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Original Article

Antimicrobial activity and Safety aspect of a multiple enterocin-producing *Enterococcus lactis* 4CP3 strain isolated from a fresh shrimp (*Palaemon serratus*)

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Abstract

Screening for bacteriocinogenic strains of lactic acid bacteria from fresh shrimps (*Palaemon serratus*) resulted in the detection of an *Enterococcus lactis* strain designated as 4CP3. The isolate 4CP3 demonstrated a bacteriocin-like activities against Gram-positive bacteria (e.g. *Listria* spp.), Gram-negative bacteria (e.g. *P. aeruginosa* ATCC 27853) and fungi (e.g. *Aspergillus niger* A79). Antimicrobial activity of the cell-free supernatant was stable over a wide range of pH (2-10) and after heating at 100°C for 15 min, whereas it was lost after protease treatments with proteinase K and trypsin. *Enterococcus lactis* 4CP3 was shown to produce the enterocins A, B and P. To our knowledge, this is the first report on multiple enterocin-producing *Enterococcus lactis* newly isolated from fresh shrimps. The mode of action of the enterocins produced by 4CP3 strain was identified as bactericidal against *L. monocytogenes* EGDe 107776 and *P. aeruginosa* ATCC 27853. An enzymatic study revealed *Enterococcus lactis* 4CP3 as high producer of β-galactosidase with moderate peptidases and proteases activities which are relevant properties in the development of organoleptic quality of fermented food. *Enterococcus lactis* 4CP3 was not haemolytic, sensitive to vancomycine, gelatinase negative and free of common virulence factors and antibiotic resistance genes. Therefore, it may be useful as safe natural additive or probiotic in food and feed.

Mots clés :
Bactéries lactiques
Enterococcus lactis
Entérocine
Activité antimicrobienne
Innocuité
Crevettes

Résumé

Activité antimicrobienne et Innocuité d'une souche *Enterococcus lactis* 4CP3 multi-productrice d'entérocines isolée de crevette fraiche (*Palaemon serratus*).
Une recherche de souches de bactéries lactiques bactériocinogènes à partir des crevettes (*Palaemon serratus*) nous a permis d'isoler un *Enterococcus lactis* 4CP3. Cet isolat est doté d'une activité inhibitrice contre des bactéries à Gram-positif (comme *Listeria.* spp) et à Gram-négatif (*P. aeruginosa* ATCC 27853) et des champignons (comme *Aspergillus niger* A79). L'activité antimicrobienne du surnagent de culture est stable à une large gamme de pH (2-10) et à un traitement thermique de 100°C pendant 15 min, tandis qu'elle n'est plus détectée après l'ajout de la protéinase K et la trypsine. *Enterococcus lactis* 4CP3 est capable de produire les entérocines A, B et P. Ainsi, notre étude est la première à décrire

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une souche d'*Enterococcus lactis* multi-productrice d'entérocines nouvellement isolée de crevettes fraiches. Le mode d'action des entérocines est bactéricide contre *L. monocytogenes* EGDe 107776 et *P. aeruginosa* ATCC 27853. L'étude des activités enzymatiques a montré qu'*Enterococcus lactis* 4CP3 possède une activité élevée de β-galactosidase et des activités modérées des peptidases et des protéases ce qui représente des propriétés importantes dans le développement de la qualité organoleptique des produits fermentés. D'autre part, il s'est avéré que la souche 4CP3 est non hémolytique, sensible à la vancomycine, gélatinase négative et dépourvue des facteurs de virulence et des gènes d'antibio-résistance. Par conséquent, cette souche pourrait être utilisée ultérieurement comme un additif naturel et sûr ou comme probiotique.

