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ORIGINAL PAPER

Bacteriocinogenic properties of *Escherichia coli* P2C isolated from pig gastrointestinal tract: purification and characterization of microcin V

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Abstract
The aim of this study was to isolate and investigate the bacteriocinogenic and probiotic potential of new Gram-negative isolates. Of 22 bacterial isolates from pig intestine and chicken crops, ten isolates had demonstrated a good activity, and the most potent five strains were identified as four *E. coli* and one as *Proteus* sp. No virulence factors were detected for *E. coli* strains isolated from pig intestine. The semi-purified microcins proved to be resistant to temperature and pH variation, but sensitive to proteolytic enzymes. Of particular interest, strain *E. coli* P2C was the most potent, free of virulence genes and sensitive to tested antibiotics. Purification procedure revealed the presence of a single pure peak having a molecular mass of 8733.94 Da and matching microcin V (MccV). The sequence obtained by LC–MS/MS confirmed the presence of MccV. Purified MccV showed a good activity against pathogenic coliforms, especially *E. coli* $O_1K_1H_7$ involved in avian colibacillosis. The present study provides evidence that *E. coli* strains isolated from pig intestine produce microcin-like substances. *E. coli* P2C is a safe MccV producer that could be a good candidate for its application as novel probiotic strain to protect livestock and enhance growth performance.

Keywords Bacteriocin · Probiotic · HPLC · Microcin V · Antimicrobial activity

Introduction

Bacterial resistance to antibiotics has become today a major public health issue. It is that the abuse of conventional antibiotics for decades, associated with many bacterial genetic mutations, has led to the emergence of resistant bacterial strains, which has become a widespread phenomenon (Davies and Davies 2010). It is clear that the characterization of natural molecules having anti-infective properties is an urgent need and a promising discovery strategy. In this perspective, probiotics and their secreted antimicrobial peptides stand as promising candidates with high potential of application due to their ability to overcome the emergence of antibiotic-resistant bacteria (Andersson et al. 2016; Deslouches et al. 2015).

Probiotics are defined as "live microorganisms, which, when consumed in adequate amounts, confer a health benefit on the host" (Shanahan 2010). Among the Gram-negative bacteria with probiotic properties, *E. coli* strain Nissle 1917 is probably the most intensively investigated bacterial strain today (Jacobi and Malfertheiner 2011). The *E. coli* species are diverse and include commensal, beneficial and pathogens strains. The mechanisms of probiotic actions of *E. coli* have been demonstrated by an increasing number of clinical trials and investigations, which had generated interest in this species as an appropriate probiotic. Indeed, several members of *E. coli* produce antimicrobial peptides (bacteriocins), which are being considered an important trait in the selection of probiotic candidates (Dobson et al. 2012).

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Bacteriocins produced by Gram-negative bacteria are diverse, ribosome-encoded, active against phylogenetically related bacterial strains, and classified into two main families: colicins and microcins (Drider and Rebuffat 2011). Microcins are highly stable hydrophobic peptides having molecular masses below 10 kDa unlike colicins, and secreted under stressful conditions of nutrients depletion (Duquesne et al. 2007). There are two classes of microcins, Class I which encompasses lower-molecular-mass peptides (<5 kDa) including microcin B17 (MccB17), MccC7/C51, MccJ25, and MccD93 that are highly post-translationally modified, and Class II which includes higher (5–10 kDa) molecular-mass peptides (Morin et al. 2011; Pons et al. 2002). Microcin V, previously known as colicin V (Zhang et al. 1995) belongs to this second class, synthesized using a non-SOS inducible system, and does not require a lysis protein for its release, thus making the protein non-lethal for the producing cells (Pinou and Riley 2001).

Adverse effects of *E. coli* use as probiotic is mainly due to the frequent presence of several virulence factors on its genome such as P. fimbriae, the temperature-sensitive hemagglutinin (*Tsh*) and aerobactin (Mainil 2013). P fimbriae is a virulence gene that mediates bacterial adherence to human epithelial cells via digalactoside-specific binding to the *P* blood group antigens, which are expressed throughout the urinary tract and facilitate ascending infection of the ureter and kidney (Johnson et al. 1988). *Tsh* gene encodes an autotransporter protein secreted by avian-pathogenic coliforms and is used to distinguish innocuous from pathogenic strains that colonize the respiratory tract leading to airsacculitis, pericarditis, and colisepticemia (Kostakioti and Stathopoulos 2004). Aerobactin is a bacterial siderophore associated with uropathogenic *E. coli* strains responsible for iron uptake in an iron-poor environment (Gao et al. 2015). The presence of aerobactin system was presumed sometimes to indicate the production of microcin V (Waters and Crosa 1991). Similarly, *Stx1* and *Stx2* genes encode shiga toxins, giving *E. coli* the capacity to cause a disease. The *eae* is one of the main genes conserved within LEE (locus of enterocyte effacement) and encodes intimin, a bacterial outer membrane protein involved in the close bacteria adhesion to enterocytes (Chandran and Mazumder 2013).

This work aimed at evaluating the bacteriocinogenic and probiotic potential of five strains previously isolated from pig intestine (Verso et al. 2017) and chicken crops. The inhibitory activity and safety of these strains were investigated. Additionally, the structure and antimicrobial activity of produced bacteriocin are also reported.

Materials and methods

Bacterial strains, media, culture conditions

Producer strains were previously isolated from pig intestine (Verso et al. 2017) and chicken crops, and are summarized in Table 1. These strains were selected among several other co-isolated Gram-negative bacteria for their antibacterial potency. All indicator strains were obtained from the stock cultures of the Dairy Science and Technology Research Centre (STELA), Université Laval, Canada. All strains were grown and maintained at 37 °C in LB broth or agar unless otherwise stated.

