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

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5

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10

11 **Keywords:** *Lacticaseibacillus paracasei*, class II bacteriocins, heterologous expression, RT-  
12 qPCR

13

14 **Abstract**

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

26

## 27        **1. Introduction**

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

41    Antimicrobial peptides (AMPs) can help curb the AMR crisis. AMPs are present in all living  
42    cells [5,6], as part of the first line defence against foreign attacks. Many AMPs have the  
43    particularity of being cationic and can bind and interact with the negatively charged bacterial  
44    cell membranes, causing leakage of intracellular constituents, ATP depletion and cell death [7].  
45    Bacteriocins are a class of AMPs ribosomally synthesized by Gram-positive and Gram-negative  
46    bacteria [8,9], and are steadily reported for their positive clinical outcomes [10]. These safe  
47    molecules [11–13] can be of narrow or broad spectrum [14] which offers numerous advantages  
48    over clinical antibiotics. The classification of bacteriocins has regularly been updated taking  
49    into consideration recent achievements and new knowledge. Currently there is no any  
50    universally accepted scheme of bacteriocins classification. Nevertheless, we refer here to the  
51    classification proposed by Alvarez-Sieiro et al, [15], which includes 3 main classes. Class I

52 contains all peptides that undergo post-translational enzymatic modifications during their  
53 biosynthesis, leading to unusual amino acids, such as lanthionine. Class II includes unmodified  
54 bacteriocins with a low molecular weight (<10 kDa); and class III contains unmodified  
55 bacteriocins with a molecular mass higher than 10 kDa, and endowed with bacteriolytic or non-  
56 bacteriolytic modes of action.

57 Bacteriocins among Gram-positive bacteria are most often produced by lactic acid bacteria  
58 (LAB). These bacteriocins are usually active against other Gram-positive bacteria, and seldom  
59 against Gram-negative bacteria, which are recalcitrant because of structure of their outer-  
60 membrane. The discovery and characterization of LAB-bacteriocins possessing activity against  
61 Gram-negative bacteria is of paramount interest for clinical issues.

62 In regard to characterization of LAB-bacteriocins endowed with activity against Gram-negative  
63 bacteria, our previous work reported the isolation of *Lacticaseibacillus paracasei* CNCM I-  
64 5369 strain carrying five genes (*orf010*, *orf012*, *orf023*, *orf030* and *orf038*) coding for class II  
65 bacteriocins [16]. These bacteriocins were heterologously expressed in *E. coli* BL21 and shown  
66 to be active against *E. coli* ATCC 8739 [16]. The present study is a continuation, which is aimed  
67 at evaluating the expression of each gene coding for these bacteriocins and defining the  
68 appropriate conditions for their effective purification.

69

## 70 **2. Materials and Methods**

### 71 *2.1. Bacterial strains, culture conditions and plasmids*

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

77

## 78 *2.2.Total RNA extraction and cDNA synthesis*

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

88

## 89 *2.3.In-silico transcriptional analysis*

90 *In-silico* prediction of Rho-Independent terminator for each bacteriocin gene was performed  
91 using the Findterm tool ([http:// softberry.com](http://softberry.com)) [18]. The putative promoter region of each gene  
92 was suggested based on the consensus sequences for -10 box (TATAAT) and -35 box  
93 (TTGACA) and considering their position upstream of the start codon.