INTRODUCTION

Lactic acid bacteria (LAB) are known to produce different antimicrobial agents such as bacteriocins which are antimicrobial peptides able to inhibit the growth of pathogens and undesirable microorganisms. Enterococci belong to the LAB group with *E. faecium* and *E. faecalis* the most prevalent species isolated from different sources (Giraffa, 2002). Bacteriocin-producing enterococci were previously isolated from vegetables (Zendo *et al*., 2005), fermented sausages (Cocolin *et al*., 2007), fish viscera (Migaw *et al.,* 2013), raw milk cheeses (Morandi *et al*., 2012) and raw bovine milk (Gaaloul *et al*., 2014a; Gaaloul *et al.*, 2014b). Enterococci were used in different fermented technologies and in probiotic therapy thanks to their beneficial properties (Kayser, 2003). However, they are considered as opportunistic pathogens causing endocarditis and urinary tract infections (Safdar *et al*., 2002). Hence, the assessment of the safety aspect of enterococci is recommended to select safe strains for food production or probiotic applications. This present work reports on the characterization of bacteriocinogenic *Enterococcus lactis* strain newly isolated from fresh shrimp (*Palaemon serratus*) and the evaluation of its safety aspect.

MATERIAL AND METHODS

Culture media used were obtained from Biokar Diagnostics (Beauvais, France) and BIO-RAD (Marnes-la-Coquette, France), while all chemicals were purchased from Sigma Aldrich (USA).

Sampling and LAB isolation

Fresh shrimps (*Palaemon serratus*) were collected from retail seafood markets in the Tunisian's coast. Strain 4CP3 was isolated from a shrimp sample according to Nair and Surendran (2005) method using MRS (Man, Rogosa and Sharpe) agar with slight modifications. The strain was purified by repeated streaking onto MRS agar. A single colony was picked up, transferred to MRS broth and incubated 24 h at 30°C. 4CP3 strain was kept in MRS broth containing 20% glycerol at -80°C.

Phenotypic and molecular identifications

The isolate 4CP3 was identified by microbiological, physiological, biochemical and molecular methods. The phenotypic tests were: Gram staining, production of catalase and oxidase, growth at different temperatures (4, 10, 15, 25, 30, 37 and 45°C), NaCl concentrations (2-6,5 and 8%) and pH (4-9,6) according to Schleifer *et al*. (1984), growth on BEA (Bile Esculin Agar) medium and fermentation of carbohydrates using API 50 CHL system (bioMérieux, Marcy-l'Étoile, France). Moreover, the isolate 4CP3 was assayed for the antibiotic susceptibility testing using the disk diffusion method on Mueller Hinton agar. Fourteen commercial antibiotics from BIO-RAD (Marnes-la-Coquette, France) were tested : amoxicillin (25 µg), piperacillin (75 µg), imipenem (10 µg), cefoxitin (30 µg), cefotaxim (30 µg), gentamicin (15 µg), kanamycin (30 µg), tobramycin (10 µg), chloramphenicol (30 µg), tetracyclin (30 UI), vancomycin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg) and ofloxacin (5 µg). The results were interpreted according to the recommendations published by the Antibiogram Committee of the French Microbiology Society (CA-SFM, 2010).

The molecular identification was carried out by 16S rRNA gene sequencing using the universal primers P8FLP and P806R (Ben Braïek *et al*., 2017). Genomic DNA used for PCR amplifications was extracted from overnight culture in M17 broth using the protocol as

described by Cremonesi *et al*. (2006). The thermal conditions were according to Morandi *et al*. (2015). Amplified PCR products were analyzed by 2% agarose gel electrophoresis stained with ethidium bromide (0.05 mg/ml) using TAE 1X buffer at 100 V for 30 min and then visualized by UV trans-illumination. DNA sequencing was performed by Macrogen Europe (Amsterdam, the Netherlands).

Detection of inhibitory activity

Antimicrobial and antifungal activities were carried out by the well diffusion assay as described by Tagg *et al.* (1976). A volume of 20 µl of cell-free culture supernatants obtained by centrifugation at 10,000 x g for 10 min was deposited into wells (4 mm) made in soft agar (0.8%) seeded with 1% (*v/v*) of bacterial strains or 10^4 spore/ml of fungi listed in Table 1. Plates were incubated for 24 h or 72 h at appropriate temperatures of the bacteria and fungi indicator strains respectively. The presence of an inhibition zone around the well of at least 2 mm indicated a positive result and the diameter was measured. The inhibitory activity was quantified by the critical dilution method according to Ghrairi *et al*. (2005) and expressed as arbitrary units per milliliter (AU/ml).

