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Expression of the hybrid bacteriocin Ent35-MccV in *Lactococcus lactis* and its use for controlling *Listeria monocytogenes* and *Escherichia coli* in milk

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ABSTRACT

Bacteriocins are antimicrobial peptides synthesised ribosomally. One of the main drawbacks of bacteriocins as food biopreservative agents is their restricted action spectrum. To obtain a broad-spectrum bacteriocin-producing lactic acid bacterium, we engineered a fusion protein between the enterocin P signal peptide and the broad-spectrum chimerical bacteriocin Ent35-MccV. *Lactococcus lactis* NZ9000 transformed with the recombinant plasmid (pRUK) was tested to identify the bacteriocin production by antimicrobial activity and colony MALDI-TOF mass spectrometry. Additionally, we performed mixed cultures in skim milk to determine the inhibition of *Lc. lactis* NZ9000 (pRUK) on both *Listeria monocytogenes* and *Escherichia coli* strains isolated from food. The results showed that *Lc. lactis* NZ900 (pRUK) was able to diminish the growth of both *L. monocytogenes* and *E. coli* in skim milk due to Ent35-MccV production. Ent35-MccV is the first linear, genetically engineered bacteriocin produced in situ in food products used effectively to control Gram-negative and Gram-positive foodborne pathogens.

1. Introduction

Pathogenic bacteria cause an important percentage of foodborne illnesses resulting from consumption of contaminated food (Alegbeleye, Singleton, & Sant'Ana, 2018). Among them, *Escherichia coli* and *Listeria monocytogenes* are responsible for a large number of outbreaks and deaths (WHO, 2015). In this regard, the implementation of appropriate safety instrumented systems in the food industry has decreased the incidence of foodborne illnesses, the application of hurdle technologies being an effective approach to food processing (Leistner, 2000). The aim of this strategy is to increase the shelf life and the microbiological safety of processed foods without affecting their nutritional and organoleptic qualities. For this reason, the use of bacteriocins in combination with different gentle physical treatments has been increasingly described as an adequate alternative in food preservation (Gálvez, Abriouel, Benomar, & Lucas, 2010; Gálvez, Abriouel, López, & Ben Omar, 2007).

Bacteriocins are bacterial antimicrobial peptides synthesised ribosomally (Cotter, Hill, & Ross, 2005), which are capable of inhibiting many foodborne pathogens and food spoilage bacteria. In particular, bacteriocins from lactic acid bacteria (LAB) have been widely studied and considered candidates for use as preservatives in food products (Johnson et al., 2017). Bacteriocins can be used as additives or produced in situ in the food (Alvarez-Sieiro, Montalbán-López, Mu, & Kuipers, 2016; Camargo, Todorov, Chihib, Drider, & Nero, 2018). The in situ production can be performed in several ways: (i) as protective culture in non-fermented food; (ii) as starter culture employing the bacteriocinogenic strain; and (iii) as adjunct with the starter culture. The use of bacteriocin-producing bacteria as starter culture requires these bacteria to carry out the fermentation process and produce enough quantities of bacteriocin to preserve

the food. Instead, if the bacteriocin-producing strain is added as co-culture, its participation in the fermentation process is not required, and the strain should not interfere with the process. In any case, cultures can be used as biopreservative agents inhibiting the growth of undesirable bacteria during the shelf life of foods, LAB being widely used for this purpose in the food industry.

Despite recent advances in food applications of antimicrobial peptides, a major limitation of LAB bacteriocins is their activity primarily against related Gram-positive bacteria. This condition makes these bacteriocins unsuccessful against Gram-negative bacteria such as *E. coli* and/or *Salmonella* spp. Therefore, searching for alternatives to control pathogenic Gram-negative bacteria is necessary (Chalón, Acuña, Morero, Minahk, & Bellomio, 2012). To this end, we constructed a chimeric bacteriocin called Ent35-MccV (Acuña, Picariello, Sesma, Morero, & Bellomio, 2012), where the bacteriocin enterocin CRL35, produced by *Enterococcus mundtii* CRL35, was attached to the linear microcin V, produced by *E. coli*. This hybrid bacteriocin gathers in a single molecule the anti-listerial and anti-*E. coli* activity derived from its parental antimicrobial peptides. This new bacteriocin proved to be active against Gram-positive and Gram-negative emergent foodborne pathogens and spoilage strains and to be capable of controlling *E. coli* an *L. monocytogenes* pathogenic strains in skim milk and hamburgers (Acuña et al., 2015).

Our specific aims were to obtain a strain of *Lactococcus lactis* able to produce the hybrid bacteriocin Ent35-MccV through the extracellular space using a signal peptide (SP) recognised by the general Sec-dependent system (Cintas, Casaus, Håvarstein, Hernández, & Nes, 1997) and to evaluate its use against foodborne pathogens. Our interest in this bacterium lies in the fact that it is a well-known food-grade LAB widely used in the production of some dairy products, where it is

responsible for flavor and textural properties as well as food preservation (Leroy & De Vuyst, 2004; Tarazanova, Huppertz, Kok, & Bachmann, 2018; Topisirovic et al., 2006). Interestingly, *Lc. lactis* is generally recognised as a safe (GRAS) bacterium, which highlights its potential for in vivo delivery of bacteriocins in fermented food and for its use as a host for in situ production (Dal Bello et al., 2012; Renye & Somkuti, 2010). Thereby, we constructed a plasmid vector to produce Ent35-MccV using the enterocin P signal peptide (SPentP) for extracellular expression and taking advantage of the constitutive promoter P32. We transformed a *Lc. lactis* strain to evaluate the heterologous bacteriocin expression and the in situ potential use against foodborne pathogenic bacteria. We confirmed the Ent35-MccV expression by partial purification, antimicrobial activity characterisation, and MALDI-TOF mass spectrometry. Furthermore, using co-culture systems in sterile skim milk as a dairy food model, we found that *Lc. lactis* producing the hybrid bacteriocin inhibited the normal growth of food isolate strains of *E. coli* and *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacteria used in this work are listed in Table 1. The *E. coli* DH5α strain was used for routine DNA transformation and plasmid propagation. *E. coli* strains were grown in Luria Bertani (LB) medium (Sigma Chemical Co., St Louis, USA) and incubated at 37 °C, 150 rpm or on LB with 1.5% agar. *Lc. lactis* NZ9000 was grown in M17 broth (Difco-BD, Franklin Lakes, NJ, USA) with 0.5% glucose (GM17) or LAPTg [peptone, 15 g L^{-1}; tryptone, 10 g L^{-1}; yeast extract, 10 g L^{-1}; glucose, 10 g L^{-1}; Tween

80, 0.1% (v/v; Sigma Chemical Co., St Louis, USA)] at 30 °C without agitation. When required, erythromycin (Sigma Chemical Co) was used at a concentration of 100 µg mL^{-1} for *E. coli* and 5 µg mL^{-1} for *Lc. lactis*. Strains of *L. monocytogenes* used as indicators in the bacteriocin sensitivity assays were grown in BHI medium (Oxoid, Hampshire, England) at 37 °C. All strains were maintained at 4 °C and –70 °C for short and long periods, respectively.