Molecular identification of bacteriocin producers by 16S rDNA sequencing

The genomic DNA was extracted from an overnight-grown culture using a genomic DNA purification kit (Promega, USA). Genotypic identification of the isolates based on 16S rRNA was carried out as described by Hanchi et al. (2014) using the primers 27F:5′-AGAGTTTGATYMTGGCTCAG and 1492R:5′-TACCTTGTTAYGACTT. Amplification was done in a PCR recycler (Eppendorf Mastercycler Gradient, Hamburg, Germany) and the amplified products were sequenced at the plateforme de séquençage et de génotypage des génomes, CHUL, Canada. The obtained 16S rRNA sequences were examined for similarities in the GenBank nucleic acid database.

Bacteria growth and inhibitory activity production kinetics

The growth kinetics for the selected five strains were studied simultaneously with measuring bacteriocin activity. 500 ml of M63 broth [KH_2Po_4 3 g l^{-1}, K_2HPO_4 7 g l^{-1}, $(NH_4)H_2PO_4$ 2 g l^{-1}, casamino acids 1 g l^{-1}, autoclaved at 121 °C for 15 min, then supplemented with glucose 20% (10 ml l^{-1}), $MgSO_4$ 20% (1 ml l^{-1}) and thiamin 1‰ (1 ml l^{-1})] were inoculated at 1% with an overnight (18 h) culture grown in LB broth at 37 °C and then incubated at 37 °C for 24 h with agitation. Samples were taken every 2 h for the first 14 h and then were taken at 24 h. For each sample, bacterial counts and bacteriocin production were determined in duplicate. Bacterial counts were determined using the drop plate method. Samples were tenfold serially diluted with 0.15% (v/v) peptone water (Difco Laboratories) and 20 µl of each dilution was then plated in duplicate on LB agar. Plates were left to dry and incubated at 37 °C for 24 h. Cell counts were expressed as log CFU ml^{-1}. One milliliter of each culture was centrifuged at 10,000×*g* for 10 min, and

Table 1 Screening of the antimicrobial activity of cell-free supernatants from isolated strains by agar diffusion assay

Indicator strains	Antimicrobial activity (Ø mm)				
	Producer strain				
	P2C	P2M	P6C	P3M	I 1006
Escherichia coli O_{157}:H_7[a]	10	13	10	13	10
Escherichia coli ATCC 35150	0	12	0	0	12
Escherichia coli *$O_{26}HM$*[a]	10	10	0	7	9
Escherichia coli O11 NM[a]	0	0	0	0	0
Escherichia coli O_{18}[a]	14	11	10	10	15
Escherichia coli O_8 K_{25}[a]	0	0	0	0	0
Escherichia coli O_{78} K_{80}[a]	0	0	0	0	0
Escherichia coli $O_1K_1H_7$[a]	12	15	0	9	12
Escherichia coli $O_2K_1H_4$[a]	0	0	0	0	0
Escherichia coli ATCC 25922	0	0	0	0	0
Escherichia coli ATCC 35695	18	17	13	14	17
Vibrio parahaemolyticus ATCC 17802	0	0	0	0	0
Yersinia O:5[a]	0	0	0	0	0
Pseudomonas aeruginosa ATCC 15442	0	0	0	0	0
Salmonella enterica subsp. *enterica* ATCC 9700	0	0	0	0	0
Salmonella enterica subsp. *enterica* ATCC 8400	0	0	0	0	0
Salmonella enterica subsp. *enterica* ATCC 29628	0	0	0	0	0
Salmonella enterica subsp. *enterica* ATCC 14028	0	0	0	0	0
Salmonella enterica subsp. *enterica* ATCC 9607	0	0	0	0	0
Salmonella enterica subsp. *enterica* ATCC 8387	0	0	0	0	0
Salmonella enteritidis MNHN (clinical isolate)[a]	0	0	0	0	0

ATCC American Type Culture Collection

[a]Our culture collection

resulting supernatant filtered through 0.22 µm cellulose acetate membrane (VWR, Mississauga, ON, Canada), and stored at − 80 °C for later use.

Determination of antimicrobial activity

Agar diffusion method

The agar diffusion method was used as described in Hammami et al. (2009). Briefly, LB agar (Difco Laboratories, Sparks, MD) containing 0.8% (w/v) agar was autoclaved, cooled to 45 °C, seeded with 150 µl of an overnight culture of the indicator strain (Table 1) and poured into sterile Petri plates (25 ml each). After solidification, wells were bored in the agar using the wide end of a 5-ml sterile pipette, followed by dispensing 80 µl of each sample into the wells. The plates were then incubated at 37 °C for 18 h to develop inhibition zones and diameters of these zones were measured.

Critical-dilution method and MIC determination

This bioassay was done using polystyrene micro-assay plates (96-well Microtest, Becton–Dickinson Labware, Sparks, MD, USA) as described in Hammami et al. (2009). Cell-free supernatants were diluted twofold with LB broth and transferred to wells, which were subsequently seeded with approximately 1×10^4 CFU of indicator strain per well using log-phase culture diluted in LB to $0.5–1.0 \times 10^6$ CFU ml^{-1}. Microplates were incubated for 12 h, and the absorbance at 595 nm was measured every 20 min using an Infinite® F200 PRO photometer (Tecan US inc., Durham, NC). Antimicrobial activity was expressed in arbitrary units per milliliter (AU ml^{-1}) and calculated as follows:

AU $ml^{-1} = (1000/125) \times 2^n$, where n = number of inhibited wells.

Minimum inhibitory concentration (MIC) values of pure bacteriocins were expressed in micrograms per milliliter and corresponded to the lowest concentration that inhibited the growth of target organism (OD_{595} nm) after 8 h of incubation. MccJ25 purified in the laboratory from *E. coli* ATCC 35695 MC4100 harboring pTUC202 as described by Hanchi et al. (2017) and colistin sulfate (Sigma, USA) were used as controls. The MIC values are reported as means of two independent experiments in duplicate.