Characterization of the inhibitory substance

Cell-free culture supernatants containing the bacteriocin were submitted to different tests such as; heating at 60°C for 30 min, 100°C for 15 min and 121°C for 15 min, adjusting the pH from 2 to 10 using HCl 1M or NaOH 1M with incubation at 30°C for 2 h and adding proteinase K and trypsin (1 mg/ml) with incubation at 37°C for 1 h. The residual activity was tested by the agar well diffusion method after each treatment. Untreated cell-free supernatants were used as controls and *Lactococcus garvieae* ATCC 43921 was used as an indicator strain.

Mode of action of the bacteriocin

L. monocytogenes EGDe 107776 and *P. aeruginosa* ATCC 27853 were used as indicator strains in order to determine whether the bacteriocin produced by 4CP3 strain acts as bacteriostatic or bactericidal bioactive peptide. Cell-free supernatant (10 ml) of 4CP3 strain was added at 1400 AU/ml to 50 ml each of BHI cultures of *L. monocytogenes* EGDe 107776 and *P. aeruginosa* ATCC 27853 at an early exponential growth phase and then incubated at 37°C for 6 h. Controls cells were treated with the inactive bacteriocin preparation (15 min at 121°C). The inhibitory effect was examined by taking samples every hour for measurement of the absorbance at 600 nm and determination of viable cell counts of *L. monocytogenes* EGDe 107776 and *P. aeruginosa* ATCC 27853 (CFU/ml) on BHI agar.

Enzymatic activities

Enzymatic profile of *E. lactis* 4CP3 strain was assessed using the API ZYM kit (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. Enzymatic activities were graded from 0 to 5 according to color reaction chart supplied by the API ZYM system and then approximately converted to number of free nanomole hydrolyzed substrate: 0, no activity; 1, release of 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol and 5, ≥ 40 nmol.

Enterocin typing

Specific primers coding for known enterocins (A, B, P, LB50A and LB50B) were used for gene amplification (Ben Braïek *et al*., 2017). PCR amplifications were performed in a total volume of 25 µl (10.1 µl RNase water free (Qiagen), 12.5 µl HotStarTaq Master Mix (Qiagen), 0.2 µl of each primer and 2 µl DNA). The thermal programs were run as described by Ben Braïek *et al*. (2017). Amplicons were analysed as described above.

Detection of antibiotic resistance and virulence genes

Genomic DNA was used in specific PCR reactions to detect the following genes encoding for antibiotic resistance and virulence factors: *HLG* (gentamicin), *vanA* and *vanB* (vancomycin), *blaZ* (β-lactamase-mediated ampicillin), *cylA* and *cylB* (cytolysin activator), *esp* (enterococcal surface protein), *efaAfs* (endocarditis antigen), *ddl* (D-Ala ligase), *gls 24-like* (stress and starvation protein), *nucl* (nuclease), *psaA* (metal-binding protein), *ace* (collagen-binding protein) and *asa1* (aggregation substance). Primers used were reported by Ben Braïek *et al.* (2017) and the conditions of PCR amplifications were previously described by El-Ghaish *et al.* (2011) and Gaaloul *et al*. (2014a). Amplified PCR products were analysed as described above.

Production of haemolysin and gelatinase

Haemolytic activity was tested by streaking fresh culture of 4CP3 strain on sheep blood agar. After an aerobic incubation at 37°C for 48 h, the production of haemolysin was examined by the presence of hydrolysis zone around the colonies. The hydrolysis of gelatin was assayed with Nutrient gelatin

medium according to Harrigan (1998). The production of gelatinase was evaluated by the liquefaction of the test medium while the uninoculated one remains solid.