94

## 95 *2.4.Primer design*

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

102

103 *2.5. Quantitative PCR (qPCR) conditions and assessment of gene expression*

104 Each RT-qPCR reaction was performed in triplicate in a final volume of 25  $\mu$ l, containing 12.5  
105  $\mu$ l of 2X Brilliant III SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara,  
106 USA), 1.25  $\mu$ l of each appropriate primer, 2  $\mu$ l of cDNA and 8  $\mu$ l of nuclease free water. The  
107 RT-qPCR reactions were performed in the “CFX Connect Real-Time PCR Detection System”  
108 thermocycler (BIO-RAD, Hercules, USA) under conditions including an initial step at 95°C for  
109 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  
110 step starting between 90°C and 58°C was performed to establish a melting curve and check the  
111 specificity of each primer pair [20]. Threshold cycle (Ct) values for each sample were obtained  
112 using Bio-Rad's CFX Manager software. The Ct value is the basis for the calculation of the  
113 relative quantification, corresponding to the expression of the target gene compared to the  
114 house-keeping gene. The analysis of the relative expression of the target genes was determined  
115 using the  $2^{-(\Delta\Delta C_t)}$  method [21]. To know the reproducibility of the data, the efficiency was  
116 determined according to the following equation  $E = [10^{-1/s} - 1] * 100$ , with s corresponding to the  
117 standard curve obtained from several dilutions of the cDNA [22].

118

119 *2.6. Heterologous production and purification of bacteriocins*

120 Each gene was cloned under the control of the inducible T7 promoter as recently reported [16],  
121 using e-Zyvec technology. Each recombinant plasmid (Table 1) was recovered and used to  
122 transform the *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3) competent cells by a heat shock  
123 method (42°C). Briefly, SOC medium (Super Optimal broth with Catabolic repression, Sigma  
124 Aldrich, St Louis, USA) was added and the transformed cells were incubated at 37°C for 1 hour  
125 with shaking (160 rpm). After overnight growth at 37°C, the *E. coli* transformants were selected  
126 on the LB agar medium supplemented with ampicillin (100  $\mu$ g/ml) and chloramphenicol (30

127  $\mu\text{g/ml}$ ) (Sigma Aldrich, St Louis, USA). To produce the recombinant bacteriocins, overnight  
128 cultures of *E. coli* Rosetta (DE3) and *E. coli* BL21 (DE3) strain harbouring recombinant  
129 plasmids were diluted to 1% (vol/vol) in 100 mL LB broth containing ampicillin (100  $\mu\text{g/ml}$ )  
130 and chloramphenicol (30  $\mu\text{g/ml}$ ) and then were grown aerobically at 37°C with shaking at 160  
131 rpm until they had reached an OD<sub>600nm</sub> of 0.8. Expression was induced by addition of 0.5 mM  
132 (IPTG) isopropyl -D-1-thiogalactopyranoside (Sigma-Aldrich, St Louis, USA) and the cells  
133 were incubated for an additional 3-5 hours at 37°C with shaking at 160 rpm.  
134 After production, the cells were recovered by centrifugation (4700 g for 20 min), resuspended  
135 in a lysis buffer containing 20 mM TRIS-HCl pH 8 and 300 mM NaCl supplemented with 10  
136 mM imidazole (W10). Then, the cells were lysed in the cell disruptor (CSL One Shot Machine  
137 2700 bars, CellD, France) by exerting a pressure of 1.035 bar to release the cytoplasmic  
138 proteins. Three cycles were repeated for the same method. The lysate was then centrifuged at  
139 11,000 g for 1 hour following purification of bacteriocins by Ni-NTA affinity chromatography.  
140 To this end, the supernatants were loaded onto a column containing Nickel resin grafted on a  
141 nitrilo-tri-acetic (Ni-NTA) matrix (HisPur™ Ni-NTA Spin Columns, 1 mL, de Thermo  
142 SCIENTIFIC, Waltham, United States) previously equilibrated with TRIS-HCl buffer (20 mM  
143 TRIS-HCl pH8 and 300 mM NaCl). Bacteriocins have a 6-histidine (6-His) tag at their N-  
144 terminal site (Table 1). The 6-His tag interacts with Ni<sup>2+</sup> ions, and only the proteins of interest  
145 remain attached to the resin. The resin was washed with 16 mL of the same buffer supplemented  
146 with 30 mM imidazole and the peptides were eluted with 1 mL of TRIS-HCl buffer  
147 supplemented with 400 mM imidazole. A desalting step was performed using PD miditrap  
148 columns (GE Healthcare Life Science, Pollard, United Kingdom) to remove the imidazole. The  
149 purity is verified by Tricine-SDS-PAGE [23].