Safety evaluation of bacteriocinogenic strains

Antibiotic susceptibility

The selected strains (except *Proteus* sp. P6C) were tested for susceptibility to 16 antibiotics from different classes. For each antibiotic, the stock solutions were prepared as described by Andrews (2001). Antibiotic susceptibility was determined in duplicate according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2012) using broth microdilution method. Bacterial concentration was adjusted to 5×10^5 CFU ml^{-1} in Mueller–Hinton broth. MIC value was determined after incubating the plates for 18 h at 37 °C and defined as the lowest concentration of the antibiotic that visibly inhibited bacteria growth. Breakpoints recommended by CLSI (2012) and EUCAST (2016) for Enterobacteriaceae were used to classify tested bacteria as resistant, intermediate, or sensitive.

Serotype identification and detection of virulence genes

A total of 85 O antisera were used for serotype identification of the selected strains. Genes amplification and serotype determination were performed at the Reference Laboratory for *E. coli*, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, QC. For the four strains of *E. coli*, 12 known virulence genes in *E. coli* were targeted namely *Aero* (gene coding for aerobactin), *LT* (heat-labile enterotoxin), *STb, STa* (heat-stable enterotoxins), *Stx1, Stx2* (Shiga toxins), *Eae* (attaching and effacing), *F4, F18* (fimbriae), *P* (adhesin fimbriae), *CNF* (cytotoxic necrotizing factor) and *Tsh* (temperature-sensitive hemagglutinin).

Bacteriocin characterization and identification

Cross-immunity test

Cross-immunity test between bacteriocin producers was performed against the following recombinant strains: *E. coli* MC4100 pTUC202 (MccJ25), *E. coli* MC4100 pMM39 (MccB17, Class I), *E. coli* MC4100 pL102 (MccL, Class IIa) and *E. coli* MC4100 93F (MccD93) (Duquesne et al. 2007), obtained from the stock cultures of the Dairy Science and Technology Research Centre (STELA), Université Laval, Canada. Cross-immunity was determined for the ten strains initially selected in duplicate by an agar-well diffusion assay as described above.

Effect of organic solvents, enzymes, pH, and heat treatment on bacteriocin stability

The effect of organic solvents on the antimicrobial activities of the five active fractions eluted from Sep-Pack columns (pH 2.32), which were considered as semi-purified bacteriocins (SPB), were assessed by adding individually (SPB/solvent) 1:1 (v/v) methanol, acetonitrile, propanol, hexane and chloroform (Fisher Scientific, ON, Canada). Samples were incubated at 37 °C for 2 h, and solvents were then removed by evaporation using Speed-Vac® concentrator (Model SC110A, Savant Instruments Inc., Farmingdale, NY). The thermal stability of the five active SPB was assessed using different time–temperature combinations (10 min at 80 and 100 °C; 30 and 60 min at 100 °C and 15 min at 121 °C), followed by residual activity test. The stability of the SPB activity was tested at pH range 2–12 (with increments of two pH units) adjusted with sterile 1 mol l^{-1} NaOH or HCl. Samples were incubated at room temperature (25 °C) for 2 h, and residual activity was subsequently measured after pH adjustment to 6.0. Sensitivity of SPB to proteolytic enzymes including subtilisin A, α-chymotrypsin, trypsin, pepsin, and proteinase K (Sigma, USA) was performed by dissolving enzymes in phosphate buffer (pH 7.5), Tris–HCl buffer (pH 8), phosphate sodium buffer (pH 7.6), HCl 10 mmol l^{-1} and phosphate buffer (pH 7.5), respectively, to a final concentration of 2 mg ml^{-1} at room temperature. Enzyme solutions were filter-sterilized through 0.2 µm cellulose acetate membrane (Fisher Scientific). Aliquots of SPB were mixed with equal volume of each enzyme solution, incubated at 25 or 37 °C for 2 h according to the used enzyme. The enzymatic reactions were then stopped by heating samples in boiling water for 5 min. After each treatment, the remaining activity was determined in duplicate by the critical microdilution method as described above. Untreated supernatants were used as controls.

Bacteriocin purification

A total of 3 L M63 broth were seeded with a 1% inoculum from an overnight culture of *E. coli* P2C and incubated at 37 °C for 12 h with shaking. The culture was centrifuged (10,000×*g*, 20 min), and the resulting supernatant was applied to a Sep-Pak C18 cartridge column (Waters, Milford, MA, USA), and eluted with increasing concentration of acetonitrile (0, 20, 60 and 100% v/v) in water containing 0.5 mmol l^{-1} of HCl. The recovered fractions were dried in a rotary evaporator to evaporate the excess of the solvent and then tested for their antimicrobial activity against *E. coli* ATCC 35695. The active fraction was loaded to a preparative C18 column (Luna 10 µm, 250×21.10 mm, Phenomenex, CA, USA) using a Beckman Gold System (Beckman Coulter, Mississauga, ON, Canada), and a linear gradient from 20 to 40% of acetonitrile in water (containing 0.5 mmol l^{-1} of HCl) at a flow rate of 10 ml min^{-1}. The eluted peaks were collected, solvent-evaporated, and tested. The active fraction was then injected into an analytic C18 reverse-phase column (Aeris™ 3.6 µm PEPTIDE XB-C18, 250×4.6 mm,

Phenomenex, CA, USA). The elution was performed at a flow rate of 1 ml min^{-1} using the following gradient of acetonitrile in water containing 0.5 mmol l^{-1} of HCl: 29% from 0 to 3 min; 29–38% from 3 to 21 min; 38–100% 21–23 min; and 100% acetonitrile 23–24 min. Peptides were detected at 214 nm and manually collected. The acetonitrile solvent was nitrogen dried (Praxair, Canada) before antibacterial activity assays using the critical-dilution method. The purified fractions were pooled and stored at − 20 °C. The concentration of proteins at each step of purification was determined using a Lowry protein assay kit (Bio-Rad, Canada), as described by Hanchi et al. (2014).