2.2. Vector design and DNA manipulation

We constructed a lactococcal expression vector using polymerase chain reactions (PCR) to fuse the regions of (i) *P32* promoter, (ii) *SPentP* encoding for the signal peptide of enterocin P, and (iii) *munA-cvaC* encoding for Ent35-MccV.

General molecular biology techniques were carried out by standard procedures. *E. coli* plasmids were isolated using a Wizard plasmid DNA purification system (Promega, Madison, WI, USA). AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen) was used for PCR amplifications. The PCR products were purified using a Gel Band Purification Kit (GE Healthcare Life Sciences, Pittsburgh, USA). T4 DNA Ligase was purchased from Promega and restriction endonucleases from New England Biolabs (Ipswich, England).

The primers used are listed in Table 2, and the procedure is shown schematically in Fig. 1. The *P32* promoter was amplified using pMG36e as a template with the primers P32FSal and P32EntSac. DNA coding for enterocin P (a generous gift from Carmen Herranz-Sorribes, Universidad Complutense de Madrid, Spain) was used as the template to amplify the signal peptide of enterocin P. The sequence *munA-cvaC* coding for Ent35-MccV was amplified from pMA24 (Acuña et al., 2012). The PCR

amplifications of all sequences were performed as follows: an initial step at 94 °C for 5 min, continued by 30 cycles of 50 °C for 30 s, 68 °C for 1 min, and 94 °C for 1 min, and finally 1 cycle at 68 °C for 5 min (final elongation step). The resulting genes were purified and used as templates for asymmetric reactions (Kitazono, Tobe, Kalton, Diamant, & Kron, 2002) to fuse P32 with *SPentP* and *munA-cvaC*. The final construction *P32-SPentP-munAcvaC* was performed by a sequential gene fusion as follows: the PCR products of *SPentP* and *munA-cvaC* (1 µL each one in a 50 µL reaction) were used as a template for the first reaction, while EntPSacPlactF and NusColVR as primers at 0.4 µM and the internal primer EntPRmunA at 0.4 nM were introduced to obtain the fusion *SPentP-munAcvaC*. Then, *P32* and *SPentP-munA-cvaC* amplification products were used as templates for the subsequent reaction. P32FSal and NusColVR were used as primers (0.4 µM), and P32EntSac was used as an internal fusion primer (0.4 nM). The program for asymmetric PCRs was as previously reported (Acuña et al., 2012) with some modifications: 94 °C 5 min, followed by 10 cycles (94 °C 1 min, 55 °C 1 min, 68 °C 40 s), 20 cycles (94 °C 1 min, 40 °C 1 min, 68 °C 40 s) and 68 °C 10 min. The *P32-SPentP-munAcvaC* chimeric gene was digested with SalI and HindIII, ligated in pAK80 (Israelsen, Madsen, Vrang, Hansen, & Johansen, 1995), which has two origins of replication, one for *E. coli,* and the other for *Lc. lactis* and transformed into *E. coli* DH5α Δ*sdaC::km* to obtain the plasmid named pRUK. Clones exhibiting erythromycin resistance were screened by PCR to confirm the presence of the chimeric gene. The insert of pRUK was sequenced by CERELA Sequencing Service using an ABI/Hitachi Genetic Analyzer 3130 to confirm its identity.

2.3. Expression and extracellular production of Ent35-MccV in Lc. lactis *NZ9000*

The hybrid bacteriocin Ent35-MccV was purified as previously described (Acuña et al., 2012) and titrated against the *E. coli* ATCC 13706 strain. Ent35-MccV was tested for its antimicrobial activity observing that it was unable to inhibit the growth of *Lc. lactis* NZ9000 in LAPTg agar plates, even at the highest dose tested (500 AU mL^{-1}). So, the recombinant plasmid (pRUK) was used to electro transform *Lc. lactis* NZ9000 (Holo & Nes, 1989). The cross-streaking method was performed to test the antimicrobial activity of the transformed strain. An overnight culture of *Lc. lactis* NZ9000 (pRUK) was centrifuged (10 min 10,000 × g) and diluted in phosphate-buffered saline (PBS) to inoculate a centre streak on a BHI agar plate. The plate was incubated at 30 °C for 24 h, and after growth, the bacteria were killed by exposure to chloroform vapours for 10 min. Then, the plate was cross-streaked against each testing strain from the edge to the centre of the plate and incubated at 37 °C for 24 h. The clear zone of inhibition from the vertical streak to the closest edge of each cross-streaked bacterium was observed.

2.4. Partial purification and identification of Ent35-MccV produced by Lc. lactis *NZ9000*

To test the expression of Ent35-MccV in *Lc. lactis*, antimicrobial assays were performed by a spot-on-lawn method. The strain *Lc. lactis* NZ9000 (pRUK) was cultured at 30 °C for 16 h in LAPTg medium without antibiotics. The culture was centrifuged (10 min for 10,000 × *g*), and the cell-free supernatant was adjusted to pH 7 and precipitated with ammonium sulphate to a final concentration of 70% (w/v). Proteins were harvested by centrifugation (30 min, 20,000 × *g*) and resuspended with PBS. The resuspended pellet was heated (10 min, 100 °C) and centrifuged (10 min,

20,000 × *g*). Finally, to confirm the peptide nature of the inhibitory substance, the supernatant was treated with chymotrypsin (10 IU; Sigma) and incubated at 37 °C for 2 h. Aliquots (20 µL) of each fraction were spotted onto wells of BHI and LB agar plates. After the drops had dried, the plates were overlaid with 8 mL of soft agar (0.6%) containing the sensitive indicator strain *L. monocytogenes* CECT 4032 or *E. coli* ATCC 700728, in BHI or LB plates, respectively. The plates were incubated at 37 °C for 12 h before examination. The supernatant from *Lc. lactis* NZ9000 (pAK80) was used as control.