150

151

152        *2.7.Solubilization, separation and detection of bacteriocins*

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

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

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

166

167        *2.8.Mass spectrometry*

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

174

175        *2.9.Bacteriocin activity*



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

186

### 187 **3. Results**

#### 188 *3.1. In-silico prediction and locations of putative promoters and terminator regions*

189 The nucleotide and protein sequences for the five bacteriocins of *L. paracasei* CNCM I-5369  
190 strain were identified using Bagel 4 software [16]. The putative sequences of promoter regions  
191 are depicted for each gene in Figure 1. The Rho-independent terminator is based on a  
192 palindrome sequence downstream from the target gene. The results of the analysis performed  
193 showed no palindrome sequences, indicating that the termination of these genes is regulated by  
194 a Rho-dependent mechanism.

195 The distance between the *orf012* and *orf010* is very short, (28 nucleotides) and therefore we  
196 could not identify any putative promoter sequences for gene *orf10* and terminator sequences for  
197 gene *orf012*. These *orfs* could be co-transcribed, and in order, to confirm this hypothesis, we  
198 performed a PCR assay using the cDNA of the *L. paracasei* CNCM I-5369 strain as a template,  
199 and the primers: *forward primer-orf012* and *reverse primer orf010* (Table 2). We obtained an

200 amplicon of about 300 bp which favourably supported a co-transcription of *orf10* and *orf12*  
201 (data not shown).

202

203 *3.2.All genes coding for these five class II bacteriocins are concomitantly expressed in the*  
204 *natural host*

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

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

217

218 *3.3.Optimization of the production of bacteriocins*

219 Plasmids carrying the bacteriocin genes were used to transform the Rosetta and BL21 *E. coli*  
220 strains. Growth of the transformed Rosetta strain was compared with that of the BL21 strain in  
221 LB broth. No significant difference in the growth between the strains and the growth rate were  
222 observed (data not shown).

223 The expression of *orf010*, *orf012*, *orf023*, *orf030* and *orf038* was carried out in *E. coli* Rosetta  
224 and *E. coli* BL21, by using RT-qPCR method. As shown in Table 3, the expression was

225 remarkable in the *E. coli* Rosetta strain, where the highest production levels were obtained  
226 (Figure 3). Finally, the *E. coli* Rosetta strain was selected to perform the bacteriocin production.  
227 The conditions producing the highest bacteriocin yields were observed following induction with  
228 0.5 mM IPTG for 5 hours at 37°C.

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

243 The concentrations of bacteriocins obtained during the purification steps after urea treatment,  
244 were quantified with BiCinchoninic acid assay (BCA assay) (Table 5). Then, the bioactivity of  
245 each bacteriocin was tested at pH 5 (Figure 6) and their minimal inhibitory concentrations are  
246 given in Table 5. After the desalting step, we noted that concentration of bacteriocin ORF030  
247 was the highest one. All these 5 bacteriocins were active against *E. coli* ATCC 8739. Due to its  
248 highest MIC, bacteriocin ORF030 exhibited the lowest specific activity, while bacteriocins  
249 ORF010 and ORF012 exhibited the highest one. Importantly, the assessment of antimicrobial

250 activity with all purified bacteriocins revealed a synergistic effect with an improved activity, as  
251 depicted on Figure 6. Consequently, the combination of these 5 purified bacteriocins at 80  
252 mg/ml each, underpinned a significant total activity of 3,200 AU/ml. Similarly, the combination  
253 of bacteriocins ORF010 and ORF012 also revealed a synergistic interaction (Figure 6).