Mass spectrometry and amino acid sequence determination

LC–MS/MS analyses were performed on a Thermo EASY nLC II LC system coupled to a Thermo LTQ Orbitrap Velos mass spectrometer equipped with a nanospray ion source (Thermo Fisher Scientific). To determine amino acid sequences, fragmentations of the peptides were performed using CID, HCD, and ETD, separately. In each run, about 1/30 of the total sample was injected onto a 10 cm × 75 µm column in-house packed with C18 Jupiter 5 µm 300 Å reverse-phase material (Phenomenex). The MS data were processed using Thermo Proteome Discoverer software (v2.1) with the SEQUEST search engine against an *E. coli* database (downloaded from UniProt). The enzyme for database search was chosen as no enzyme. Only unique peptides with high confidence (false discovery rate < 1%) and with at least two PSM (peptide spectrum match) were considered and reported after manual verifications. Same MS data were also processed using a FASTA file containing 115 sequences of proteins recognized as common contaminants in proteomic studies (http://www.thegpm.org/cRAP/) plus BACTIBASE database (Hammami et al. 2010). The proteomic analyses were performed at the Centre for Biological Applications of Mass Spectrometry (CBAMS) located at Concordia University (Canada).

Results

Screening, molecular identification and safety characterization of the bacteriocinogenic strains

Cell-free supernatant from 22 isolates previously collected from chicken crops and pig gastrointestinal tract were screened against a wide range of Gram-negative bacteria by the agar-well diffusion assay (preliminary results). Ten isolates showed antibacterial activity against at least one indicator strain (data not shown), and the most active five strains were selected for further characterization. The isolates were identified as *E. coli* P2C, *E. coli* P2M, *Proteus* sp. P6C, *E. coli* P3M, and *E. coli* I1006. 16S rRNA gene sequences were submitted to gene bank under respective accession numbers MF355370, MF355371, MF355372, MF355373, and MF355374. The cell-free supernatants of the five strains were active, with strains *E. coli* P2C, *E. coli* P2M and *E. coli* I1006 being the most inhibitory against *E. coli* O_{157} H_7, *E. coli* O_{18}, *E. coli* $O_1K_1H_7$, *E. coli* O_{26} and *E. coli* ATCC 35695 (Table 1). Figure 2a illustrates the antimicrobial activity of cell-free supernatants from the five strains against *E. coli* ATCC 35695, with those recovered from P2C and P2M strains being the most potent. As shown in Table 2, strains P2C and P2M of *E. coli* belonging to serotypes O_{15}, while P3M strain belongs to serotype O_8. No serotype was obtained for the strain *E. coli* I1006 due to the absence of agglutination with the tested 86 antisera. The strain P6C was identified as *Proteus* sp. and was not serotype investigated due to its presumed pathogenicity. Besides, all selected strains were screened for the presence of 12 different virulence genes in *E. coli* that are known to be pathogenic to various animal species and humans (Table 2). The *E. coli* I1006 had two genes coding for adhesin fimbriae (*P*) and temperature-sensitive hemagglutinin (*Tsh*), while the other *E. coli* strains did not contain any of the tested virulence genes. Table 3 summarizes the sensitivity of selected strains to antibiotics. While *E. coli* P2C and P2M strains were sensitive to either colistin sulfate, nalidixic acid, chloramphenicol, gentamicin and ampicillin, *E. coli P3M* and *E. coli* I1006 showed resistance to ampicillin. No breakpoints were provided by CLSI and EUCAST to the other tested antibiotics for which only their MIC values were provided (Table 3).

Bacterial growth kinetics and inhibitory activity production

Figure 1 illustrates the growth kinetics of the five selected strains having a logarithmic curve. The stationary phase was attained after 8 h of incubation, reaching a maximum of 3×10^9 CFU ml^{-1}, and cell number remained stable up

Table 2 Serotype identification and detection of virulence factors in isolated strains

	Identification	Serotype	Gene of pathogenicity
P2C	*Escherichia coli*	O_{15}	–
P2M	*Escherichia coli*	O_{15}	–
P6C	*Proteus* sp	ND[a]	ND
P3M	*Escherichia coli*	O_8	–
I1006	*Escherichia coli*	UT[a]	*P, Tsh*

[a]ND not determined, UT untyped

Table 3 Antibiotic susceptibility of the selected strains with interpretation

Antibiotic	Breakpoint	*E. coli* P2C		*E. coli* P2M		*E. coli* P3M		*E. coli* I1006	
		MIC (µg ml^{-1})	Interpretation	MIC (µg ml^{-1})	Interpretation	MIC (µg ml^{-1})	Interpretation	MIC (µg ml^{-1})	Interpretation
Colistin sulfate	2–32[a]	0.39	S	0.39	S	0.39	S	1.56	S
Novobiocin		12.50	\	50.00	\	25.00	\	12.50	\
Cloxacillin		50.00	\	50.00	\	50.00	\	50.00	\
Streptomycin		25.00	\	25.00	\	25.00	\	50.00	\
Neomycin		25.00	\	25.00	\	25.00	\	12.50	\
Chloramphenicol	≤8–16–≥32[b]	6.25	S	6.25	S	12.50	I	3.12	S
Kanamycin		12.50	\	12.50	\	12.50	\	6.25	\
Erythromycin		50.00	\	50.00	\	50.00	\	50.00	\
Dicloxacillin		50.00	\	50.00	\	50.00	\	50.00	\
Vancomycin		50.00	\	50.00	\	50.00	\	50.00	\
Paromomycin		25.00	\	25.00	\	12.50	\	12.50	\
Nalidixic acid	≤16–≥32[b]	3.12	S	3.12	S	3.12	S	3.12	S
Polymyxin B		0.78	\	0.78	\	0.78	\	1.56	\
Gentamycin	≤4–8–≥16[b]	3.12	S	3.12	S	12.50	I	3.12	S
Penicillin G		12.50	\	12.50	\	12.50	\	50.00	\
Ampicillin	≤8–16–≥32[b]	6.25	S	6.25	S	50.00	R	50.00	R

R resistant, *I* intermediate, *S* sensitive

[a](EUCAST 2016)

[b](CLSI 2012)

to 24 h. Inhibitory activity was detected in supernatants after 6 h of incubation and reached a maximum of 256 AU ml^{-1} after 10 h of fermentation. The maximum production of antagonistic substances was quite similar for all active strains at the stationary phase of the culture. For the strains *E. coli* P2C, *E. coli* P3M and *E. coli* I1006, the activity slightly decreased after 12 h of incubation but remained stable for the strains *E. coli* P2M and *Proteus* sp P6C (Fig. 1).