2.4. MALDI-TOF MS and proteomic analysis of Lc. lactis *Ent35-MccV-producing strain*

Lc. lactis NZ9000 transformed with either pRUK or pAK80 was grown on BHI plates for 24 h supplemented with erythromycin. Then, a 1 µL loop was collected and placed in 100 µL of a solution consisting of 50% acetonitrile (ACN) (Merck, Darmstadt, Germany), 49% water, and 1% aqueous trifluoracetic acid (TFA) (Acros Organics, Morris Plains, NJ, USA). The protein extraction protocol was as described elsewhere (Böhme et al., 2010; Fernández-No et al., 2010), with some modifications. Briefly, three extractions were performed for every strain and all extracts were measured in duplicate, resulting in six spectra per bacterial strain. Mass spectra were obtained using a Voyager "DE STR MALDI-TOF" Mass Spectrometer (Applied Biosystems, Foster City, CA, USA) as previously described (Böhme et al., 2010; Böhme, Fernández-No, Gallardo, Cañas, & Calo-Mata, 2011). Each spectrum was the accumulated sum of 1,000 laser shots, obtained from 10 regions manually selected from each sample in a range of 1500–15,000 Da. After six spectral profiles for the same bacterial strain were obtained,

the mass spectra were analysed with Data Explorer® software (Version 4.0), applying baseline correction and noise filter. Data lists containing *m/z* values with relative intensities higher than 2% were taken from mass spectral data. The acquired peak mass lists were examined and compared using values in the mass range of 2000–10,000 Da due to the reproducibility of the spectral profile in that range. Mass lists were additionally processed with the free web-based application SPECLUST, available at "http://co.bmc.lu.se/speclust/" (Alm et al., 2006). The application permitted calculation of the differences between peak lists and the resolution of common peak masses. This tool was used to analyse the six spectra of each sample, considering the representative peaks present in all six spectra and a peak match score greater than 0.7, corresponding to a measurement error of ± 5 Da. Peak masses were analysed using the Expasy search engine (https://web.expasy.org/findpept/). As we worked with whole bacteria cells to detect soluble cytoplasmic proteins, we considered the presence of the signal peptide before cleavage (SPentP-Ent35-MccV) in our analysis.

2.5. In situ inhibition of food pathogen isolates by Ent35-MccV-producing Lc. lactis

Since *Lc. lactis* NZ9000 (pRUK) could have application as a bioprotective product, co-cultures with the *L. monocytogenes* CECT 4032, Li01, Li02, Li03, Li04, *E. coli* ATCC 700728, D05, D41, D73, and D79 were performed in skim milk. We conducted an experimental trial where each tube was composed by ~10^6 cfu mL^{-1} of the *Lc. lactis* strain (pRUK bacteriocinogenic or pAK80 non-bacteriocinogenic control) and ~10^4 cfu mL^{-1} of one of the pathogenic strains mentioned above. The effect of Ent35-MccV-producing *Lc. lactis* NZ9000 (pRUK) on the pathogenic strains was compared with a non-bacteriocin producing control *Lc. lactis* NZ9000 (pAK80). Experimental

data are shown as means of triplicate measurements (Fig. 5). *Lc. lactis* NZ9000 (pRUK) and NZ9000 (pAK80) cells cultured overnight were separately inoculated in sterile skim milk, where a strain of *L. monocytogenes* or *E. coli* isolated from food was previously seeded. The mixed cultures were incubated at 37 °C for 72 h without agitation, and viable bacteria were measured by cfu counting at 0, 3, 7, 10, 24, 48, and 72 h. Cell counts were performed by serial dilution in physiological saline solution (0.9% NaCl) and enumeration on Palcam *Listeria* agar base (Merck) and Fluorocult Violet Red Bile agar (VRB) (Merck) for *L. monocytogenes* and *E. coli* strains, respectively. Microbial strains isolated from foods and used for evaluation of antimicrobial activities in the co-culture assays are listed in Table 1.

3. Results

3.1. Construction of an expression vector for bacteriocins in Lc. lactis

Lc. lactis is a GRAS bacterium commonly used in the food industry capable of delivering antimicrobial peptides into fermented foods (Dal Bello et al., 2012). The main objective of this study was to obtain a *Lc. lactis* strain able to produce the hybrid bacteriocin Ent35-MccV to promote its biotechnological applications in food preservation. Plasmid pAK80 was used to introduce our genetic construct. However, the vector lacked the promoter that allows the transcription by the RNA polymerase. Thus, a hybrid gene was prepared by asymmetric PCRs (Fig. 1), including the following sequences from the 5' extremity: (i) the constitutive promoter P32 flanked by restriction sites for SalI (5´) and SacI (3´); (ii) the gene portion coding for the secretory signal peptide of enterocin P with its ribosome-binding site for efficient translation and

secretion; and (iii) the hybrid gene of Ent35-MccV (134 amino acid residues) with a restriction site for HindIII (3´). The construction was successfully cloned into pAK80, yielding a new plasmid called pRUK. Fig. 1 shows the complete DNA sequence of the insert and its translation, the construction of the complete vector, DNA fusion reactions, and the electrophoresis gel, which verifies the construction.

3.2. Antimicrobial activity of Ent35-MccV in Lc. lactis

Lc. lactis NZ9000 strain was transformed with pRUK. To verify the secretion and functional expression of the hybrid bacteriocin Ent35-MccV, we first analysed the antimicrobial activity through the cross-streaking method. Fig. 2 shows that *Lc. lactis* NZ9000 (pRUK) suppressed the growth of Ent35-MccV sensitive strains *E. coli* ATCC 700728, MC4100, *L. monocytogenes* CECT 4032, and *Listeria innocua* 7 (Acuña et al., 2015, 2012). No inhibition was observed against the MccV- and enterocin CRL35-producing strains *E. coli* MC4100 (pHK11) and *Ent. mundtii* CRL35, respectively, which are also immune to Ent35-MccV (Acuña et al., 2012) (Fig. 2). In addition, no growth inhibition was observed against the previously reported Ent35-MccV- and MccV-Gram-negative resistant strains (Acuña et al., 2012), bearing mutations in the *cirA*, *tonB*, or *sdaC* genes, nor against the Ent35-MccV- and enterocin CRL35-Gram-positive resistant strains *L. innocua* 4L1Pe, SR 215, and *L. monocytogenes* EGDE (Fig. 2) (Acuña et al., 2012, 2015).