254

#### 255 **4. Discussion**

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

265 Previously, we have already reported the ability of the multiproducing strain *Lactobacillus*  
266 *paracasei* CNCM I-5369 (now renamed as per new nomenclature of classification *L. paracasei*  
267 CNCM I-5369), to produce five distinct class II bacteriocins [16], with activity against *E. coli*  
268 strains including those exhibiting resistant to colistin, an antibiotic used as a last-line option to  
269 treat infections caused by antibiotic-resistance bacteria such as *Acinetobacter baumannii*,  
270 *Pseudomonas aeruginosa*, *E. coli*, and *Klebsiella* spp [31]. The multi bacteriocin producing  
271 strain expressed activity against *E. coli* only when supernatant was adjusted to pH between 4.5  
272 to 5. Similar results were observed when each bacteriocin was recovered from the insoluble  
273 fraction of the heterologous strain *E. coli* Rosetta. The genes *orf010*, *orf012*, *orf023*, *orf030* and  
274 *orf038* coding for five putative bacteriocins previously identified [32], were expressed after 24

275 hours of growth, from the multi-bacteriocin producing strain *L. paracasei* CNCM I-5369. The  
276 RT-qPCR data reveals the overexpression of the above studied genes. The expression observed  
277 at the end of the logarithmic growth phase, was correlated with the bioactivity, delineating a  
278 possible link between mRNA stability and production of these bacteriocins, as reported for  
279 different regulations systems [20,33,34].

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

284 Purification of bacteriocins from the natural hosts are usually tedious and typically only low  
285 yields are recovered at the end of the purification process [35]. The heterologous production of  
286 bacteriocins is a more suitable approach as genes coding for these bacteriocins can be placed  
287 under inducible promoters and controlled growth conditions allowing the isolation of greater  
288 quantities of bacteriocins [36]. Of note, bacteriocins from Gram-positive bacteria can be  
289 heterologously produced from synthetic DNA leading as well to larger amounts of bacteriocins  
290 than produced by the native host [37].

291 Here, the expression of each gene was studied in *E. coli* Rosetta DE3 and *E. coli* BL21. Overall,  
292 the bacteriocins produced in such a manner have shown a better expression level, when they  
293 were produced in *E. coli* Rosetta (DE3), a strain that harbours a plasmid (pRARE) coding rare  
294 tRNAs. The analysis of each bacteriocin gene revealed the presence of rare codons (AGG,  
295 AGA, CGG, CGA AUA, CUA, CCC, and GGA) which could affect the translation outcome  
296 [38]. *E. coli* Rosetta possess the required tRNAs for the codons AGG, AGA, AUA, CUA, CCC,  
297 GGA and ensures efficient translation of the recombinant bacteriocins [39]. In the purification  
298 process developed here only the bacteriocin encoded by *orf030* gene was obtained in the soluble  
299 fraction, the other ones were recovered from the insoluble fraction. Fahnert et al. [40] reported

300 that overexpression of recombinant proteins can be lethal for the *E. coli* host, yielding to  
301 inactive inclusion bodies associated with the specific folding of these proteins [40]. The  
302 formation of the inclusion bodies may be an advantage for bacteriocin purification as they can  
303 remain protected from the deleterious actions of the host proteases [36]. In addition to these  
304 two hypotheses, expression of these bacteriocins in other hosts like lactic acid bacteria, and  
305 yeasts could be advantageous.

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

312

## 313 5. Conclusions

314 The *in-silico* analysis and expression in the native system of the 5 bacteriocin genes of *L.*  
315 *paracasei* CNCM I-5369 strain was studied. Their expression was successfully conducted  
316 heterologously using *E. coli* as a host system. Each bacteriocin heterologously produced was  
317 purified, characterized by MALDI TOF Mass spectrometry and tested against *E. coli* ATCC  
318 8739. A synergistic activity was observed when these five bacteriocins were tested  
319 concomitantly on this target strain.

