



Expression of five class II bacteriocins with activity against *Escherichia coli* in *Lactocaseibacillus paracasei* CNCM I-5369, and in a heterologous host

Désiré Madi-Moussa, Françoise Coucheney, Djamel Drider*

UMR Transfrontalière BioEcoAgro1158, Univ. Lille, INRAE, Univ. Liège, UPJV, YNCREA, Univ. Artois, Univ. Littoral Côte d'Opale, ICV – Institut Charles Viollette, F-59000 Lille, France



ARTICLE INFO

Article history:

Received 20 March 2021

Received in revised form 16 May 2021

Accepted 17 May 2021

Keywords:

Lactocaseibacillus paracasei

Class II bacteriocins

Heterologous expression

RT-qPCR

ABSTRACT

Five open reading frames viz *orf010*, *orf12*, *orf023*, *orf030* and *orf038* coding class II bacteriocins in *Lactocaseibacillus paracasei* CNCM I-5369 strain previously isolated from an Algerian dairy product, were found to be expressed after 24 h of growth. The strain has also shown anti-*E. coli* activity in a narrow pH range between 4.5 and 5. Then, expression and purification of these bacteriocins was conducted in the heterologous host *E. coli*. This strategy enabled us to purify the peptide encoded by *orf030* in large quantities, in contrast to other peptides that were produced but required to be released from the insoluble fraction following 4 M urea and desalting treatments. All peptides heterologously produced were characterized by MALDI TOF Mass spectrometry and successfully tested for their anti-*E. coli* activity. Furthermore, *in silico* transcriptional analysis was determined by Findterm tool and with Bagel4 software permitted to locate potential promoters and co-transcription events.

© 2021 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

In the 20th century, the discovery of antibiotics enabled a major decline in bacterial infection-related mortality and morbidity. Antibiotics impacted on the whole practice of medicine by minimizing the risk of infectious diseases. The overuse and misuse of antibiotics in human and veterinary medicine has led to the development of resistant strains with compromised therapeutic options, tending to threaten human health. Bacteria have developed a plethora of mechanisms of resistance including (i) the production of enzymes that degrade or modify antibiotics, (ii) the development of efflux pumps that drive antibiotics outside the cell, (iii) the modification of the targeted sites. Genes involved in intrinsic resistance to antibiotics of different classes, such as β -lactams, fluoroquinolones and aminoglycosides have been identified. These genes can be transferred between bacteria [1], and usually using mobile genetic elements such as plasmids, integrons and introns [2,3]. The World Health Organization (WHO) is continuously warning on this threat and encourages new strategies and interventions to tackle drug-resistance [4].

Antimicrobial peptides (AMPs) can help curb the AMR crisis. AMPs are present in all living cells [5,6], as part of the first line defence against foreign attacks. Many AMPs have the particularity of being cationic and can bind and interact with the negatively charged bacterial cell membranes, causing leakage of intracellular constituents, ATP depletion and cell death [7].

Bacteriocins are a class of AMPs ribosomally synthesized by Gram-positive and Gram-negative bacteria [8,9], and are steadily reported for their positive clinical outcomes [10]. These safe molecules [11–13] can be of narrow or broad spectrum [14] which offers numerous advantages over clinical antibiotics. The classification of bacteriocins has regularly been updated taking into consideration recent achievements and new knowledge. Currently there is no any universally accepted scheme of bacteriocins classification. Nevertheless, we refer here to the classification proposed by Alvarez-Sieiro et al, [15], which includes 3 main classes. Class I contains all peptides that undergo post-translational enzymatic modifications during their biosynthesis, leading to unusual amino acids, such as lanthionine. Class II includes unmodified bacteriocins with a low molecular weight (<10 kDa); and class III contains unmodified bacteriocins with a molecular mass higher than 10 kDa, and endowed with bacteriolytic or non-bacteriolytic modes of action.

Bacteriocins among Gram-positive bacteria are most often produced by lactic acid bacteria (LAB). These bacteriocins are

* Corresponding author.

E-mail address: djamel.drider@univ-lille.fr (D. Drider).

usually active against other Gram-positive bacteria, and seldom against Gram-negative bacteria, which are recalcitrant because of structure of their outer-membrane. The discovery and characterization of LAB-bacteriocins possessing activity against Gram-negative bacteria is of paramount interest for clinical issues.

In regard to characterization of LAB-bacteriocins endowed with activity against Gram-negative bacteria, our previous work reported the isolation of *Lacticaseibacillus paracasei* CNCM I-5369 strain carrying five genes (*orf010*, *orf012*, *orf023*, *orf030* and *orf038*) coding for class II bacteriocins [16]. These bacteriocins were heterologously expressed in *E. coli* BL21 and shown to be active against *E. coli* ATCC 8739 [16]. The present study is a continuation, which is aimed at evaluating the expression of each gene coding for these bacteriocins and defining the appropriate conditions for their effective purification.

2. Materials and methods

2.1. Bacterial strains, culture conditions and plasmids

Bacterial strains used in this work are listed in Table 1. The strain *L. paracasei* CNCM I-5369 was grown in de Man, Rogosa and Sharpe medium (MRS, from SIGMA-ALDRICH, St. Louis, USA) supplemented with 0.1 % Tween 80 at 37 °C [17]. All the *E. coli* strains were grown in Luria-Bertani broth (LB) or Brain Heart Infusion (BHI, SIGMA-ALDRICH Saint-Louis, USA) at 37 °C with shaking at 160 rpm.

2.2. Total RNA extraction and cDNA synthesis

Cells from *L. paracasei* CNCM I-5369 were harvested by centrifugation from a culture of 10 ml at the beginning and at the end of the logarithmic growth phase. Total RNA was extracted using the NucleoSpin RNA Set for NucleoZOL (MACHERY-NAGEL, Düren, Germany). Then, 1 µg of total RNA from each sample was treated with DNase (Thermo Fisher Scientific, Waltham, United States) in order to remove all traces of DNA. After inactivation of DNase with 50 mM EDTA (Thermo Fisher Scientific, Waltham, United States), the total RNA was converted to complementary DNA (cDNA) using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, United States), following the recommended instructions.

2.3. In-silico transcriptional analysis

In-silico prediction of Rho-Independent terminator for each bacteriocin gene was performed using the Findterm tool ([http://](http://softberry.com)

Table 1
Strains and plasmids used in this study.