To better assess the presence of Ent35-MccV in the extracellular space, we also measured the antimicrobial activity against the pathogenic strains *E. coli* CECT 4622 and *L. monocytogenes* CECT 4032 (Fig. 3). Cell-free supernatant from a *Lc. lactis* NZ9000 (pAK80) culture was used as a control. Consistent with previous results,

295 inhibition halos were observed only in wells with the neutralised supernatant of NZ9000
296 (pRUK) culture, on both *E. coli* and *L. monocytogenes* indicator strains (Fig 3). To
297 confirm the peptide nature of the compound that produced the inhibition, the
298 supernatant was precipitated with ammonium sulphate to a final concentration of 70%
299 (w/v) and centrifuged. The antimicrobial activity was detected in the pellet after
300 resuspension but not in the supernatant. The resuspended active pellet was heated (10
301 min, 100 °C), and antimicrobial activity against both indicator strains remained. Finally,
302 after hot treatment, we treated the sample with 10 IU of chymotrypsin for 2 h. The
303 protease treatment led to the disappearance of inhibition zones (Fig. 3). Altogether,
304 these results suggest that the transformed strain produces and releases an active
305 antimicrobial peptide against Gram-positive and Gram-negative bacteria, which
306 indicates the presence of Ent35-MccV in the extracellular space.
307
308 *3.3. MALDI-TOF MS- based characterisation of* Lc. lactis *NZ9000 (pRUK)*
309
310 Characterisation of *Lc. lactis* NZ9000 (pAK80) and NZ9000 (pRUK) was
311 performed by MALDI-TOF MS. Six spectra were obtained for each strain. The
312 common peak masses in the spectra were searched using the SPECLUST application.
313 Table 3 shows the mass peak lists for these two strains. The spectral profiles include 19
314 peak masses for *Lc. lactis* NZ9000 (pRUK) and 15 peak masses for the control strain
315 NZ9000 (pAK80). All peaks found in the mass spectrometry profile of the control strain
316 are present in the strain that produces the hybrid bacteriocin. The four peak masses that
317 appear only in the MALDI spectra of NZ9000 (pRUK) are presented in Fig. 3. Peaks
318 were analysed using the Expasy search engine and, interestingly, the four signals can be

assigned to fragments that arise from proteolysis of SPEntP-Ent35-MccV (Table 4), which supports the identification of the bacteriocin.

3.4. In situ inhibition of food pathogen isolates co-cultured with Lc. lactis *expressing Ent35-MccV*

The final purpose of this work was to know whether *Lc. lactis* NZ9000 (pRUK) producing Ent35-MccV could inhibit or decrease the growth of pathogenic bacteria in foods. To address this question, we studied isolated, food-related *E. coli* and *L. monocytogenes* in sterile skim milk co-cultured with *Lc. lactis* NZ9000 (pRUK). *E. coli* ATCC 700728 and *L. monocytogenes* CECT 4032 were used as reference strains. As a control, the same assays were carried out in co-cultivation with *Lc. lactis* NZ9000 (pAK80) strain. Fig. 4 shows the growth rate of all pathogenic strains co-cultured either with Ent35-MccV-producing strain or with the non-producing strain. The viable cell count (cfu mL^{-1}) for both pathogenic strains, *L. monocytogenes* and *E. coli*, was approximately two orders of magnitude lower when they grew up in the presence of NZ9000 (pRUK) than in the presence of NZ9000 (pAK80). The average growth of both *L. monocytogenes* and *E. coli* is represented in Fig. 4A and B, reflecting their inhibition growth under the action of the hybrid bacteriocin.

4. Discussion

The hybrid bacteriocin Ent35-MccV is formed by enterocin CRL35 followed by a hinge of 3 Gly residues and microcin V. Our results indicate that expression and secretion of Ent35-MccV hybrid bacteriocin by *Lc. lactis* can decrease the bacterial load

of Gram-positive and Gram-negative pathogens when co-cultured with the bacteriocin-producing strain. The genetic cassette includes a 5´ P32 constitutive *Lc. lactis* promoter followed by sequences of enterocin P signal peptide and mature Ent35-MccV region. In this way, the secretion of Ent35-MccV was achieved through Sec-system.

Most of the described LAB bacteriocins are secreted by specific ABC transporters or devoted transport systems (McCormick, Klaenhammer, & Stiles, 1999; Pugsley, 1993; van Belkum, Worobo, & Stiles, 1997). Only a few bacteriocins, such as enterocin P, are synthesised with an N-terminal signal peptide recognised by the general Sec-dependent system (Cintas et al., 1997). The similarity in certain N-terminal ends between bacteriocins allows us to believe that their processing and secretion systems could be interchangeable. Different strategies have been employed to improve bacteriocin production, mainly by analysing transporter systems and exchanging leader peptides (Biet, Berjeaud, Worobo, Cenatiempo, & Fremaux, 1998; Martín et al., 2007a; McCormick, Worobo, & Stiles, 1996). In this sense, many researchers have studied the heterologous production of bacteriocins by exchanging the leader sequences with SP recognised by the Sec pathway (Freudl, 2018; McCormick et al., 1996; Worobo et al., 1995). Previous studies on *Lc. lactis* enterocin P high-level heterologous production suggested that fusions between SPentP and other bacteriocins may permit *Lc. lactis* to produce bacteriocins without the presence of specific secretion and immunity proteins (Gutiérrez, Larsen, Cintas, Kok, & Hernández, 2006; Martín et al., 2007a). Consequently, the use of *Lc. lactis* as a producer of antimicrobial peptides has been reported (Arqués, Rodríguez, Gasson, & Horn, 2008; Martín et al., 2007b; Reviriego, Fernández, & Rodríguez, 2007; Ribeiro, O´Connor, Ross, Stanton, & Silva, 2016).