320

321 **Author Contributions:** “Conceptualization, DMM, FC, DD; experimental procedures, DMM;  
322 writing—review and editing, DMM, FC DD; supervision FC, DD; funding acquisition, DD. All  
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335

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1

## 2 **FIGURE CAPTIONS**

3 **Figure 1:** *In silico* prediction of promoter regions for each bacteriocin coding gene. The  
4 putative sequences of promoter regions (-10 and -35) are depicted for each bacteriocin gene

5

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

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13 **Figure 3:** Bacteriocins electrophoretic patterns on SDS-PAGE (12 % polyacrylamide). **M:**  
14 Markers of molecular weights, **B:** *E. coli* strain BL21, **R:** *E. coli* strain Rosetta; The arrows  
15 indicated the position of each bacteriocin. The molecular weight of each bacteriocin with His-  
16 Tag are: 8136.10 Da for ORF010, 8 417.59 Da for ORF012, 5035.80 Da for ORF023, 14088.07  
17 Da for ORF030 and 12 230.92 Da for ORF038.

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

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

25 (B) MALDI-TOF spectra of purified bacteriocins, ORF010, ORF012, ORF023, ORF030 and  
26 ORF038. The molecular weight of each bacteriocin with His-Tag are 8149.1 Da for ORF010,  
27 8431.1 Da for ORF012, 5075.2 Da for ORF023, 14119.4 Da for ORF030 and 10281.6 Da for  
28 ORF038.

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30 **Figure 6:** Agar diffusion test on *E. coli* ATCC 8739 using purified bacteriocins produced by  
31 *Escheichia coli* Rosetta strain.

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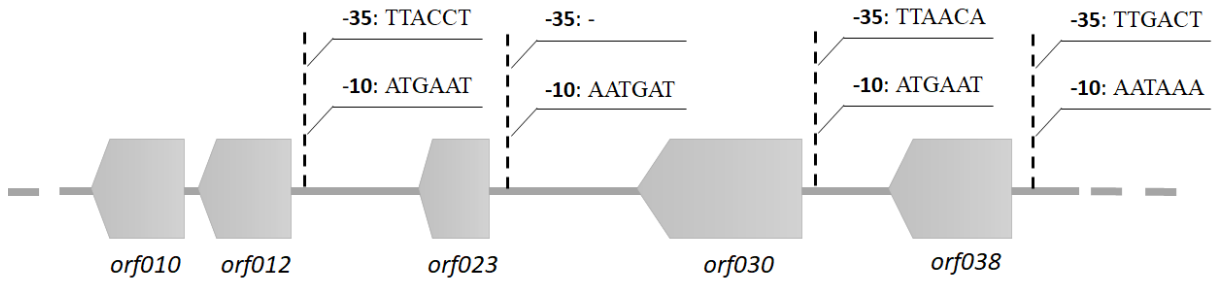
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47 **FIGURES**