Strains and plasmids	Source/references
Strains	
Producer strain	
<i>Lacticaseibacillus paracasei</i> CNCM I-5369	[16]
Target strain	
<i>Escherichia coli</i> ATCC 8739	Manassas, VA (USA)
Heterologous producer strain	
<i>E. coli</i> Rosetta (DE3)	Promega, Madison, United States
<i>E. coli</i> BL21 (DE3)	Merck Millipore, Burlington, United States
Expression Plasmids	
pT7-6HIS-010	[16]
pT7-6HIS-012	[16]
pT7-6HIS-023	[16]
pT7-6HIS-030	[16]
pT7-6HIS-038	[16]

softberry.com) [18]. The putative promoter region of each gene was suggested based on the consensus sequences for -10 box (TATAAT) and -35 box (TTGACA) and considering their position upstream of the start codon.

2.4. Primer design

The nucleotide sequences of the bacteriocin genes of *L. paracasei* CNCM I-5369 were previously determined with Bagel 4 software [16]. The house-keeping genes used to standardize the expression of bacteriocin genes were the *gyrA* and 16S rRNA genes [19]. The oligonucleotides used for RT-qPCR (Table 2) in this work were designed using the Primer 3 tool (<http://primer3.ut.ee/>). The specificity and the quality of the primer pairs have been verified with NetPrimer (<http://www.premierbiosoft.com/netprimer/>).

2.5. Quantitative PCR (qPCR) conditions and assessment of gene expression

Each RT-qPCR reaction was performed in triplicate in a final volume of 25 µl, containing 12.5 µl of 2X Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, USA), 1.25 µl of each appropriate primer, 2 µl of cDNA and 8 µl of nuclease free water. The RT-qPCR reactions were performed in the "CFX Connect Real-Time PCR Detection System" thermocycler (BIO-RAD, Hercules, USA) under conditions including an initial step at 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 58 °C for 1 min, and 72 °C for 30 s. An additional step starting between 90 °C and 58 °C was performed to establish a melting curve and check the specificity of each primer pair [20]. Threshold cycle (Ct) values for each sample were obtained using Bio-Rad's CFX Manager software. The Ct value is the basis for the calculation of the relative quantification, corresponding to the expression of the target gene compared to the house-keeping gene. The analysis of the relative expression of the target genes was determined using the $2^{-(\Delta\Delta Ct)}$ method [21]. To know the reproducibility of the data, the efficiency was determined according to the following equation $E = [10^{-1/s} - 1]^{-1} * 100$, with s corresponding to the standard curve obtained from several dilutions of the cDNA [22].

2.6. Heterologous production and purification of bacteriocins

Each gene was cloned under the control of the inducible T7 promoter as recently reported [16], using e-Zyvec technology. Each recombinant plasmid (Table 1) was recovered and used to transform the *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3) competent cells by a heat shock method (42 °C). Briefly, SOC medium (Super Optimal broth with Catabolic repression, Sigma Aldrich, St Louis, USA) was added and the transformed cells were incubated at 37 °C for 1 h with shaking (160 rpm). After overnight growth at 37 °C, the *E. coli* transformants were selected on the LB agar medium supplemented with ampicillin (100 µg/mL) and chloramphenicol (30 µg/mL) (Sigma Aldrich, St Louis, USA). To produce the recombinant bacteriocins, overnight cultures of *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3) strain harbouring recombinant plasmids were diluted to 1% (vol/vol) in 100 mL LB broth containing ampicillin (100 µg/mL) and chloramphenicol (30 µg/mL) and then were grown aerobically at 37 °C with shaking at 160 rpm until they had reached an OD_{600nm} of 0.8. Expression was induced by addition of 0.5 mM (IPTG) isopropyl -D-1-thiogalactopyranoside (Sigma-Aldrich, St Louis, USA) and the cells were incubated for an additional 3–5 hours at 37 °C with shaking at 160 rpm.

After production, the cells were recovered by centrifugation (4700 g for 20 min), resuspended in a lysis buffer containing 20 mM TRIS-HCl pH 8 and 300 mM NaCl supplemented with

Table 2
Sequences of oligonucleotide primers used in this study.

Target gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Theoretical and expected amplicon size (bp)
<i>orf010</i>	AGATCACTGGCGGTTTTGC	GCACCACCAGCAATTCATCT	100
<i>orf012</i>	AGCTTGGAAAAGATTGCTGCT	CAGACTACCAGTAAGCACGC	99
<i>orf023</i>	ACGTAAATTCCTGACAATGCTGA	GCCCTGCCATCCCTTTAAAA	100
<i>orf030</i>	TCCAACGATCAAAGCAGAGC	ACAGCCGTCACATAAGCCCTT	99
<i>orf038</i>	CGGCAGGTATTGGATCAGGA	ACCCAACCGCTCCTAAGTTT	99
<i>gyrA</i>	CTTCCACGCATGATGTCCTG	AATCCCTTTGCGGTTCGAC	101
16S rRNA	GTAGGTGGCAAGCGTTATCC	GATGCGCTTCCTCGGTTAAG	101

10 mM imidazole (W10). Then, the cells were lysed in the cell disruptor (CSL One Shot Machine 2700 bars, CellID, France) by exerting a pressure of 1.035 bar to release the cytoplasmic proteins. Three cycles were repeated for the same method. The lysate was then centrifuged at 11,000 g for 1 h following purification of bacteriocins by Ni-NTA affinity chromatography. To this end, the supernatants were loaded onto a column containing Nickel resin grafted on a nitrilo-tri-acetic (Ni-NTA) matrix (HisPur™ Ni-NTA Spin Columns, 1 mL, de Thermo SCIENTIFIC, Waltham, United States) previously equilibrated with TRIS–HCl buffer (20 mM TRIS–HCl pH8 and 300 mM NaCl). Bacteriocins have a 6-histidine (6-His) tag at their N-terminal site (Table 1). The 6-His tag interacts with Ni²⁺ ions, and only the proteins of interest remain attached to the resin. The resin was washed with 16 mL of the same buffer supplemented with 30 mM imidazole and the peptides were eluted with 1 mL of TRIS–HCl buffer supplemented with 400 mM imidazole. A desalting step was performed using PD miditrap columns (GE Healthcare Life Science, Pollard, United Kingdom) to remove the imidazole. The purity is verified by Tricine-SDS-PAGE [23].

2.7. Solubilization, separation and detection of bacteriocins

The inclusion bodies were resolubilized with 50 mM TRIS–HCl pH8 buffer containing 4 M urea and incubated for 1.5 h at room temperature with shaking at 160 rpm followed by centrifugation at 11,000 g for 1 h. The resolubilized proteins contained in the supernatant were purified by Ni-NTA chromatography (as described above) to renature the proteins and to isolate those of interest.