The Ent35-MccV action spectrum against a great number of Gram-negative and Gram-positive bacteria was previously reported (Acuña et al., 2012, 2015). Taking

advantage of this knowledge, we characterised the Ent35-MccV production from *Lc. lactis* by antimicrobial assays against Gram-negative and Gram-positive strains already known as sensitive, immune, or resistant to the hybrid bacteriocin (Acuña et al., 2012, 2015). From these results, we deduced that the transformed strain released a substance with antimicrobial activity compatible with the Ent35-MccV hybrid bacteriocin. Accordingly, the culture supernatant of *Lc. lactis* NZ9000 (pRUK) displayed antimicrobial activity against the Ent35-MccV sensitive *E. coli* ATCC 700728 and *L. monocytogenes* CECT 4032 strains. We also observed the antimicrobial activity against both indicator strains after a partial purification performed by ammonium precipitation and heat treatment. By contrast, the supernatant of the control strain (NZ9000 containing the empty vector) was unable to inhibit any of the strains, supporting the hybrid bacteriocin identification in the *Lc. lactis* NZ9000 (pRUK) derived cell-free supernatant. Thus, we demonstrated the Ent35-MccV release by a naturally resistant *Lc. lactis* strain without the presence of specific immunity proteins. This is in agreement with the fact that both parental bacteriocins of Ent35-MccV were heterologously produced in LAB without requiring its specific immunity gene (McCormick et al., 1999; Saavedra, 2005; van Belkum et al., 1997). However, the possibility of producing Ent35-MccV in larger quantities by including immunity gene, as previously reported for pediocin PA-1, is not totally excluded.

We further characterised the bacteriocin-producing strain by MALDI-TOF-MS. To identify the presence of the hybrid bacteriocin, the obtained MS spectra were compared with those obtained for the control strain. When performing analyses, we found that all unique peak signals corresponding to the producer strain were compatible with fragments of Ent35-MccV. These results together indicate that Ent35-MccV is expressed and transported to the extracellular medium. As previously reported for other

Gram-positive bacteriocins, the enterocin P signal peptide was effective at secreting the hybrid bacteriocin by means of the Sec-dependent secretion system (Herranz & Driessen, 2005; Martín et al., 2007b; Natale, Brüser, & Driessen, 2008). This system seems to be the most versatile and advantageous among heterologous expression systems (Borrero et al., 2011; Freudl, 2018; Zheng & Sonomoto, 2018).

To evaluate the performance of *Lc. lactis* NZ9000 (pRUK), co-cultures with *L. monocytogenes* and *E. coli* strains were carried out in milk. Interestingly, *E. coli* ATCC 700728 and *L. monocytogenes* CECT 4032 significantly decreased the growth rate and bacterial load at 48 h when co-cultured with Ent35-MccV-expressing strain. Our results indicate that continuous production of hybrid bacteriocin in milk controls the presence of pathogenic Gram-positive and Gram-negative microorganisms. The main limitation of the heterologously expressed bacteriocin is based on their industrial use, which could be limited by restrictive legal regulations (Benmechernene et al., 2013). Furthermore, although Ent35-MccV *Lc. lactis* expression is not capable of completely restricting pathogen growth, the bacteriocin is likely to contribute to their total elimination when used in addition to control processes such us osmotic shock, low pH exposure, or Gram-negative lipopolysaccharide shed inducing agents (Ananou, Gálvez, Martínez-Bueno, Maqueda, & Valdivia, 2005; Deegan, Cotter, Hill, & Ross, 2006; Sobrino-López & Martín-Belloso, 2008). However, the presence of an antibiotic resistance gene in a strain that could be used in food preservation is not an ideal feature. On the contrary, the study of bacteriocin production in an in situ system co-cultured with isolated species of contaminated foods reinforces the feasibility of the use of these substances in biopreservation.

To conclude, Ent35-MccV hybrid bacteriocin was successfully expressed and released by GRAS bacteria through a Sec-mediated secretion pathway. Ent35-MccV

was able to diminish the *E. coli* and *L. monocytogenes* growth in skim milk. Thus, these results are promising enough to justify further studies aimed at the application of broad-spectrum bacteriocins like Ent35-MccV and the optimisation of antimicrobial activity and production in dairy products.

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Figure legends

Fig. 1. Genetic constructs for Ent35-MccV production in *Lc. lactis*. Panel A: nucleotide sequence of the 655-bp fragment containing the P32 promoter sequence, the sequence codifying for enterocin P signal peptide and the *munA-cvaC* hybrid gene; amino acid sequences of SPentP-Ent35-MccV are shown below the DNA sequence, the cleavage site of the pre-bacteriocin is indicated by a vertical arrow, the enzyme restriction sites are underlined, the ribosome binding site (RBS) is overlined and the hinge region between *munA* and *cvaC* is in bold and underlined. Panel B: schematic representation of the PCR strategy used to fuse *P32*, *SPentP* and *munA-cvaC*. Panel C: electrophoretic analysis of the fusion gene into pRUK in 1% agarose gel stained with (GelredTM (Biotium); lane 1, DNA step ladder 1 Kb (Promega); lanes 2, 3, 4, and 5 show amplified DNA fragments from *P32*, *P32-SPentP*, *P32-SPentP-munA*, and *P32-SPentP-munA-cvaC*, respectively.

Fig. 2. Cross-streaking assay demonstrating the heterologous production and release of the hybrid bacteriocin Ent35-MccV. *Lc. lactis* NZ9000 (pRUK) strain was streaked across a BHI, glucose 0.6% agar plate. On the left side, *E. coli* strains [(sensitive or resistant to Ent35-MccV and MccV or immune (MccV-producing)] were streaked perpendicular to the NZ9000 (pRUK): a, ATCC 700728; b, MC4100; c, MC4100 (pHK11); d, MC4100 Δ*sdaC::km*; e, MC4100 Δ*tonB::km*; f, MC4100 Δ*cirA::km*. On the right side, Gram-positive strains [(sensitive or resistant to Ent35-MccV and enterocin CRL35 or immune (CRL35-producing)] were streaked: g, *L. monocytogenes* CECT 4622; h, *L. innocua* 7; i, *Enterococcus mundtii* CRL35; j, *L. innocua* 4L1Pe; k, *L. innocua* SR 215; l, *L. monocytogenes* EGDE.

Fig. 3. Antimicrobial activity of extracellular Ent35-MccV produced by *Lc. lactis* as determined by the agar well diffusion test. The sensitive strains *L. monocytogenes* CECT 4032 (left) and *E. coli* ATCC 700728 (right) were used as Gram-positive and Gram-negative indicator microorganisms, respectively: a and b, neutralised cell-free supernatants derived from non-bacteriocinogenic strain *Lc. lactis* NZ9000 (pAK80) and*Lc. lactis* NZ9000 (pRUK), respectively; c and d, supernatant and resuspended pellet, respectively, obtained after protein precipitation of sample b; e, sample c after hot treatment; f, sample d after chymotrypsin treatment. Pure Ent35-MccV was used as a positive antimicrobial control.

Fig. 4. MALDI-TOF MS spectral profile of *Lc. lactis* NZ9000 (pRUK). Specific peaks missing in *Lc. lactis* NZ9000 (pAK80) are indicated by vertical arrows and they are zoomed in the upper panels. The other signals are shown in Table 3.