Cross-immunity test

Cross-immunity test between bacteriocin producers was performed against the recombinant *E. coli* strains producing MccJ25, MccB17 (Class I), MccL (Class IIa) and MccD93, and presented in Table 4. Immunity tests of the supernatant of the producing strains were positive against MccJ25, D93 and B17 producing strains. The zones of inhibition vary from 7 to 19 mm. The supernatants of the bacteriocin producers were not active against *E. coli* pL102, the producing strains of Mcc L, except for P2C and P6C.

Sensitivity of bacteriocins to enzymes, pH, thermal treatment and organic solvents

The effects of proteolytic enzymes, pH, and temperature on bacteriocin activity were evaluated and summarized in Table 5. A partial inactivation of the inhibitory molecule was observed from 80 °C 10 min for P2C, P2M and from 100 °C 30 min for P6C and I1006. In addition, the active substances were strongly inactivated after the treatment at 121 °C for 15 min (Table 5). The bacteriocin activity was conserved at pH 2, but it decreased when the pH increased excluding *E. coli* P2C and *E. coli* P3M that kept a residual activity even at pH 12. The antimicrobial activity was completely sensitive to all tested proteases. Conversely, the SPB retained their antimicrobial activity when exposed to methanol, acetonitrile, propanol, and hexane organic solvents except for chloroform (Table 5).

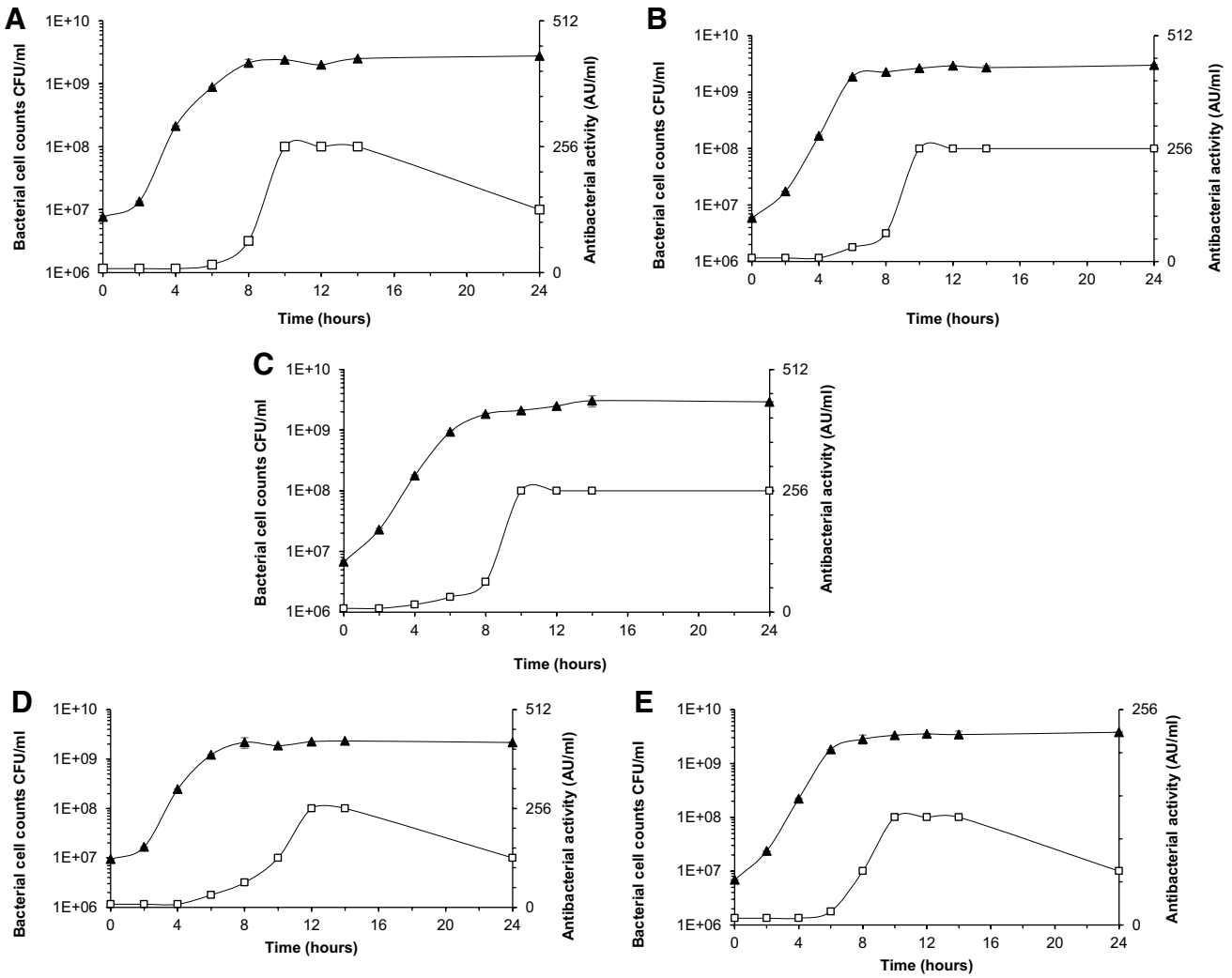

Fig. 1 Curves of cell growth of inhibitory strains (triangle), and their production of inhibitory substance (square) in M63 broth at 37 °C. **a** *Escherichia coli* P2C; **b** *Escherichia coli* P2M; **c** *Proteus* sp P6C; **d** *Escherichia coli* P3M; **e** *Escherichia coli* I1006. Each value is the mean of two independent repetitions, bars represent standard errors