RESULTS

Phenotypic and molecular identifications

The isolate 4CP3 was characterized as member of the genus *Enterococcus* based on the following criteria: Gram positive coccus, catalase and oxidase negative with ability to grow at 10°C and 45°C in MRS broth containing 6.5% NaCl, at pH 9.6 and on BEA medium. Moreover, 4CP3 isolate was sensitive to the antibiotics tested by the disk diffusion method. Carbohydrates fermentation profile (data not shown) and 16S rRNA gene sequencing identified the 4CP3 strain as *E. lactis*. The 16S rRNA gene sequence of 4CP3 strain was deposited to the NCBI Gene Bank with accession number KX819270.

Inhibitory activity and identification of the bioactive substance

The inhibitory spectrum of cell-free supernatants of *E. lactis* 4CP3 strain assayed by the well diffusion method against different microorganisms is shown in Table 1.

The 4CP3 strain was active against Gram-positive bacteria such as *L. garvieae* ATCC 43921 and *L. monocytogenes* EGDe 107776, the Gram-negative bacterium *P. aeruginosa* ATCC 27853 and fungi such as *A. niger* A79 and *F. equiseti* F6. The inhibitory activity of cell-free supernatant of 4CP3 strain was stable after incubation at pH values between 2 and 10, after heat treatments at 65°C for 30 min and at 100°C for 15 min, while it was lost after treatment with proteolytic enzymes (proteinase K and trypsin). This indicates that bioactive substance produced by 4CP3 strain is proteinaceous in nature and thus could be assigned as bacteriocin.

To identify the produced bacteriocin, structural genes of known enterocins were detected in *E. lactis* 4CP3 strain using specific PCRs. Agarose gel electrophoresis revealed three amplicons of 130 bp, 132 bp and 180 bp in DNA of 4CP3 strain corresponding to *entA*, *entP* and *entB* genes (Figure 1). This result indicates that *E. lactis* 4CP3 is a multiple enterocin-producing strain.

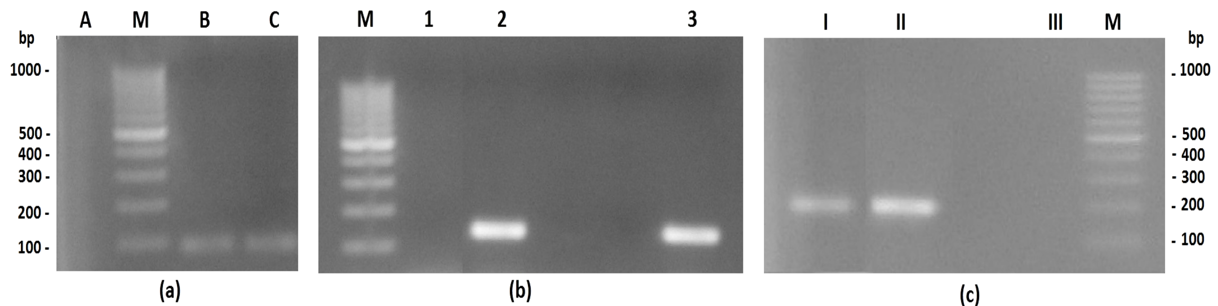

Figure 1. PCRs detection of genes encoding enterocin P (132 bp) **(a)**, enterocin A (130 bp) **(b)** and enterocin B (180 bp) **(c)** in *E. lactis* 4CP3 strain. M: molecular marker (100 bp); Lanes A, 1 and III: negative controls, Lane B: enterocin P positive control (*E. faecium* VC87), Lanes 2 and II: enterocins A and B positive control (*E. faecium* MMT21); Lanes C, 3 and I: *E. lactis* 4CP3 strain.

Mode of action

The addition of cell-free supernatant from *E. lactis* 4CP3 strain to exponentially growing cultures of *L. monocytogenes* EGDe 107776 and *P. aeruginosa* ATCC 27853 caused a decrease in both optical density at 600 nm and viable cells number (Figure 2).

