH and tested against *C. jejuni* NCTC 11168. Neutralized and filtered supernatant of L. *Lb. salivarius* NRRL B-30514 was used as positive control. Next, the soluble peptides were isolated from the supernatant by ammonium sulfate precipitation to produce a crude antimicrobial preparation, which was then filtered through 0.22 μm filters (Millipore, Bedford, MA). Collected fractions were assayed for antimicrobial activity against *C. jejuni* NCTC 11168.

The pH of the active fraction was adjusted to 3 by adding 5% TFA to obtain a final concentration of 0.1%. Sample was applied on a reversed phase SPE UPTI-CLEAN cartridge (REC18-1G/6 ml; D94, Interchim) equilibrated with 20% H_2O-TFA 0.1% (trifluoroacetic acid), 80% acetonitrile. Elution was performed in steps using different concentrations of acetonitrile (32%, 48% and 80%).

Fractions (5 ml) were collected and acetonitrile was removed by a Speed-Vac concentrator (SC110A, Savant). Before testing the antimicrobial activity against *C. jejuni* NCTC 11168, the pH was adjusted to 6.8 using NaOH. The active fraction was further purified by reversed phase-high performance liquid chromatography (RP-HPLC) using a Waters Alliance apparatus with Millennium software (Millford, MA, USA). One hundred microliters of concentrated bacteriocin was injected into an analytical RP Nucleosil C_{18} column (Symmetry 300 Å C_{18}, 5 μm, 2.1 × 150 mm, Macherey Nagel, France) equilibrated with solvent A. Elution was performed at a flow-rate of 0.2 ml/min with a linear gradient from 40% to 100% solvent B in 40 min. The eluted peaks were detected by spectrophotometry, measuring the absorbance between 210 and 300 nm with a photodiode array detector (PDA 996; Waters), and were collected manually. The active fractions were then concentrated in a Speed-Vac concentrator. All fractions were recovered and re-suspended in 50 μl phosphate buffer (50 mM K_2HPO_4, 50 mM KH_2PO_4, pH 7) and tested against *C. jejuni* NCTC 11168. The active fraction was re-injected onto the same column in the same conditions in order to improve the quality of the purification, and checked again for its activity.

The bacteriocin activity in arbitrary units (AU) was calculated as described by (Batdorj et al., 2006). Serial two-fold dilutions of samples from different steps of the purification (Table 2) were performed with phosphate buffer and filtered through a 0.2 μm membrane (Sartorius). These samples were tested by the agar diffusion test on the *C. jejuni* NCTC 11168 strain, as described previously (Messaoudi et al., 2011). For each sample, one AU was defined as the reciprocal of the lowest dilution that inhibited the growth of the target strain.