The purification steps were analyzed on the Tricine-SDS-PAGE [23]. Samples diluted in Laemmli Buffer [24] were heated at 90 °C for 10 min before loading onto the gel. Migration was performed at a constant voltage of 120 V and initial amperage of approximately 50 mA per gel for 45 min. After migration, the gels were stained with InstantBlue™ (Expedeon, Cambridge, United Kingdom) for 20 min.

The final concentration of each bacteriocin after purification was determined with the BCA (bicinchoninic acid) Assay protein kit (Sigma-Aldrich, St Louis, MO, USA), as recommended by the supplier.

2.8. Mass spectrometry

Mass spectra were obtained with a MALDI-TOF mass spectrometer. The purified bacteriocin contained in the elution fractions, was detected by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS). The analysis was carried out using the Autoflex Speed MALDI TOF/TOF equipment (Bruker Daltonics, Germany) and spectra were obtained using flexAnalysis softwear (Bruker Daltonics, Germany). When required, the samples were concentrated using Pierce™ C18 tips (Thermo Scientific).

2.9. Bacteriocin activity

The antimicrobial activity of the bacteriocins was tested by the agar diffusion test method [25], using *E. coli* ATCC 8739 as the target strain. This strain does not contain the *stx1* or *stx2* genes coding for Shiga toxins [26]. To titrate the antibacterial activity of purified bacteriocins, the serial dilution method on agar medium was used. Thus, purified bacteriocins were acidified to a pH between 4.5 and 5 and sterilized by filtration. Serial double dilutions of the samples were performed and was added to the wells of BHI soft agar containing the *E. coli* ATCC 8739 indicator strain. The Petri dishes were then incubated at 37 °C for 18 h. The antibacterial activity was then determined in Arbitrary Units per millilitre (AU/mL) according to the following formula: 2ⁿ × (1000 μl/ deposited volume), with n corresponding to the highest number of dilution at which growth inhibition of the sensitive strain is observed [27].

3. Results

3.1. In-silico prediction and locations of putative promoters and terminator regions

The nucleotide and protein sequences for the five bacteriocins of *L. paracasei* CNCM I-5369 strain were identified using Bagel 4 software [16]. The putative sequences of promoter regions are depicted for each gene in Fig. 1. The Rho-independent terminator is based on a palindrome sequence downstream from the target gene. The results of the analysis performed showed no palindrome

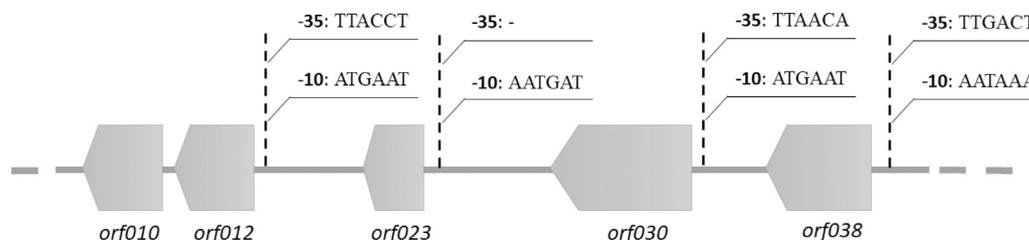


Fig. 1. In silico prediction of promoter regions for each bacteriocin coding gene. The putative sequences of promoter regions (–10 and –35) are depicted for each bacteriocin gene.

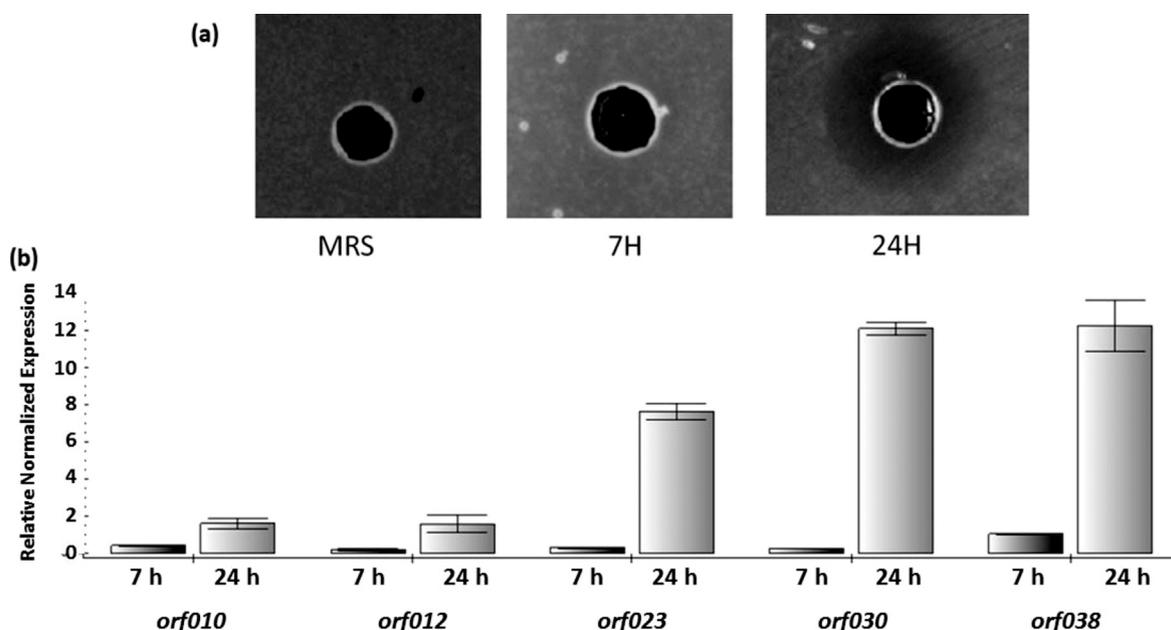


Fig. 2. (a) Agar diffusion test against *E. coli* ATCC 8739 using the *Lacticaseibacillus paracasei* CNCM I-5369 supernatant withdrawn after 7 and 24 h of growth. MRS broth, adjusted to pH 5, was used as control. (b) Expression of *orf010*, *orf012*, *orf023*, *orf030* and *orf038* genes coding for five distinct class II bacteriocins in *Lacticaseibacillus paracasei* CNCM I-5369. The black and the grey bars indicate the RT-qPCR data after 7 and 24 h of growth respectively. The error bars represent standard deviation.

sequences, indicating that the termination of these genes is regulated by a Rho-dependent mechanism.