There were approximately 5 and 6 log reductions in viable cells of the indicator strains *L. monocytogenes* EGDe 107776 and *P. aeruginosa* ATCC 27853 respectively, six hour after the addition of bacteriocinogenic supernatant from 4CP3 strain. This result indicates a bactericidal mode of action of the bacteriocin produced by *E. lactis* 4CP3 strain.

Enzymatic activities

The results of the enzymatic activities revealed by the API ZYM system are given in Table 2. The enzymatic profile of *E. lactis* 4CP3 shows that alkaline phosphatase, lipase (C14), α-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase and β-fucosidase were not detected. The activities of esterase (C4), α-chymotrypsin, acid phosphatase, β-glucosidase and N-acetyl- β-glucosaminidase were

weak with 5 nmol of hydrolyzed substrate. The other enzymes showed moderate activities estimated to be in the range of 10-20 nmol and only β-galactosidase had interesting activity with ≥40 nmol of released substrate.

Table 1. Spectrum of inhibitory activity of *E. lactis* 4CP3 strain

Indicator strain		Source[a]	Media[b]	T(°C)[c]	Activity[d]
Gram-positive bacteria					
Enterococcus faecium	MMT21	LBT-LR01ES05	MRS	30	++
Enterococcus faecalis	JH22	LBT-LR01ES05	MRS	30	+
Enterococcus durans	TO1	LBT-LR01ES05	MRS	30	-
Lactococcus lactis ssp *cremoris*	11603	ATCC	BHI	30	+
Lactococcus garvieae	43921	ATCC	BHI	30	+++
Micrococcus luteus	10240	ATCC	BHI	30	-
Carnobacterium maltaromaticum	14829	NRRLB	MRS	30	-
Lactobacillus brevis	F145	LBT-LR01ES05	MRS	30	++
Lactobacillus delbrueckii	20081	DSM	MRS	30	-
Lactobacillus bulgaricus	340	LBT-LR01ES05	MRS	30	-
Staphylococcus saprophiticus	-	LBT-LR01ES05	BHI	37	-
Staphylococcus aureus	6538	ATCC	BHI	37	-
Listeria ivanovii	BUG496	LBT-LR01ES05	BHI	37	++
Listeria monocytogenes	EGDe 107776	CIP	BHI	37	++
Bacillus thuringiensis	-	LBT-LR01ES05	BHI	30	+++
Gram-negative bacteria					
Escherichia coli	PUC18	LBT-LR01ES05	LB	37	-
Salmonella enterica	8297	CIP	BHI	37	-
Salmonella typhimurium	-	LBT-LR01ES05	BHI	37	-
Pseudomonas aeruginosa	27853	ATCC	BHI	37	++
Acinetobacter baumanni	-	LBT-LR01ES05	BHI	37	-
Klebsiella pneumoniae	-	LBT-LR01ES05	BHI	37	-
Fungi					
Aspergillus flavus	A1	LBT-LR01ES05	MEA	25	-
Aspergillus flavus	A6	LBT-LR01ES05	MEA	25	-
Aspergillus flavus	A20	LBT-LR01ES05	MEA	25	-
Aspergillus niger	A60	LBT-LR01ES05	MEA	25	-
Aspergillus niger	A79	LBT-LR01ES05	MEA	25	++
Aspergillus niger	A88	LBT-LR01ES05	MEA	25	-
Fusarium equiseti	F6	LBT-LR01ES05	MEA	25	++
Fusarium equiseti	F56	LBT-LR01ES05	MEA	25	-
Fusarium equiseti	F85	LBT-LR01ES05	MEA	25	-
Fusarium verticillioides	F97	LBT-LR01ES05	MEA	25	++
Fusarium verticillioides	F99	LBT-LR01ES05	MEA	25	-
Fusarium verticillioides	F123	LBT-LR01ES05	MEA	25	-