Fig. 5. In situ growth inhibitory effect of Ent35-MccV in sterile skim milk against food pathogens. Curves show the growth of different strains of *L. monocytogenes* (A) and *E. coli* (B) co-cultured with *Lc. lactis* NZ9000 (pRUK) expressing Ent35-MccV (empty symbols) or *Lc. lactis* NZ9000 (pAK80) as control (filled symbols) Panel A: ●, ○, 4032; ▲, △, Li01; ◆, ◇, Li02; ■, □, Li03; ▼, ▽, Li04. Panel B: ●, ○, 700728; ▲, △, D05; ◆, ◇, D41; ■, □, D73; ▼, ▽, D79. Cell viability was expressed as cfu mL^{-1} as the mean of three independent experiments. The insets show the mean growth ± SEM of all strains evaluated, exposed (empty circle) and not exposed (filled circles) to Ent35-MccV.

Table 1

Bacterial strains used in this study. [a]

Strain	Source, relevant genotype or reference
Gram-positive	
Lc. lactis NZ9000	Kuipers, de Ruyter, Kleerebezem, & de Vos (1998)
Ent. mundtii CRL35	CERELA; (Farías, Farías, de Ruiz Holgado, & Sesma (1996)
L. innocua 7	INRA
L. innocua 4L1Pe	INRA
L. innocua SR 215	IHT
L. monocytogenes EGDE	ATCC: BAA-679
L. monocytogenes 4032	CECT: 4032 – CI ; Bannister (1987)
L. monocytogenes Li01	LHICA - FI
L. monocytogenes Li02	LHICA – FI
L. monocytogenes Li03	LHICA – FI
L. monocytogenes Li04	LHICA – FI
Gram-negative	
E. coli DH5α	Invitrogen
E. coli O157:H7	ATCC: 700728
E. coli MC4100	CGSC
E. coli MC4100 (pHK11)	Gilson, Mahanty, & Kolter (1987)
E. coli LA1	FBQF; MC4100 Δ*sdaC*::*km*; (Acuña et al. (2012)
E. coli LA2	FBQF; MC4100 Δ*cirA*::*km*; Acuña et al. (2012)
E. coli NC1	FBQF; MC4100 Δ*tonB*::*km*; Acuña et al. (2012)
E. coli D05	LHICA-FI
E. coli D41	LHICA-FI
E. coli D73	LHICA-FI
E. coli D79	LHICA-FI

[a] Abbreviations are: CERELA, Centro de Referencias para Lactobacillos, Tucumán, Argentina; INRA, Jouy-en-Josas, France; IHT, Institute of Hygiene and Toxicology, Karlsruhe, Germany; ATCC, American Type Culture Collection; CGSC, E. coli Genetic Stock Center; FBQF, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina; LHICA, Laboratorio de Higiene, Inspección y Control de Alimentos, USC, Spain; CI, clinical isolate; FI, food isolate.

Table 2

Synthetic oligonucleotides used in this study. [a]

Primer	Specificity and direction	Nucleotide sequence (5'→ 3')	Sites inserted
NusColVR	*cvaC* (R)	GGTGGTAAGCTTTTATAAACAAACATCACTAAG	*Hind*III
P32FSal	*P32* (F)	GGTGGTGTCGACATAGTTTTAGCTATTAATCTTTTT	*Sal*I
P32EntSacR	P32 (R)	**ACCTCCTTGAGCTCC**CGAATATTTTTTTACCTACCTAG	*Sac*I / fusion fragment with EntPSacPlactF
EntPSacPlactF	*SPentP* (F)	**GGAGCTCAAGGAGGT**ATTGATTTATGAGAAAAAAATTATTTAGTTTAGC	*Sac*I / fusion fragment with P32EntSacR
EntPRMunA	*SPentP* (R)	**CCGTAGTATTTTGCATCAAC**TTTTGTACCAAAATTTG	Fusion fragment with MunAFEntP
MunAFEntP	*munA* (F)	**GTTGATGCAAAATACTACGG**TAATGGAGTCTC	Fusion fragment with EntPRMunA

[a] F and R in parentheses indicate the forward (F) or reverse (R) amplification direction; the underlined sequences are the restriction sites; the sequences in bold represent the fusion and overlapping fragments of complementary genetic sequences with those from another primer.

Table 3

Characteristic peak masses of transformed *Lc. lactis* NZ9000. [a]

pAK80		pRUK	
Mass (average)	*m/z* (std)	Mass (average)	*m/z* (std)
2037.98	1.47	2038.23	0.73
2075.41	1.17	2075.98	0.65
2108.36	1.66	2107.86	1.00
2124.98	1.05	2124.31	0.91
2210.45	0.24	2210.90	0.19
2224.22	1.20	2224.20	0.53
2649.42	1.18	2648.77	0.92
		3092.39	0.61
3225.59	1.03	3224.13	1.19
		3520.72	1.69
3868.24	0.84	3867.25	0.49
3906.33	0.63	3905.64	1.03
3930.29	0.08	3928.83	2.09
		4118.69	1.49
4421.37	0.95	4419.77	0.62
4459.67	1.09	4457.76	1.4
5983.37	1.43	5983.34	2.03
		6060.04	0.93
8425.43	0.35	8422.61	1.99

[a] Peak masses are presented as $[M+H]^+$ values; specific peaks are highlighted in bold.

Table 4

MALDI-TOF MS assignment of SPentP-Ent35-MccV proteolytic fragments for specific peaks of *Lc. lactis* NZ9000 (pRUK). [a]

Measured *m/z*	Expected *m/z*	Δ mass (daltons)	Assignment	Position
3092.39	3092.47	0.083	(G)NGVSCNKKGCSVDWGRAIGIIGNNSAANLAT(G)	32-62 (Ent35)
	3092.47	0.083	(N)GVSCNKKGCSVDWGRAIGIIGNNSAANLATGG(A)	33-64 (Ent35)
3520.70	3520.94	0.244	(S)AANLATGGAAGWKSGGGASGRDIAMAIGTLSGQFVAGGI(G)	57-95 (Ent35-MccV)
4118.69	4118.57	-0.117	(A)ANLATGGAAGWKSGGGASGRDIAMAIGTLSGQFVAGGIGAAAGGVAGG(A)	58-105 (Ent35-MccV)
	4118.57	-0.117	(A)NLATGGAAGWKSGGGASGRDIAMAIGTLSGQFVAGGIGAAAGGVAGGA(G)	59-106 (Ent35-MccV)
	4118.61	-0.073	(T)GGAAGWKSGGGASGRDIAMAIGTLSGQFVAGGIGAAAGGVAGGAGTIK(Q)	63-110 (Ent35-MccV)
	4118.63	-0.058	(G)TKVDAKYYGNGVSCNKKGCSVDWGRAIGIIGNNSAANLAT(G)	23-62 (SP-Ent35)
6060.04	6059.81	-0.232	(A)SGRDIAMAIGTLSGQFVAGGIGAAAGGVAGGAGTIKQKPEGIPSEAWNYAAGRLCNWSPNNL(S)	75-136 (Ent35-MccV)
	6059.81	-0.232	(S)GRDIAMAIGTLSGQFVAGGIGAAAGGVAGGAGTIKQKPEGIPSEAWNYAAGRLCNWSPNNLS(D)	76-137 (Ent35-MccV)