The distance between the *orf012* and *orf010* is very short, (28 nucleotides) and therefore we could not identify any putative promoter sequences for gene *orf010* and terminator sequences for gene *orf012*. These *orfs* could be co-transcribed, and in order, to confirm this hypothesis, we performed a PCR assay using the cDNA of the *L. paracasei* CNCM I-5369 strain as a template, and the primers: *forward primer-orf012* and *reverse primer orf010* (Table 2). We obtained an amplicon of about 300 bp which favourably supported a co-transcription of *orf010* and *orf012* (data not shown).

3.2. All genes coding for these five class II bacteriocins are concomitantly expressed in the natural host

The growth of a culture of *L. paracasei* CNCM I-5369 was monitored during 24 h. At the beginning (7 h) and at the end of the logarithmic growth phase (24 h), the culture supernatant was recovered, adjusted to a pH between 4.5 and 5 and tested against *E. coli* ATCC 8739 as a target strain (Fig. 2a). It should be noted that only the supernatant withdrawn at the end of the logarithmic growth phase displayed bioactivity. Of note, this bioactivity was abolished following treatment of the supernatant with proteolytic enzymes like papain and proteinase K at a final concentration of 2 mg/mL, indicating a proteinaceous nature of the molecule(s) responsible of this bioactivity.

At 7 and 24 h of growth, cells were collected and used to study expression of each gene coding for these bacteriocins, using the RT-qPCR method. As indicated in Fig. 2b, genes coding for these five bacteriocin genes were uniquely expressed after 24 h of growth. The RT-qPCR analysis revealed that *orf023*, *orf030* and *orf038* were overexpressed.

3.3. Optimization of the production of bacteriocins

Plasmids carrying the bacteriocin genes were used to transform the Rosetta and BL21 *E. coli* strains. Growth of the transformed Rosetta strain was compared with that of the BL21 strain in LB

broth. No significant difference in the growth between the strains and the growth rate were observed (data not shown).

The expression of *orf010*, *orf012*, *orf023*, *orf030* and *orf038* was carried out in *E. coli* Rosetta and *E. coli* BL21, by using RT-qPCR method. As shown in Table 3, the expression was remarkable in the *E. coli* Rosetta strain, where the highest production levels were obtained (Fig. 3). Finally, the *E. coli* Rosetta strain was selected to perform the bacteriocin production. The conditions producing the highest bacteriocin yields were observed following induction with 0.5 mM IPTG for 5 h at 37 °C.

The purification of bacteriocins by Ni-NTA affinity chromatography was monitored by SDS-PAGE. Only the bacteriocin coded by *orf030* was purified under the pre-determined expression condition, as shown on Fig. 4. For the other bacteriocins coded by *orf010*, *orf012*, *orf023* and *orf038*, low concentrations were found in the soluble fraction, suggesting that these bacteriocins were misfolded and forming inclusion bodies (Table 4). To verify this hypothesis, the total fraction was treated with a buffer containing 4 M urea. Then a second Ni-NTA affinity chromatography was performed in order to increase the purification yield. The SDS-PAGE analysis showed that the urea treatment increased the concentration of bacteriocins in the eluted fractions during purification (Fig. 5-A). In addition, bacteriocins were analysed by MALDI-TOF mass spectrometry and the mass spectra of 5 bacteriocins were shown on Fig. 5-B. Thus, a main peak corresponding to the expected m/z value (m/z is the mass/charge ratio, where z is usually 1 and m corresponds to the molecular weight) of each bacteriocin (8149.1 for ORF010, 8431.1 for ORF012, 5075.2 for ORF023, 14119.4 for ORF030 and 10281.6 for ORF038) was observed.

Table 3
Bacteriocin genes expression levels in *E. coli* Rosetta strain in fold of the level found in BL21 strain.

Genes	Level of expression	P-Value
<i>orf010</i>	12.75	0.15086
<i>orf012</i>	27.98	0.11891
<i>orf023</i>	47.55	0.06880
<i>orf030</i>	144.13	0.15235
<i>orf038</i>	5.54	0.05529

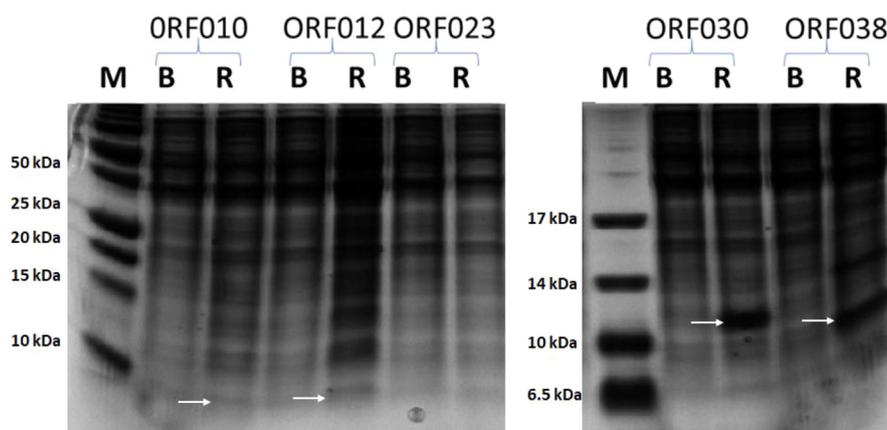


Fig. 3. Bacteriocins electrophoretic patterns on SDS-PAGE (12 % polyacrylamide). **M:** Markers of molecular weights, **B:** *E. coli* strain BL21, **R:** *E. coli* strain Rosetta; The arrows indicated the position of each bacteriocin. The molecular weight of each bacteriocin with His-Tag are: 8136.10 Da for ORF010, 8417.59 Da for ORF012, 5035.80 Da for ORF023, 14088.07 Da for ORF030 and 12230.92 Da for ORF038.

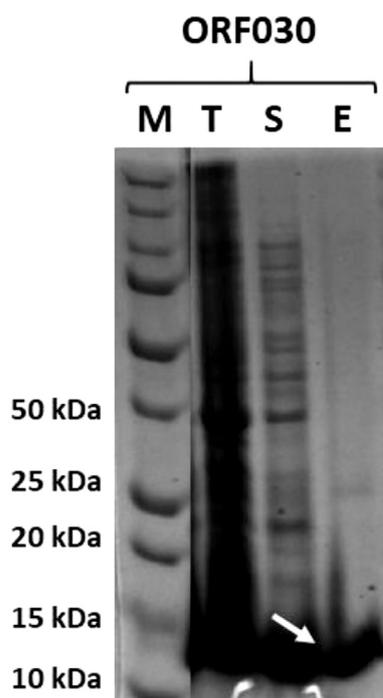


Fig. 4. Ni-NTA purification (SDS-PAGE with 12 % polyacrylamide) of the bacteriocin encoded by *orf030*. **T:** total fraction; **S:** soluble fraction; **M:** Size markers; **E:** Elution with 400 mM imidazole; The arrows indicated the position of ORF030.