[a]ATCC, American Type Culture Collection; CIP, Collection of Institut Pasteur; DSM, Deutche Sammlung von Mikroorganismen und ZellKulturen; NRRLB, North Regional Research Laboratory Bacterian Collection; LBT-LR01ES05, Laboratory of Biochemistry and Technobiology (Tunis, Tunisia)
[b]Media (Broth and agar): MRS, de Man, Rogosa and Sharpe; BHI, Brain Heart Infusion; LB, Lysogeny broth; MEA, Malt Extract Agar
[c]Growth temperature of indicator strains
[d]Activity is represented as diameters of inhibition zones in millimeters (mm):-, no inhibition; +, inhibition zone <6 mm; ++, inhibition zone 6-12 mm; +++, inhibition zone >12 mm

Safety characterization

PCR amplifications performed to detect antibiotic resistance genes (*HLG, vanA, vanB* and *blaZ*) and virulence factors (*cylA*, *cylB*, *esp*, *efaAfs*, *ddl*, *gls 24-like*, *nucl*, *psaA*, *ace* and *asa1*) in *E. lactis* 4CP3 strain confirmed the absence of these determinants (data not shown). Besides, our 4CP3 strain did not show neither haemolysin nor gelatinase productions (data not shown).

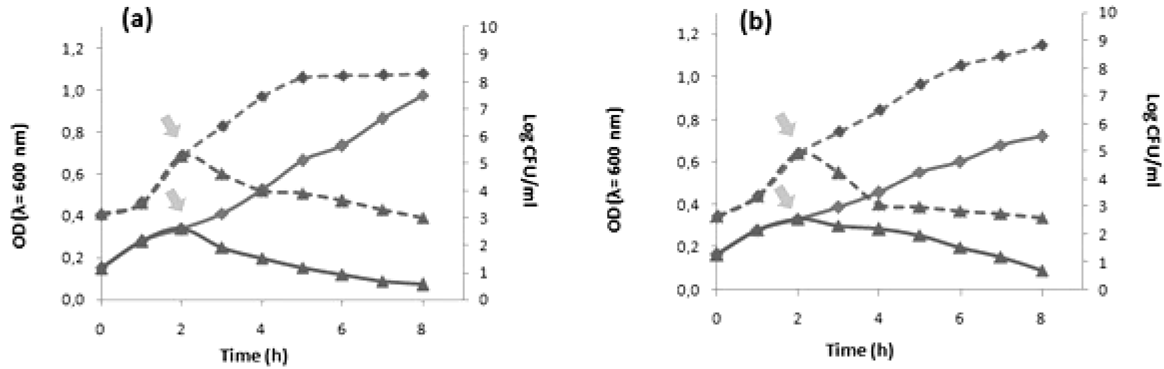

Figure 2. Mode of action of the bacteriocins produced by *E. lactis* 4CP3 against *L. monocytogenes* EGDe 107776 **(a)** and *P. aeruginosa* ATCC 27853 **(b)**. Optical density (OD) was indicated by a straight line and viability counts of both *L. monocytogenes* EGDe 107776 **(a)** and *P. aeruginosa* ATCC 27853 **(b)** were indicated by a dotted line. (♦) OD or Viable cell counts of controls (without adding the cell-free supernatant of *E. lactis* 4CP3 strain); (▲) OD or Viable cell counts of *L. monocytogenes* EGDe 107776 **(a)** and *P. aeruginosa* ATCC 27853 **(b)** incubated with cell-free supernatant of *E. lactis* 4CP3 strain. The arrow indicates the time at which the active cell-free supernatant was added.

Table 2. Enzymatic activities* of *E. lactis* 4CP3 strain revealed by API ZYM kit

Enzyme	4CP3
Alkaline phosphatase	0
Esterase (C4)	10
Esterase lipase (C8)	5
Lipase (C14)	0
Leucine arylamidase	10
Valine arylamidase	20
Cystine arylamidase	20
Trypsin	10
α-Chymotrypsin	10
Acid phosphatase	20
Naphthol-AS-BI-phosphohydrolase	20
α-Galactosidase	0
β-Galactosidase	≥40
β-Glucuronidase	0
α-Glucosidase	0
β-Glucosidase	10
N-acetyl-β-glucosaminidase	10
α-Mannosidase	0
β-Fucosidase	0

*Enzymatic activity is shown in nanomole (nmol) of chromophore released after incubation at 37°C for 4 h.