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49 **Figure 1**

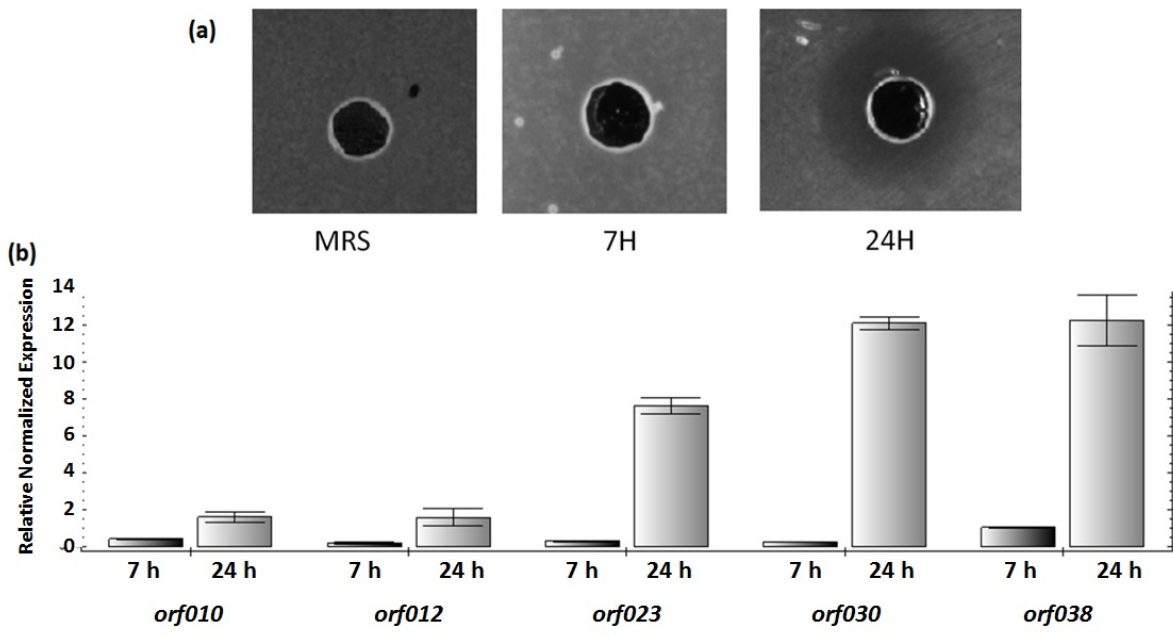


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53 **Figure 2:**



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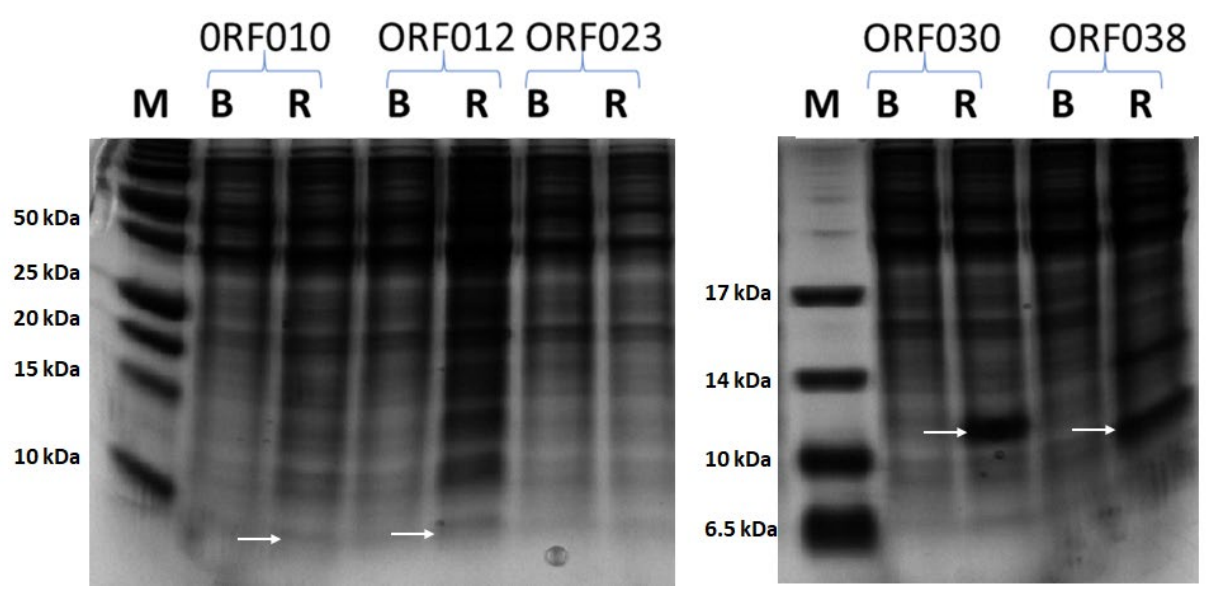
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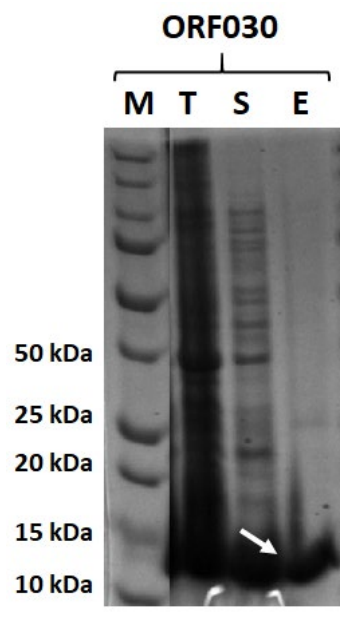
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63 **Figure 3:**