The concentrations of bacteriocins obtained during the purification steps after urea treatment, were quantified with BiCinchoninic acid assay (BCA assay) (Table 5). Then, the bioactivity of each bacteriocin was tested at pH 5 (Fig. 6) and their minimal inhibitory concentrations are given in Table 5. After the desalting step, we noted that concentration of bacteriocin

Table 4
Bacteriocins yields obtained before urea treatment.

Bacteriocin	Concentration ($\mu\text{g/mL}$)
ORF010	1.02
ORF012	1.23
ORF023	1.27
ORF030	871.15
ORF038	1.36

ORF030 was the highest one. All these 5 bacteriocins were active against *E. coli* ATCC 8739. Due to its highest MIC, bacteriocin ORF030 exhibited the lowest specific activity, while bacteriocins ORF010 and ORF012 exhibited the highest one. Importantly, the assessment of antimicrobial activity with all purified bacteriocins revealed a synergistic effect with an improved activity, as depicted on Fig. 6. Consequently, the combination of these 5 purified bacteriocins at 80 mg/mL each, underpinned a significant total activity of 3200 AU/mL. Similarly, the combination of bacteriocins ORF010 and ORF012 also revealed a synergistic interaction (Fig. 6).

4. Discussion

LAB-bacteriocins have gained attention because of their low toxicity, high potency, target specificities [28], and numerous other related biological functions [10,29,30]. LAB-bacteriocins are steadily reported as alternatives for traditional antibiotics especially in light of antimicrobial resistance (AMR), which is a global public problem amplified by the overuse of clinical antibiotics, and the lack of novel drugs in the pipeline. Therefore, demands of new molecules and interventions are urgently required. Currently the death rates are around 750,000 globally and it is expected to rise up till 10 million per year by 2050 with a cumulative cost of US \$ 100 trillion [4]. To face this postulated crisis, AMPs such as bacteriocins can act as efficient strategical plan to alleviate AMR resistance on immediate grounds.

Previously, we have already reported the ability of the multiproducing strain *Lactobacillus paracasei* CNCM I-5369 (now renamed as per new nomenclature of classification *L. paracasei* CNCM I-5369), to produce five distinct class II bacteriocins [16], with activity against *E. coli* strains including those exhibiting resistant to colistin, an antibiotic used as a last-line option to treat infections caused by antibiotic-resistance bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *E. coli*, and *Klebsiella* spp [31]. The multi bacteriocin producing strain expressed activity against *E. coli* only when supernatant was adjusted to pH between 4.5 to 5. Similar results were observed when each bacteriocin was recovered from the insoluble fraction of the heterologous strain *E. coli* Rosetta. The genes *orf010*, *orf012*, *orf023*, *orf030* and *orf038* coding for five putative bacteriocins previously identified [32], were expressed after 24 h of growth, from the multi-bacteriocin producing strain *L. paracasei* CNCM I-5369. The RT-qPCR data reveals the overexpression of the above studied genes. The expression observed at the end of the logarithmic growth phase, was correlated with the bioactivity, delineating a possible link between mRNA stability and production

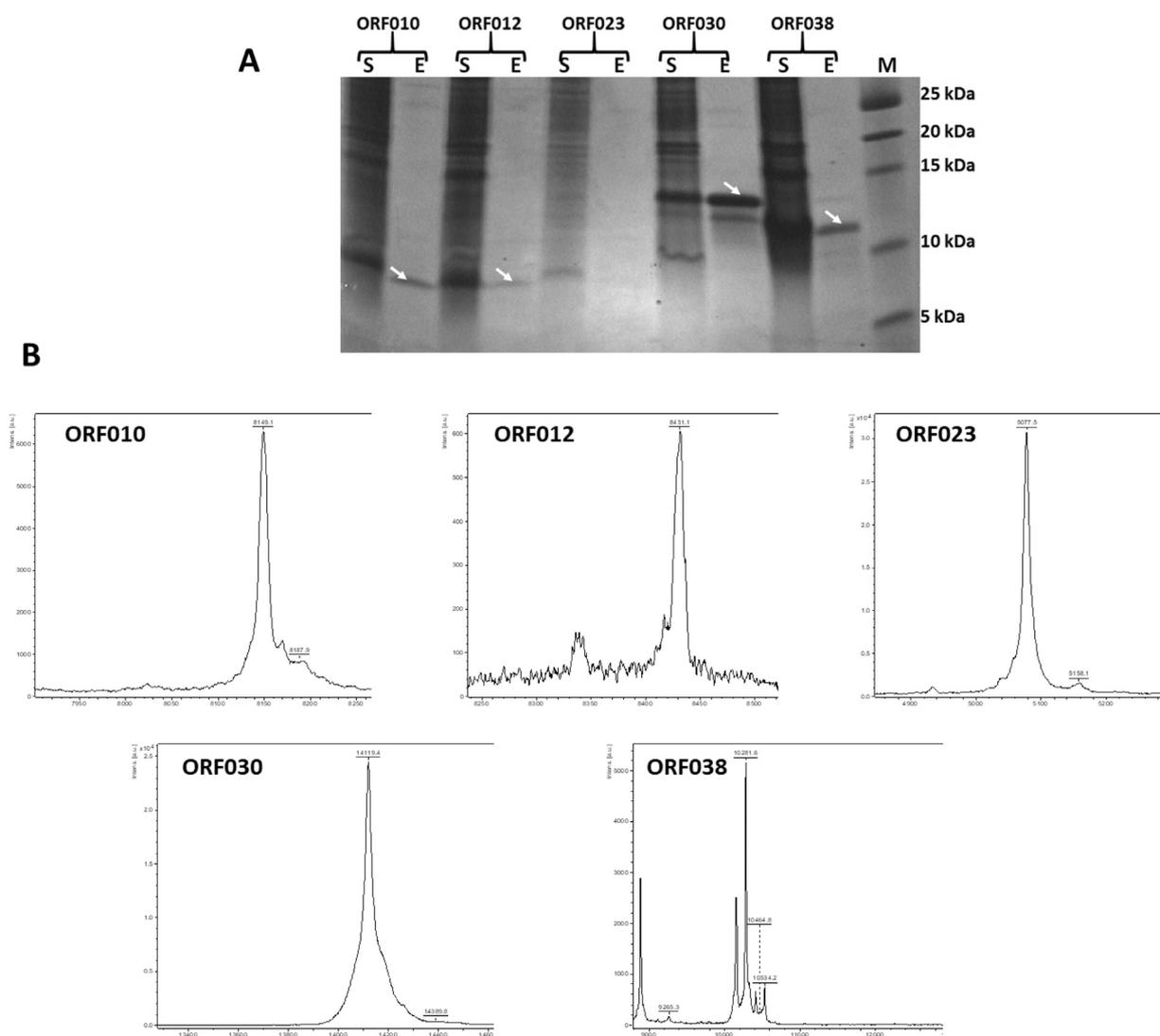


Fig. 5. (A) Ni-NTA purification (SDS-PAGE with 12 % polyacrylamide) of the 5 bacteriocins after urea treatment. **S:** soluble fraction; **M:** Size markers; **E:** Elution with 400 mM imidazole; The arrows indicated the position of each purified bacteriocin.