Table 4 Cross-immunity test of selected strains with different microcin producers on solid medium

Supernatants (Ø mm)										
Microcin producing strains	P2CCO2	P2C	P2M	P2MCo1-2C	P2MCo1	P6C	P3M	I1004	I1006	I1026
E. coli pTUC202 = MccJ25	13	14	11	10	15	10	15	10	15	13
E. coli 93F = MccD93	13	15	19	19	16	13	10	11	17	13
E. coli pL102 = MccL	0	7	0	0	0	13	0	0	0	0
E. coli pmm39 = MccB17	13	15	17	17	16	12	12	11	15	13

Microcin purification and characterization

As illustrated in Fig. 2B, for both strains *E. coli* P2C and P2M the active molecules were recovered in fractions eluted using 60% acetonitrile. The chromatographic profile obtained after the first HPLC purification step exhibited several peaks (data not shown). A single active peak was obtained in the second step and was eluted with 34.5% acetonitrile, as shown in Fig. 3a. The increase of antimicrobial activity is clearly shown in Fig. 3b, where the diameter of the inhibition zone increases in each purification step. The active peak has a single protein with a molecular mass of

Table 5 Effect of enzymes, pH, heat and organic solvents on antibacterial activity

Treatment	Residual activity (AU ml^{-1})				
	P2C	P2M	P6C	P3M	I1006
Control	512	512	128	256	128
A. Enzymes					
Proteinase K	8	8	8	8	8
Pepsin	8	8	8	8	8
Trypsin	8	8	8	8	8
Subtilisin A	8	8	8	8	8
α-chymotrypsin	8	8	8	8	8
B. pH					
2	512	512	128	256	128
4	128	64	32	256	16
6	128	32	32	256	16
8	64	32	16	256	16
10	64	32	8	128	8
12	32	8	16	32	8
C. Temperature					
80 °C 10 min	256	256	128	256	128
80 °C 30 min	256	256	128	128	128
100 °C 10 min	256	256	128	256	128
100 °C 30 min	256	128	64	128	64
100 °C 60 min	64	64	32	128	64
121 °C 15 min	32	16	16	32	16
D. Organic solvents					
Control	256	256	64	256	64
Methanol	64	128	16	128	32
Hexane	128	128	64	256	16
Propanol	128	64	32	128	32
Acetonitrile	256	128	32	128	32
Chloroform	8	8	8	8	8

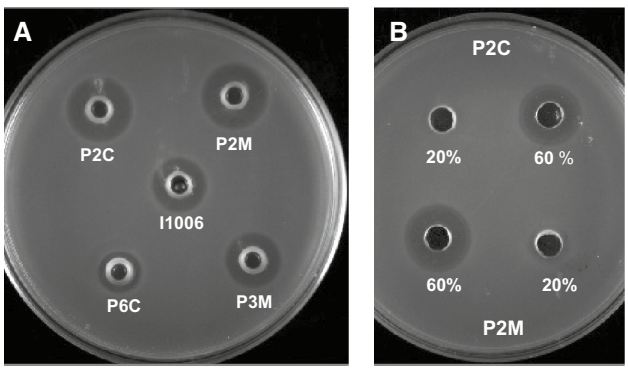

Fig. 2 Antimicrobial activity of bacteriocin-producing strains against *E. coli* ATCC 35695. **a** Activity of cell-free culture supernatants; **b** active fractions obtained from strains *Escherichia coli* P2C and P2M by low-pressure chromatography (SPB)

8733.94 Da (Fig. 3c). The final obtained purification yield was 8%, with an increased specific activity. The antimicrobial activity has increased from 256 AU ml^{-1} in the cell-free supernatant to 32,768 AU ml^{-1} in the pure active protein (Table 6). LC–MS/MS analysis of the purified microcin peak revealed 22 different fragment peptides matched to microcin V (MccV). The peptide was successfully identified and the sequence of its 62 N-terminal amino acid residues was: ASGRDIAMAIGTLSGQFVAGGIGAAAGGVAGGAIY DYASTHKPNPAMSPSGLG GTIKQKPEG. The molecular mass and the amino acids sequence of this molecule corresponded both to MccV (BACTIBASE #BAC120).

Antimicrobial spectrum of purified microcin V and MIC determination

Table 7 summarizes MIC values of purified MccV compared to MccJ25 and colistin. While purified MccV was active against some of the tested pathogenic coliforms including *E. coli* O_{157} H_7, *E. coli* ATCC 35695, *E. coli* $O_{26}HM$, *E. coli* O_{18}, *E. coli* $O_2K_1H_4$, *E. coli* ATCC 25922 and *E. coli* $O_1K_1H_7$, no activity was detected against *Salmonella, Vibrio* or *Yersinia*. MccV MIC values ranged from 0.89 to 1517.94 nM, with *E. coli* ATCC 35695 being the most sensitive strain (MIC = 0.89 nM). MccV was the most potent against *E. coli* ATCC 35695 compared to MccJ25 and colistin sulfate, which had respective MIC values of 1.67 and 270.46 nM. Conversely, MccJ25 was the most active against *E. coli* $O_1K_1H_7$ and *E. coli* O_{157} H_7 with MIC values of 3.35 and 214.19 nM, respectively. To a lesser extent, both strains were considerably less sensitive to colistin (MIC = 540.92 nM) and MccV (MIC = 1517.94 nM).