DISCUSSION

The objectives of the present study were the characterization of a bacteriocinogenic lactic acid bacterium strain isolated from a fresh shrimp (*Palaemon serratus*) and the evaluation of its safety aspect. The 4CP3 strain had been selected for phenotypic and molecular characterizations and it was identified as *E. lactis* by 16S rRNA sequencing. To our knowledge, this is the first study to deal with the isolation of *E. lactis* from fresh shrimps. Previously, Morandi *et al*. (2012) isolated atypical *Enterococcus* strains from Italian raw milk cheeses “Bitto PDO”. These were characterized as a novel enterococcal species named *E. lactis* (DSM 23655). Enterococci were commonly isolated from dairy products (Ghrairi *et al*., 2008), but our study reported isolation of *Enterococcus* spp. from non-dairy source. Similarly, previous studies reported enterococci strains from fermented meat (Belgacem *et al*., 2010), seafood (Valenzuela *et al*., 2010) and fish viscera and skin (Migaw *et al*., 2013; Ghomrassi *et al*., 2016).

The antibacterial substance produced by 4CP3 strain was thermostable and still active over a wide range of pH (2-10). This result is compatible with that found with other described bacteriocins (Cocolin *et al*., 2007; Gaaloul *et al*., 2014a; Ben Braïek *et al*., 2017). Furthermore, results of proteolytic enzymes effects on the loss of inhibitory activity of *E. lactis* 4CP3 enable us to confirm that antagonistic activity was caused by a bacteriocin. The identification of this produced bacteriocin by specific PCR amplifications revealed that *E. lactis* 4CP3 could produce the enterocins A, B and P. Thus, our study describes for the first time a multiple enterocin-producing *E. lactis* strain. In fact, it has been found earlier that *E. lactis* could produce enterocin P (Morandi *et al*. 2013). Moreover, *E. lactis* 4CP3

strain exhibited a broad-spectrum of inhibitory activity against bacterial and fungal pathogens and displays bactericidal mode of action against *L. monocytogenes* EGDe 107776 and *P. aeruginosa* ATCC 27853. This finding is quite interesting and suggests that our *E. lactis* 4CP3 strain could be promising candidate for future applications in food biopreservation. Indeed, the use of bacteriocins or producer lactic acid bacteria strains is of particular interest to the agro-food industry since it may be helpful to ensure biologically the microbial safety of the food products instead of chemical additives (Caplice and Fitzgerald, 1999).

E. lactis 4CP3 was characterized by high β-galactosidase activity and medium peptidases and proteases activities (leucine, valine, cystine arylamidases, trypsin and chymotrypsin). These traits could be important features in flavour and texture development during food fermentation and cheese ripening. However, the absence of lipase (C14) activity in *E. lactis* 4CP3 strain is in agreement with the literature.

Safety aspects of *E. lactis* 4CP3 were investigated through molecular and microbiological tests. Indeed, PCR amplifications for antibiotic resistance genes and virulence factors showed negative results. Similar findings were previously described by Morandi *et al.* (2013), Gaaloul *et al*. (2014a) and Ben Braïek *et al.* (2017). Also, our *E. lactis* appeared to be non haemolytic and gelatinase negative. These findings are in accordance with those described by Fortina *et al*. (2008), Morandi *et al*. (2015) and Ben Braïek *et al*. (2017). These tests confirm the innocuity of *E. lactis* 4CP3 strain suggesting its use as safe starter culture or co-culture or probiotic strain. Nevertheless, additional experiments need to be performed to confirm this possibility.

CONCLUSION

E. lactis 4CP3 strain isolated from a fresh shrimp (*Palaemon serratus*) have attractive properties (production of enterocins, interesting spectrum of inhibitory activity, bactericidal mode of action, safety aspects) suggesting its use as natural additive in food biopreservation to ovoid contamination and proliferation of pathogens and spoilage microorganisms or in probiotic therapy.

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