(B) MALDI-TOF spectra of purified bacteriocins, ORF010, ORF012, ORF023, ORF030 and ORF038. The molecular weight of each bacteriocin with His-Tag are 8149.1 Da for ORF010, 8431.1 Da for ORF012, 5075.2 Da for ORF023, 14119.4 Da for ORF030 and 10281.6 Da for ORF038.

Table 5

Molecular weights, concentration and antibacterial activity of the five bacteriocins after urea treatment.

Bacteriocins	Molecular weight ^a (Da)	Concentration (μg/mL)	MIC (μg/mL)	Activity (UA/mL)	Specific activity (UA/μg)
ORF010	8 136.10	21. 3	2.66	800	37.55
ORF012	8 417.59	23.4	2.92	800	34.18
ORF023	5035.80	31.7	3.96	800	25.23
ORF030	14 088.07	317.4	39.67	800	2.52
ORF038	12 230.92	44.7	11.17	400	8.94

^a The molecular weight of each bacteriocin with His-Tag was reported by Belguesmia *et al.* [16].

of these bacteriocins, as reported for different regulations systems [20,33,34].

In terms of transcriptional analysis, the *in-silico* analysis performed here enabled us to locate the putative promoters controlling expression of *orf023*, *orf030* and *orf038*. A more detailed *in silico* analysis, revealed a possible common promoter for *orf010* and *orf012*; two *orfs* that were co-transcribed according to our RT-qPCR analysis.

Purification of bacteriocins from the natural hosts are usually tedious and typically only low yields are recovered at the end of the

purification process [35]. The heterologous production of bacteriocins is a more suitable approach as genes coding for these bacteriocins can be placed under inducible promoters and controlled growth conditions allowing the isolation of greater quantities of bacteriocins [36]. Of note, bacteriocins from Gram-positive bacteria can be heterologously produced from synthetic DNA leading as well to larger amounts of bacteriocins than produced by the native host [37].

Here, the expression of each gene was studied in *E. coli* Rosetta DE3 and *E. coli* BL21. Overall, the bacteriocins produced in such a

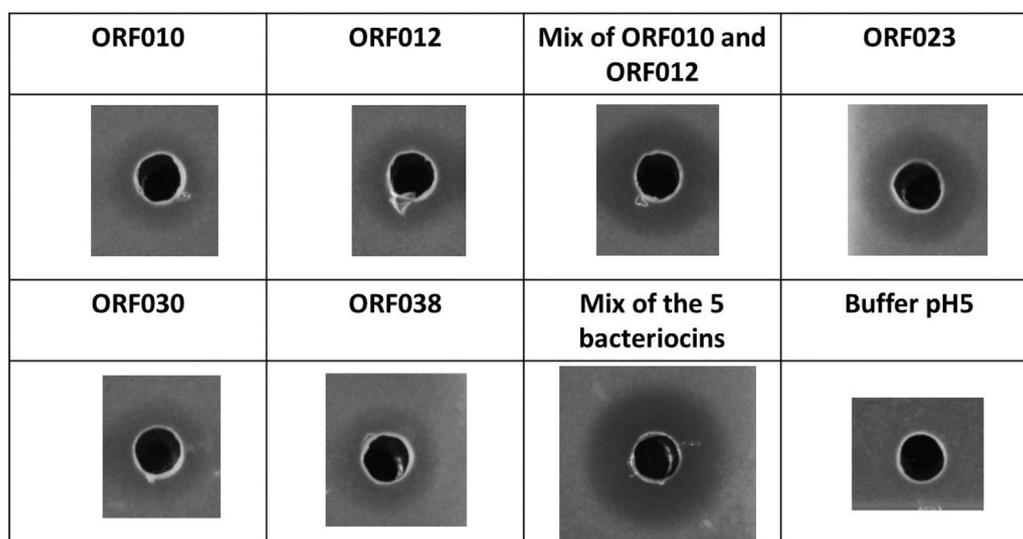


Fig. 6. Agar diffusion test on *E. coli* ATCC 8739 using purified bacteriocins produced by *Escheichia coli* Rosetta strain.

manner have shown a better expression level, when they were produced in *E. coli* Rosetta (DE3), a strain that harbours a plasmid (pRARE) coding rare tRNAs. The analysis of each bacteriocin gene revealed the presence of rare codons (AGG, AGA, CCG, CGA AUA, CUA, CCC, and GGA) which could affect the translation outcome [38]. *E. coli* Rosetta possess the required tRNAs for the codons AGG, AGA, AUA, CUA, CCC, GGA and ensures efficient translation of the recombinant bacteriocins [39]. In the purification process developed here only the bacteriocin encoded by *orf030* gene was obtained in the soluble fraction, the other ones were recovered from the insoluble fraction. Fahnert et al. [40] reported that overexpression of recombinant proteins can be lethal for the *E. coli* host, yielding to inactive inclusion bodies associated with the specific folding of these proteins [40]. The formation of the inclusion bodies may be an advantage for bacteriocin purification as they can remain protected from the deleterious actions of the host proteases [36]. In addition to these two hypotheses, expression of these bacteriocins in other hosts like lactic acid bacteria, and yeasts could be advantageous.

In this study, the inclusion bodies were solubilized with 4 M of urea. Urea is a chaotrope molecule that at high concentrations can lead to denaturation of the secondary structures of proteins. It is used to inhibit protein aggregation during the folding process, improving the recovery of bioactive proteins [41]. The solubilized inclusion body proteins are refolded by removal of the solubilisation agent. In this study, Ni-NTA affinity chromatography was used for refolding of solubilized proteins.

5. Conclusions

The *in-silico* analysis and expression in the native system of the 5 bacteriocin genes of *L. paracasei* CNCM I-5369 strain was studied. Their expression was successfully conducted heterologously using *E. coli* as a host system. Each bacteriocin heterologously produced was purified, characterized by MALDI TOF Mass spectrometry and tested against *E. coli* ATCC 8739. A synergistic activity was observed when these five bacteriocins were tested concomitantly on this target strain.