Discussion

As the incidence of disease caused by drug-resistant pathogens increases in the human population, debate grows around the systematic use of antibiotics to protect livestock and enhance growth performance. Probiotics and their secreted bacteriocins are evolving as a promising and safe alternative with high potential of application due to their ability to overcome the resistance problem in animal feed (Cotter et al. 2013). Bacteriocins possess several attractive properties including unique mechanism of action that differs from antibiotics, highly specific activity, and their low propensity to generate resistance (Hammami et al. 2013). Unlike broad-spectrum antibiotics that can alter gut microbiota communities' structure, bacteriocins are considered less disruptive to the intestinal microbiota equilibrium (Cotter et al. 2013). While numerous bacteriocins have been characterized primarily to facilitate their applications as food additives, their full potential as antimicrobial agents remains

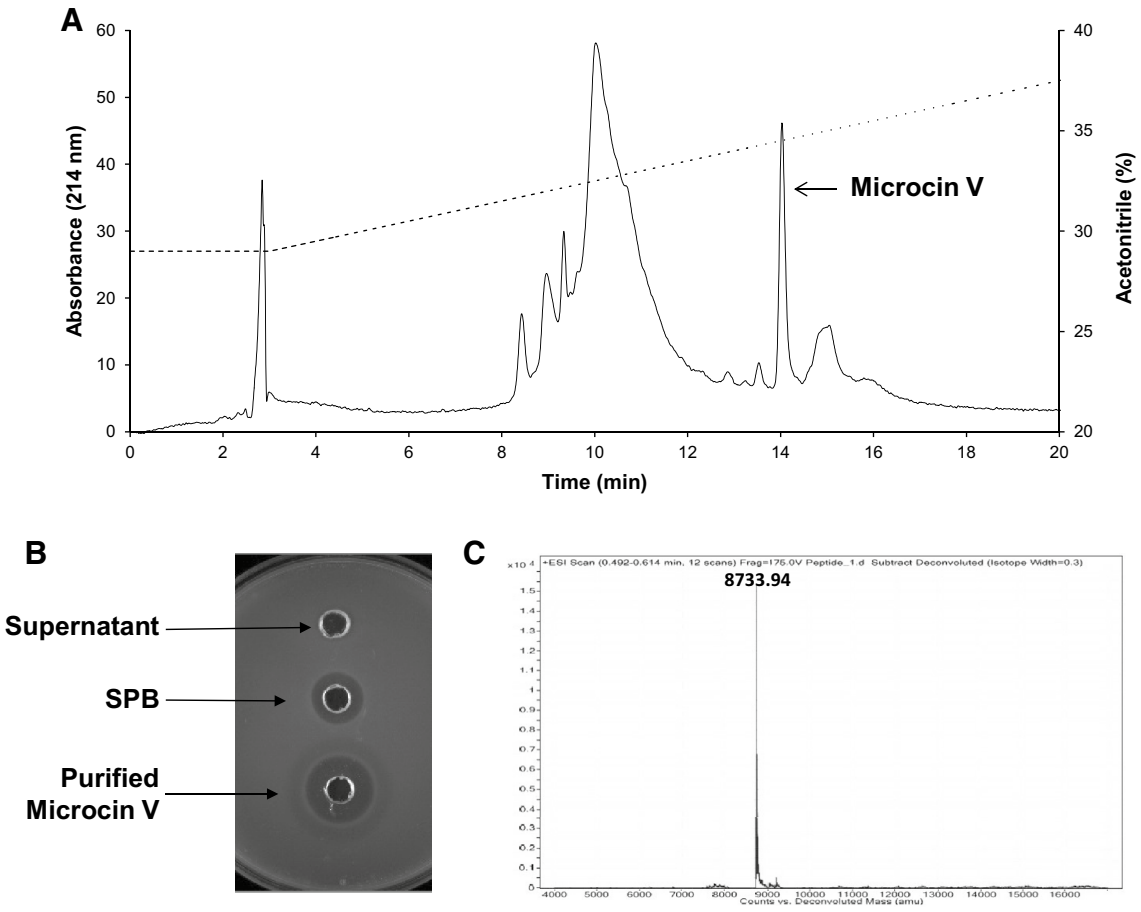

Fig. 3 Reversed-phase HPLC profile (**a**), antimicrobial activity against *E. coli* ATCC 35695 (**b**), and molecular mass determination by LC–MS (**c**) of microcin V purified from *E. coli* P2C

Table 6 Purification steps of microcin V from *E. coli* P2C

Purification steps	Volume (ml)	Protein concentration (µg ml^{-1})	Total proteins (µg)	AU (ml^{-1a})	Total activity (AU)	Specific activity (AU μg^{-1})	MIC (ng ml^{-1})	Yield (%)
Supernatant	3000	315.07	945205.48	256	7.68E+05	1	9845.89	100
Semi-purified bacteriocin (SPB)	333	84.47	28130.14	512	1.70E+05	6.06	1319.92	22.13
HPLC purification	1.88	31.96	60.09	32768	6.16E+04	1025	7.80	8.02

[a]Antimicrobial activity [in arbitrary units (AU)] was assayed by the critical control method

under-explored. Particularly, microcins are the less investigated antimicrobial peptides, due to their main production by coliforms, which are not considered as safe by the regulatory agencies. Recent advances in bacteriocin identification and characterization have renewed interest in the study of their use as therapeutic agents, and support is accumulating for their efficacy in treating infections in humans and animals (Hammami et al. 2013). Many bacteriocins produced by established or potential probiotic strains have been assessed for potential application as single or cocktail therapeutic agents. For example, durancin 61A has been shown effective against multi-resistant pathogens of clinical relevance in vitro, and to act synergistically with several antimicrobials including reuterin, pediocin PA-1, nisin and vancomycin (Hanchi et al. 2017). Similarly, Class I (nisin) or Class IIa (pediocin) bacteriocins have been shown to be synergistic

Table 7 Determination of minimal inhibition concentration (MIC) of purified microcin V (MccV) compared with microcin J25 (MccJ25) and colistin

Strains	MIC (nM)		
	MccV	MccJ25	Colistin
Escherichia coli ATCC 35695	0.89	1.67	270.46
Escherichia coli O_{11}NM	–	26.77	270.46
Escherichia coli O_{26} HM	758.97	107.09	540.92
Escherichia coli O18	1517.94	54831.66	540.92
Escherichia coli O_8K_{25}	–	–	540.92
Escherichia coli $O_{78}K_{80}$	–	–	540.92
Escherichia coli $O_1K_1H_7$	1517.94	3.35	540.92
Escherichia coli $O_2K_1H_4$	1517.94	26.77	1081.85
Escherichia coli ATCC 25922	1517.94	107.09	1081.85
Escherichia coli O_{157} H_7	1517.94	214.19	540.92

with polymyxin E against resistant variants of *Listeria monocytogenes* and *E. coli* (Naghmouchi et al. 2011).