[a] The expected *m/z* of the SP-Ent35-MccV fragments were calculated using average masses of the occurring amino acid residues, interpreting peptide masses as $[M + H]^+$, and considering Cys in reduced form. Peptides are displayed with the adjoining residues before cleavage in parentheses.

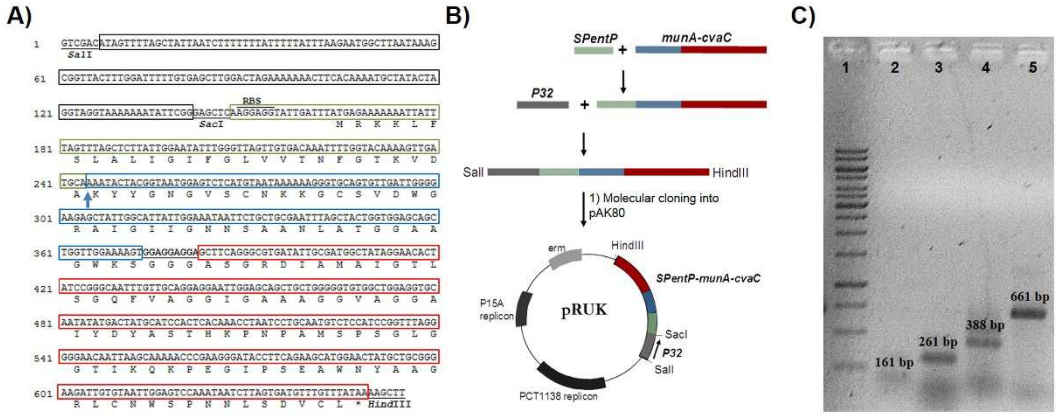

Figure 1

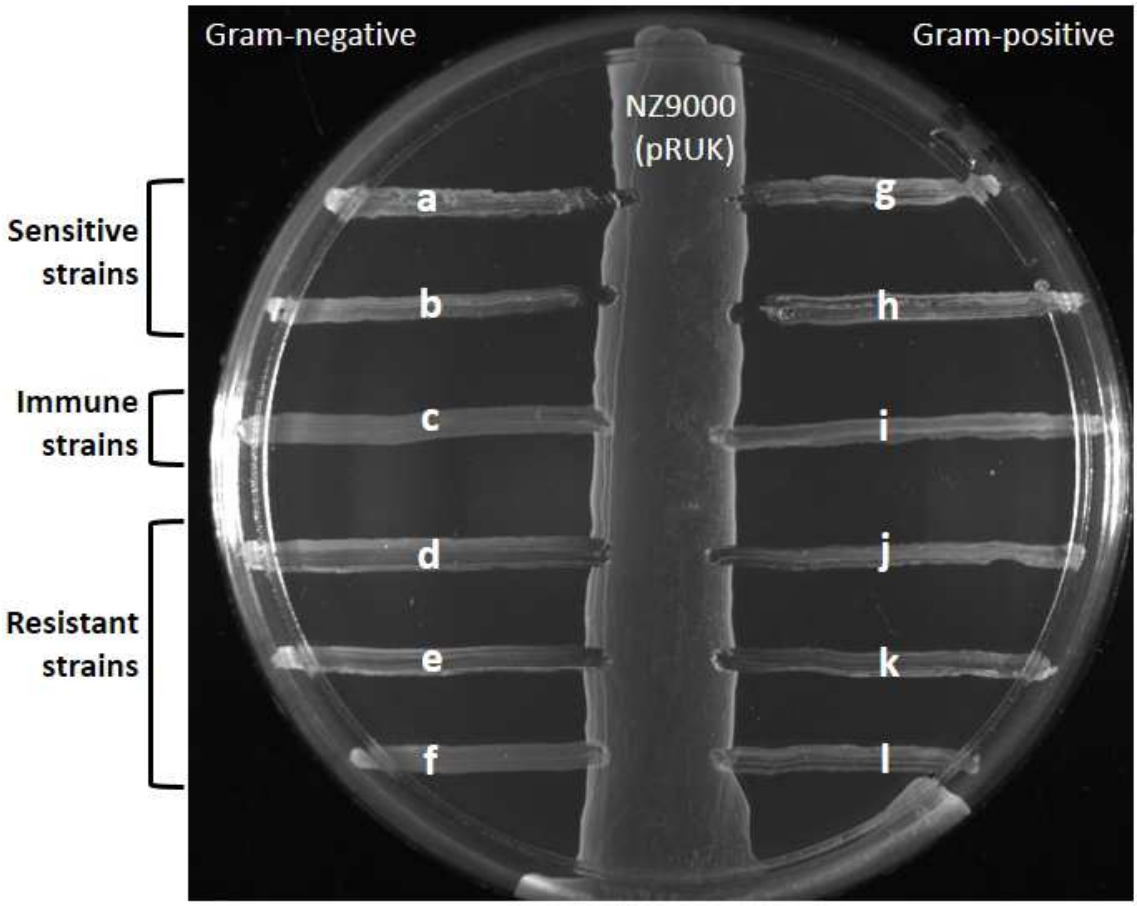

Figure 2

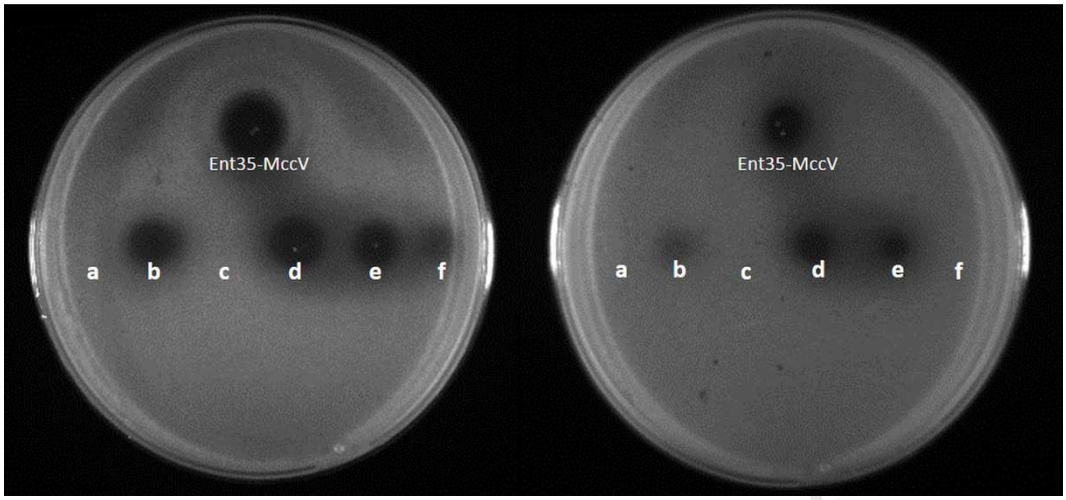

Figure 3

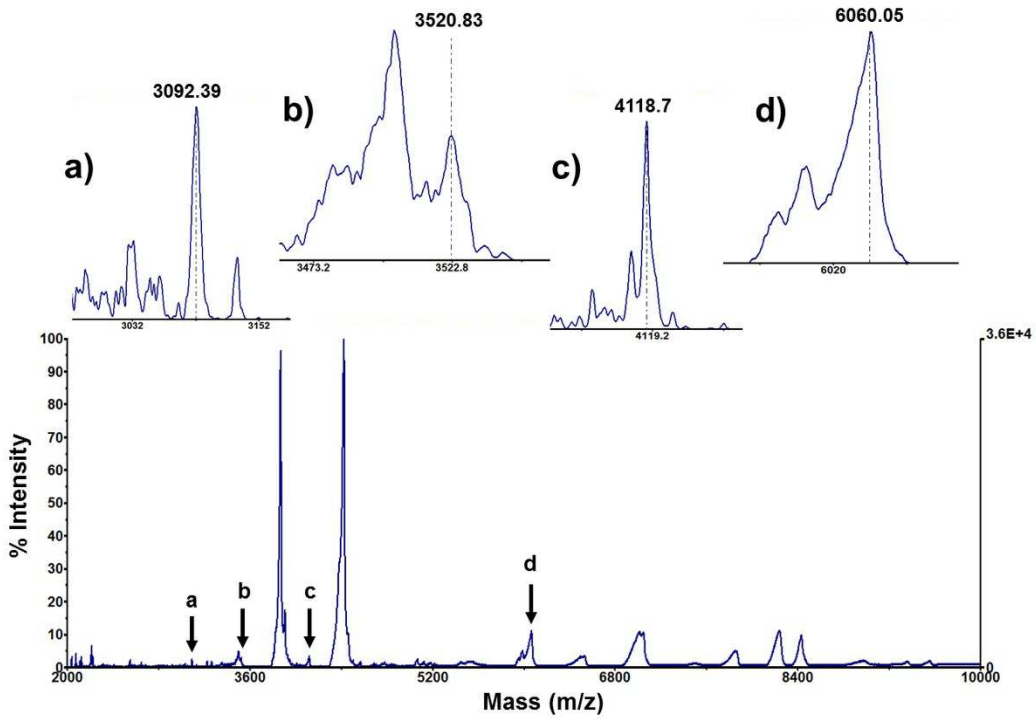

Figure 4

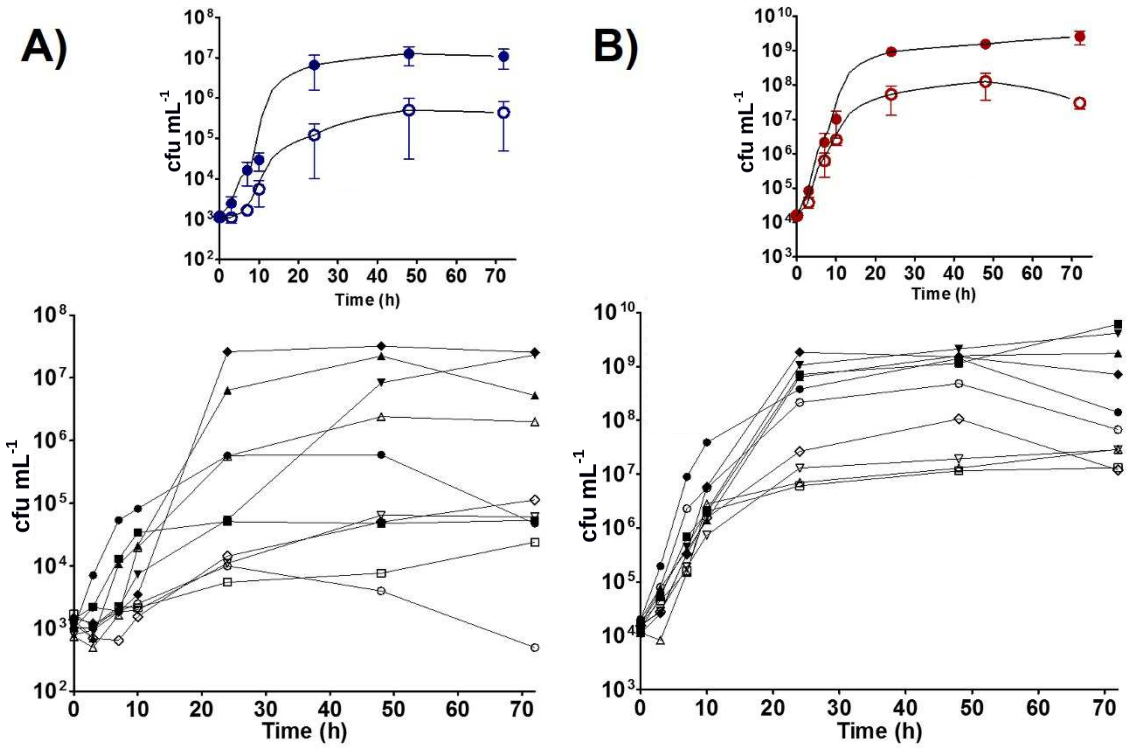

Figure 5