Author contributions

Conceptualization, DMM, FC, DD; experimental procedures, DMM; writing—review and editing, DMM, FC DD; supervision FC,

DD; funding acquisition, DD. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by La Région des Hauts-de-France, through ALIBIOTECH CPER/FEDER 2016/2021.

Declaration of Competing Interest

The authors declare do not have conflict of interest

Acknowledgments

The authors are grateful to Dr. Stephen W Elson for the critical reading of the manuscript and the English improvement of this manuscript. The MALDI-TOF/MS experiments were performed on the REALCAT platform funded by the French government grant managed by the French National Research Agency (ANR) as part of the "Investments for the Future" program (ANR-11-EQPX-0037). We also acknowledge the Hauts-de-France region, the ERDF, the Ecole Centrale de Lille, and the Foundation Centrale Initiatives for financial support with the acquisition of the REALCAT platform equipment.

References

- [1] J.M.A. Blair, M.A. Webber, A.J. Baylay, D.O. Ogbolu, L.J.V. Piddock, Molecular mechanisms of antibiotic resistance, *Nat. Rev. Microbiol.* 13 (2015) 42–51, doi:<http://dx.doi.org/10.1038/nrmicro3380>.
- [2] B.H. Normark, S. Normark, Evolution and spread of antibiotic resistance, *J. Intern. Med.* 252 (2002) 91–106, doi:<http://dx.doi.org/10.1046/j.1365-2796.2002.01026.x>.
- [3] L.F. Chen, T. Chopra, K.S. Kaye, Pathogens resistant to antibacterial agents, *Infect. Dis. Clin. North Am.* 23 (2009) 817–845, doi:<http://dx.doi.org/10.1016/j.idc.2009.06.002>.
- [4] J.N. O'Neill, *Nat. Rev. Drug Discov.* 15 (2016) 526, doi:<http://dx.doi.org/10.1038/nrd.2016.160>.
- [5] H. Jenssen, P. Hamill, R.E.W. Hancock, Peptide antimicrobial agents, *Clin. Microbiol. Rev.* 19 (2006) 491–511, doi:<http://dx.doi.org/10.1128/CMR.00056-05>.
- [6] E.M. Kościuczuk, P. Lisowski, J. Jarczak, N. Strzałkowska, A. Józwiak, J. Horbańczuk, J. Krzyżewski, L. Zwierzchowski, E. Bagnicka, Cathelicidins: family of antimicrobial peptides. A review, *Mol. Biol. Rep.* 39 (2012) 10957–10970, doi:<http://dx.doi.org/10.1007/s11033-012-1997-x>.
- [7] K. Naghmouchi, D. Drider, I. Fliss, Action of divergicin M35, a class Ila bacteriocin, on liposomes and *Listeria*, *J. Appl. Microbiol.* 102 (2007) 1508–1517, doi:<http://dx.doi.org/10.1111/j.1365-2672.2006.03206.x>.