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65 **Figure 4:**



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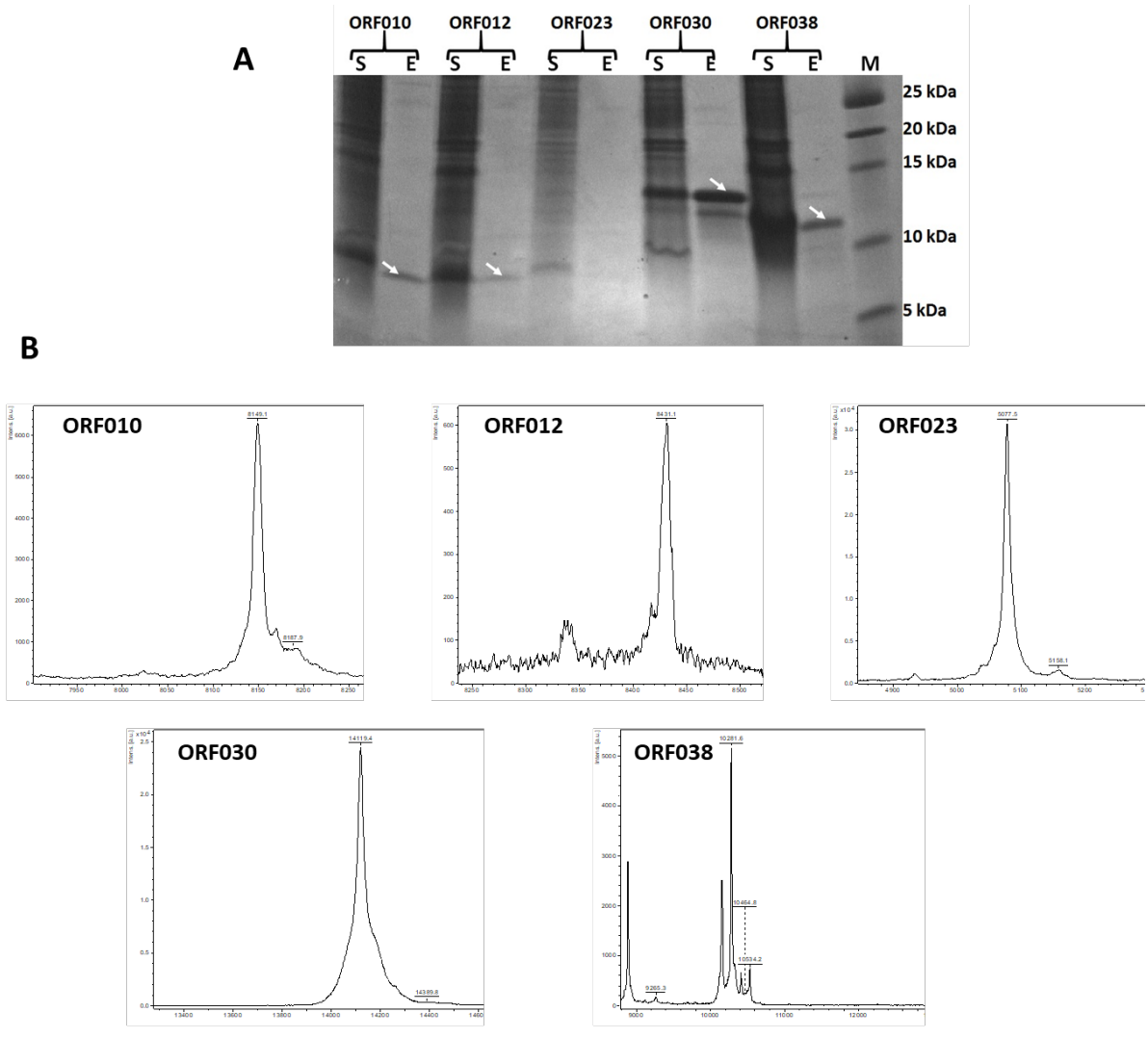
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73 **Figure 5**