The aim of this work was to evaluate the bacteriocinogenic and probiotic potential of *E. coli* strains isolated from pig intestine and chicken crops. Of the 22 strains isolated, ten isolates demonstrated a good activity, and the most potent five strains were retained for the remaining study. Four strains were identified as *E. coli* and one as *Proteus* sp. The selected strains were sensitive to all antibiotics recommended by the CLSI (2012) and EUCAST (2016) for the evaluation of enterobacteria, except *E. coli* P3M and *E. coli* I1006 that showed resistance to ampicillin. The breakpoints of certain antibiotics against enterobacteria were not provided in this study due to their absence at the CLSI and EUCAST. The observed resistance of Gram-negative bacteria to antibiotics is likely due to the mobile genes on the plasmids that can easily diffuse through bacterial populations (Kumarasamy et al. 2010). No virulence factors were detected for *E. coli* strains isolated from pig intestine. However, genes encoding for *Tsh* and *P* were found in *E. coli* I1006, which was isolated from the chicken crops. These genes are generally found in avian-pathogenic *E. coli* (APEC) (Dho-Moulin and Fairbrother 1999).

The secretion of antimicrobial agents by the selected strains was in the exponential phase, with a profile similar to most known microcins, whose activities are expressed in the exponential or the stationary growth phase. During the stationary phase for the strains *E. coli* P2C, *E. coli* P3M, and *E. coli* I1006, there was a stable production of microcins, followed by a decrease during incubation that could be related to peptide degradation by bacterial endogenous proteases (Baquero and Moreno 1984). Supernatants of the five strains were active against the producer strains of MccJ25, Mcc93F, and MccB17, suggesting that produced antimicrobial substances do not belong to any of these microcins. The absence of immunity of a given strain to a known microcin could imply that bacteria do not produce that peptide (Cherif et al. 2008; Sablé et al. 2003). The MccL-producing strain *E. coli* pL102 was found resistant to tested cell-free supernatants except those of *E. coli* P2C and P6C, but this does not necessarily mean that all these strains produce MccL. In a previous study, Sablé et al. (2003) reported that *E. coli* LR05 producing four different microcins (B17, D93, J25, and L) was resistant to MccV. This resistance was related to the co-expression of *MccL* with *cvi*, a gene encoding the MccV immunity protein. Thus, strains that are non-active against *E. coli* pL102 probably produce MccV. Based on the results of the cross-immunity test, *E. coli* P2C strain was selected as its cell-free supernatant was the most potent against the microcin producing strains (positive test against the four strains), indicating the presence of a different microcin from those tested. The five semi-purified bacterial substances were sensitive to all tested proteolytic enzyme, which indicates their proteinaceous nature. Purified bacteriocins were sensitive to high temperature at 80 °C for 10 min, stable at acidic pH (=2) but unstable at alkaline conditions (pH > 10). Moreover, the activity of bacteriocins from all the five strains decreased after exposure to organic solvents mainly chloroform. The heat-stable characteristics of these substances relay their potential as food preservatives since many food-processing procedures implicate a heating step. Their large pH-resistance is also very profitable for applications in acidic foods as well as non-acid foods. Many studies have reported that many bacteriocins were stable to temperature and acidity, but were sensitive to enzymes mainly proteinase K and alkaline pH degrees (Chalasani et al. 2014; Elayaraja et al. 2014; Gaaloul et al. 2015; Ge et al. 2016; Goh and Philip 2015; Liu et al. 2015).

Of particular interest, strain *E. coli* P2C was the most potent, free of virulence genes and sensitive to tested antibiotics. Purification procedure revealed the presence of a single pure peak having a molecular mass of 8733.94 Da and matching MccV (Duquesne et al. 2007; Håvarstein et al. 1994). The sequence obtained by LC–MS/MS confirmed the presence of MccV as reported in BACTIBASE and UniProt. Purified MccV showed a good activity against pathogenic coliforms, especially *E. coli* $O_1K_1H_7$ involved in avian colibacillosis. It was also more potent than colistin against *E. coli* ATCC 35695. Although the protein has been reported as active against clinical isolates of *Salmonella* (Chalón et al. 2012), the absence of activity in our study may be due to strain differences. The production yield of the MccV was 8% from 3 L culture broth. Similar results were previously reported by Håvarstein et al. (1994) who obtained about 0.5 mg of pure MccV from 1 L culture of LB broth. The authors have used a recombinant strain cultured in a medium supplemented with 2,2′-dipyridyl, an iron chelator that increased MccV production by sixfold. Difficulties

associated with MccV purification are mainly related to its instability and low-production rate (Chehade and Braun 1988; Håvarstein et al. 1994). The low yield may also be due to the inactivation of microcin by binding to flask surfaces or to other components in the medium (Håvarstein et al. 1994). Besides, expression of MccV genes has been reported to be repressed by excess iron (Fath et al. 1994; Håvarstein et al. 1994).

Conclusion

The present study provides evidence that *E. coli* strains isolated from pig intestine produce microcin-like substances. Of particular interest, *E. coli* P2C is sensitive to antibiotics, free from virulence genes, produces MccV, and is a good candidate for its application as novel probiotic strain to protect livestock and enhance growth performance.

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