- [8] M.A. Riley, J.E. Wertz, Bacteriocin diversity: ecological and evolutionary perspectives, *Biochimie* 84 (2002) 357–364, doi:[http://dx.doi.org/10.1016/S0300-9084\(02\)01421-9](http://dx.doi.org/10.1016/S0300-9084(02)01421-9).
- [9] D. Drider, S. Rebuffat, Prokaryotic Antimicrobial Peptides: From Genes to Applications, Springer Science & Business Media, 2011.
- [10] M.L. Chikindas, R. Weeks, D. Drider, V.A. Chistyakov, L.M.T. Dicks, Functions and emerging applications of bacteriocins, *Curr. Opin. Biotechnol.* 49 (2018) 23–28, doi:<http://dx.doi.org/10.1016/j.copbio.2017.07.011>.
- [11] S. Soltani, R. Hammami, P.D. Cotter, S. Rebuffat, L.B. Said, H. Gaudreau, F. Bédard, E. Biron, D. Drider, I. Fliss, Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations, *FEMS Microbiol. Rev.* 45 (1) (2021), doi:<http://dx.doi.org/10.1093/femsre/fuaa039>.
- [12] Y. Belguesmia, A. Madi, D. Sperandio, A. Merieau, M. Feuilleley, H. Prévost, D. Drider, N. Connil, Growing insights into the safety of bacteriocins: the case of enterocin S37, *Res. Microbiol.* 162 (2011) 159–163, doi:<http://dx.doi.org/10.1016/j.resmic.2010.09.019>.
- [13] L.M.T. Dicks, L. Dreyer, C. Smith, A.D. van Staden, A review: the fate of bacteriocins in the human gastro-intestinal tract: do they cross the gut–blood barrier? *Front. Microbiol.* 9 (2018) 2297, doi:<http://dx.doi.org/10.3389/fmicb.2018.02297>.
- [14] P.D. Cotter, R.P. Ross, C. Hill, Bacteriocins – a viable alternative to antibiotics? *Nat. Rev. Microbiol.* 11 (2013) 95–105, doi:<http://dx.doi.org/10.1038/nrmicro2937>.
- [15] P. Alvarez-Sieiro, M. Montalbán-López, D. Mu, O.P. Kuipers, Bacteriocins of lactic acid bacteria: extending the family, *Appl. Microbiol. Biotechnol.* 100 (2016) 2939–2951, doi:<http://dx.doi.org/10.1007/s00253-016-7343-9>.
- [16] Y. Belguesmia, K. Bendjedou, I. Kempf, R. Boukherroub, D. Drider, Heterologous biosynthesis of five new class II bacteriocins from *Lactobacillus paracasei* CNCM I-5369 with antagonistic activity against pathogenic *Escherichia coli* strains, *Front. Microbiol.* 11 (2020), doi:<http://dx.doi.org/10.3389/fmicb.2020.01198>.
- [17] J.C.D. Man, M. Rogosa, M.E. Sharpe, A medium for the cultivation of lactobacilli, *J. Appl. Bacteriol.* 23 (1960) 130–135, doi:<http://dx.doi.org/10.1111/j.1365-2672.1960.tb00188.x>.
- [18] V. Solovyev, A. Salamov, I. Seledtsov, D. Vorobyev, A. Bachinsky, Automatic annotation of bacterial community sequences and application to infections diagnostic, *Proceedings of the International Conference on Bioinformatics Models, Methods and Algorithms (Meta-2011)* (2011) 346–353, doi:<http://dx.doi.org/10.5220/000333703460353>.
- [19] W. Zhao, Y. Li, P. Gao, Z. Sun, T. Sun, H. Zhang, Validation of reference genes for real-time quantitative PCR studies in gene expression levels of *Lactobacillus casei* Zhang, *J. Ind. Microbiol. Biotechnol.* 38 (2011) 1279–1286, doi:<http://dx.doi.org/10.1007/s10295-010-0906-3>.
- [20] A. Hurtado, C. Reguant, A. Bordons, N. Rozès, Expression of *Lactobacillus pentosus* B96 bacteriocin genes under saline stress, *Food Microbiol.* 28 (2011) 1339–1344, doi:<http://dx.doi.org/10.1016/j.fm.2011.06.004>.
- [21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method, *Methods* 25 (2001) 402–408, doi:<http://dx.doi.org/10.1006/meth.2001.1262>.
- [22] C. Beltramo, N. Desroche, R. Tourdot-Maréchal, C. Grandvalet, J. Guzzo, Real-time PCR for characterizing the stress response of *Oenococcus oeni* in a wine-like medium, *Res. Microbiol.* 157 (2006) 267–274, doi:<http://dx.doi.org/10.1016/j.resmic.2005.07.006>.
- [23] H. Schägger, G. von Jagow, Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem.* 166 (1987) 368–379, doi:[http://dx.doi.org/10.1016/0003-2697\(87\)90587-2](http://dx.doi.org/10.1016/0003-2697(87)90587-2).
- [24] H.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685, doi:<http://dx.doi.org/10.1038/227680a0>.
- [25] J.R. Tagg, A.R. McGiven, Assay system for bacteriocins, *Appl. Microbiol.* 21 (1971) 943.
- [26] *Escherichia coli* ATCC 8739, Complete Genome, (2014). (accessed February 28, 2021) <http://www.ncbi.nlm.nih.gov/nuccore/CP000946.1>.
- [27] B. Batdorj, M. Dalgarrondo, Y. Choiset, J. Pedroche, F. Métro, H. Prévost, J.M. Chobert, T. Haertlé, Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag, *J. Appl. Microbiol.* 101 (2006) 837–848, doi:<http://dx.doi.org/10.1111/j.1365-2672.2006.02966.x>.
- [28] P.M. O'Connor, T.M. Kuniyoshi, R.P. Oliveira, C. Hill, R.P. Ross, P.D. Cotter, Antimicrobials for food and feed; a bacteriocin perspective, *Curr. Opin. Biotechnol.* 61 (2020) 160–167, doi:<http://dx.doi.org/10.1016/j.copbio.2019.12.023>.
- [29] D. Drider, F. Bendali, K. Naghmouchi, M.L. Chikindas, Bacteriocins: not only antibacterial agents, *Probiotics Antimicrob. Proteins* 8 (2016) 177–182, doi:<http://dx.doi.org/10.1007/s12602-016-9223-0>.
- [30] S.D. Todorov, H.J. Kang, I.V. Ivanova, W.H. Holzapfel, Bacteriocins from LAB and other alternative approaches for the control of *Clostridium* and *Clostridiodes* related gastrointestinal colitis, *Front. Bioeng. Biotechnol.* 8 (2020), doi:<http://dx.doi.org/10.3389/fbioe.2020.581778>.
- [31] I. Caniaux, A. van Belkum, G. Zambardi, L. Poirel, M.F. Gros, MCR: modern colistin resistance, *Eur. J. Clin. Microbiol. Infect. Dis.* 36 (2017) 415–420, doi:<http://dx.doi.org/10.1007/s10096-016-2846-y>.
- [32] Y. Belguesmia, N. Hazime, I. Kempf, R. Boukherroub, D. Drider, New bacteriocins from *Lactocaseibacillus paracasei* CNCM I-5369 adsorbed on alginate nanoparticles are very active against *Escherichia coli*, *Int. J. Mol. Sci.* 21 (2020), doi:<http://dx.doi.org/10.3390/ijms21228654>.
- [33] P. Chanos, T. Mygind, Co-culture-inducible bacteriocin production in lactic acid bacteria, *Appl. Microbiol. Biotechnol.* 100 (2016) 4297–4308, doi:<http://dx.doi.org/10.1007/s00253-016-7486-8>.
- [34] J.A. Renye, G.A. Somkuti, J.I. Garabal, D.H. Steinberg, Bacteriocin production by *Streptococcus thermophilus* in complex growth media, *Biotechnol. Lett.* 38 (2016) 1947–1954, doi:<http://dx.doi.org/10.1007/s10529-016-2184-2>.
- [35] S. Yildirim, D. Konrad, S. Calvez, D. Drider, H. Prévost, C. Lacroix, Production of recombinant bacteriocin divercin V41 by high cell density *Escherichia coli* batch and fed-batch cultures, *Appl. Microbiol. Biotechnol.* 77 (2007) 525–531, doi:<http://dx.doi.org/10.1007/s00253-007-1188-1>.
- [36] M. Mesa-Pereira, M.C. Rea, P.D. Cotter, C. Hill, R.P. Ross, Heterologous expression of biopreservative bacteriocins with a view to low cost production, *Front. Microbiol.* 9 (2018), doi:<http://dx.doi.org/10.3389/fmicb.2018.01654>.
- [37] C. Richard, D. Drider, K. Elmorjani, D. Marion, H. Prevost, Heterologous expression and purification of active divercin V41, a class IIa bacteriocin encoded by a synthetic gene in *Escherichia coli*", *J. Bacteriol.* 186 (2004) 4276–4284, doi:<http://dx.doi.org/10.1128/JB.186.13.4276-4284.2004>.
- [38] C.P. Papanephytous, G. Kontopidis, Statistical approaches to maximize recombinant protein expression in *Escherichia coli*: a general review, *Protein Expr. Purif.* 94 (2014) 22–32, doi:<http://dx.doi.org/10.1016/j.pep.2013.10.016>.
- [39] N.K. Tripathi, Production and purification of recombinant proteins from *Escherichia coli*, *ChemBioEng Rev.* 3 (2016) 116–133, doi:<http://dx.doi.org/10.1002/cben.201600002>.
- [40] B. Fahnert, H. Lilie, P. Neubauer, Inclusion bodies: formation and utilisation, *Physiological Stress Responses in Bioprocesses: -/-*, Springer, Berlin, Heidelberg, 2004, pp. 93–142, doi:<http://dx.doi.org/10.1007/b93995>.
- [41] A. Singh, V. Upadhyay, A.K. Upadhyay, S.M. Singh, A.K. Panda, Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process, *Microbial Cell Factory* 14 (2015) 41, doi:<http://dx.doi.org/10.1186/s12934-015-0222-8>.