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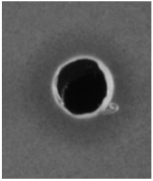
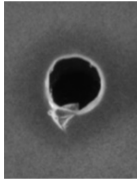
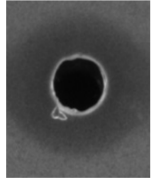
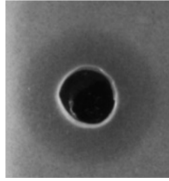
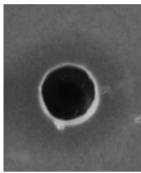
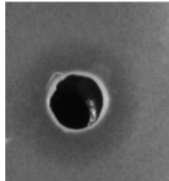
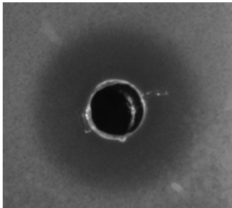
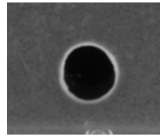
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78 **Figure 6:**



<b>ORF010</b>	<b>ORF012</b>	<b>Mix of ORF010 and ORF012</b>	<b>ORF023</b>
			
<b>ORF030</b>	<b>ORF038</b>	<b>Mix of the 5 bacteriocins</b>	<b>Buffer pH5</b>
			

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## TABLES

**Table 1** Strains and plasmids used in this study

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

**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	GCACCACCAGCAATTTTCATCT	100
<i>orf012</i>	AGCTTGGAAGATTGCTGGT	CAGACTACCAGTAAGCACGC	99
<i>orf023</i>	ACGTAAATTCCTGACAATGCTGA	GCCCTGCCATCCCTTTAAAA	100
<i>orf030</i>	TCCAACGATCAAAGCAGAGC	ACAGCCGTCACATAAGCCTT	99
<i>orf038</i>	CGGCAGGTATTGGATCAGGA	ACCCAACCGCTCCTAAGTTT	99
<i>gyrA</i>	CTTCCACGCATGATGTCCTG	AATCCCTTTTGCGGTTTCGAC	101
<b>16S rRNA</b>	GTAGGTGGCAAGCGTTATCC	GATGCGCTTCCTCGGTTAAG	101

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

<b>Genes</b>	<b>Level of expression</b>	<b>P-Value</b>
<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

**Table 4** Bacteriocins yields obtained before urea treatment

<b>Bacteriocin</b>	<b>Concentration (µg/ml)</b>
<b>ORF010</b>	1.02
<b>ORF012</b>	1.23
<b>ORF023</b>	1.27
<b>ORF030</b>	871.15
<b>ORF038</b>	1.36

**Table 5** Molecular weights, concentration and antibacterial activity of the five bacteriocins after urea treatment

<b>Bacteriocins</b>	<b>Molecular weight<sup>a</sup> (Da)</b>	<b>Concentration (µg/ml)</b>	<b>MIC (µg/ml)</b>	<b>Activity (UA/ml)</b>	<b>Specific activity (UA/µg)</b>
<b>ORF010</b>	8 136.10	21.3	2.66	800	37.55
<b>ORF012</b>	8 417.59	23.4	2.92	800	34.18
<b>ORF023</b>	5035.80	31.7	3.96	800	25.23
<b>ORF030</b>	14 088.07	317.4	39.67	800	2.52
<b>ORF038</b>	12 230.92	44.7	11.17	400	8.94

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