World J Microbiol Biotechnol (2015) 31:929–940
DOI 10.1007/s11274-015-1847-9

ORIGINAL PAPER

Bacillus subtilis subsp. *subtilis* CBMDC3f with antimicrobial activity against Gram-positive foodborne pathogenic bacteria: UV-MALDI-TOF MS analysis of its bioactive compounds

M. J. Torres[1] · G. Petroselli[2] · M. Daz[1] · R. Erra-Balsells[2] · M. C. Audisio[1]

Received: 18 December 2014 / Accepted: 21 March 2015 / Published online: 29 March 2015
© Springer Science+Business Media Dordrecht 2015

Abstract In this work a new *Bacillus* sp. strain, isolated from honey, was characterized phylogenetically. Its antibacterial activity against three relevant foodborne pathogenic bacteria was studied; the main bioactive metabolites were analyzed using ultraviolet matrix assisted laser desorption-ionization mass spectrometry (UV–MALDI MS). *Bacillus* CBMDC3f was phylogenetically characterized as *Bacillus subtilis* subsp. *subtilis* after rRNA analysis of the 16S subunit and the *gyrA* gene (access codes Genbank JX120508 and JX120516, respectively). Its antibacterial potential was evaluated against *Listeria monocytogenes* (9 strains), *B. cereus* (3 strains) and *Staphylococcus aureus* ATCC29213. Its cell suspension and cell-free supernatant (CFS) exerted significant anti-*Listeria* and anti-*S. aureus* activities, while the lipopeptides fraction (LF) also showed anti-*B. cereus* effect. The UV-MALDI-MS analysis revealed surfactin, iturin and fengycin in the CFS, whereas surfactin predominated in the LF. The CFS from CBMDC3f contained surfactin, iturin and fengycin with four, two and four homologues per family, respectively, whereas four surfactin, one iturin and one fengycin homologues were identified in the LF. For some surfactin homologues, their UV-MALDI-TOF/TOF (MS/MS; Laser Induced Decomposition method, LID) spectra were also obtained. Mass spectrometry analysis contributed with relevant information about the type of lipopeptides that *Bacillus* strains can synthesize. From our results, surfactin would be the main metabolite responsible for the antibacterial effect.

Keywords *Bacillus subtilis* subsp. *subtilis* · Foodborne pathogens · Lipopeptide homologues · UV-MALDI-TOF MS · Surfactin

Introduction

In recent decades, the demand for safer and higher-quality food products has increased. Food products contaminated with pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Bacillus cereus*, may generate severe infectious diseases (Foster 2005; Gandhi and Chikindas 2007; Bhunia 2008). To preserve and prolong the shelf life of food products, the most common method is the use of food additives or preservatives, which are mainly harmful chemicals (Pandey et al. 2014). For this reason, natural alternatives are becoming important, and research done in that sense will cooperate with both human health and the food industry.

Several *Bacillus* sp. have received considerable attention as biological control agents in the food industry because they are generally recognized as safe (GRAS) (Pedersen et al. 2002; Romero et al. 2007). Among the studied strains are *B. subtilis*, *B. licheniformis*, *B. mojavensis* and *B. amyloliquefaciens* (Gomaa 2013; Kaewklom et al. 2013; Ayed et al. 2014; Khochamit et al. 2015). In particular, *B. subtilis* is the

Electronic supplementary material The online version of this article (doi:10.1007/s11274-015-1847-9) contains supplementary material, which is available to authorized users.

✉ M. C. Audisio
audisio@unsa.edu.ar

[1] Instituto de Investigaciones para la Industria Química (INIQUI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Salta, Av.Bolivia 5150, 4400 Salta, Argentina

[2] CIHIDECAR-CONICET, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, 3 Ciudad Universitaria, 1428 Buenos Aires, Argentina

species that has received most attention; thus, three subspecies have been identified: *subtilis, spizizenii* and *inaquosorum* (Nakamura et al. 1999; Rooney et al. 2009; Freyre-González et al. 2013). Interestingly, each subspecies presents different biological properties due to differences in the synthesis of metabolites (Burkard et al. 2007). For example, it has been reported that *B. subtilis* subsp *subtilis* strains can synthesize either, the lipopeptide surfactin with anti-*Listeria* effects (Sabaté and Audisio 2013) or surfactin plus a novel bacteriocin with antibacterial properties (Compaoré et al. 2013). Meanwhile, a *B. subtilis* subsp *spizizenii* strain produces entianin, a lantibiotic with inhibitory activity against *Haemophilus parasuis* and *S. aureus* (Fuchs et al. 2011; Teixeira et al. 2013).

Lipopeptides like surfactins, fengycins, iturins and bacitracins are some of the compounds with antimicrobial activity synthetized by *B. subtilis* strains. They present several advantages over chemically synthesized products: they have low cost, do not pollute the environment and present no problems of waste management (Ongena and Jacques 2008). In natural habitats, the lipopeptides have been proposed to confer a competitive advantage in interactions with other microorganisms. Surfactins are among the most powerful biosurfactants and they exert antibacterial, antiviral and antimycoplasm activities (Seydlova and Svobodova 2008; Savadogo et al. 2011); in turn, fengycins and iturins are known mainly for their strong antifungal properties (Hiradate et al. 2002; Patel et al. 2011). Even though there are several scientific articles about the peptides and lipopeptides with antimicrobial properties synthesized by different *B. subtilis* strains, the phylogenetic characterization of those strains were only done up to the *Bacillus* species level (Lee et al. 2010; Thasana et al. 2010; Khochamit et al. 2015).

In our group, we have isolated *Bacillus* sp. strains form honey samples and from the *Apis mellifera* bee gut; some of these strains were studied due their antimicrobial properties against honeybee pathogens (Sabaté et al. 2009) and *Listeria* strains (Sabaté and Audisio 2013). In that previous work, the chemical nature of the antimicrobial compounds was investigated mainly by HPLC analyses. In the present study, we have phylogenetically characterized a new *Bacillus* sp. strain, isolated from honey in the northwest of Argentina (NOA). Its antibacterial potential has been evaluated against three relevant Gram-positive, foodborne pathogenic bacteria. The main lipopeptides involved in the biological action have been characterized using ultraviolet matrix assisted laser desorption-ionization mass spectrometry (UV–MALDI MS). This is an innovative technique that has reached relevance in the last decade because can help with the identification of different compounds with biological activity (Vater et al. 2002; Pathak and Keharia 2014).

Materials and methods

Bacterial strains and growth conditions

Bacillus CBMDC3f sp. was isolated from a honey sample in the province of Salta (Argentina). It was grown in Mueller–Hinton broth (MH, Britania, Argentina) at 37 °C and was preserved as frozen stock in MH broth plus 10 % (v/v) glycerol at −20 °C.

The strains described in Table 1 were used as indicator microorganisms. They were grown in brain–heart infusion broth (BHI, Britania, Argentina) at 37 °C without a special atmosphere. All indicator strains were kept at −20 °C in BHI broth with glycerol (10 % v/v).

Phylogenetic characterization of *Bacillus* CBMDC3f sp

DNA extraction was carried out with an active culture after incubation in 5 mL of brain–heart infusion broth (BHI, Britania, Argentina) at 37 °C for 24 h according to Miller (1972). The isolate was genetically characterized by both, the analysis of the subunit 16S of rRNA and the *B. subtilis*

Table 1 Antibacterial activity of *B. subtilis* subsp. *subtilis* CBMDC3f against Gram-positive indicator strains

Strain	Origin	Antibacterial activity[d]		
		CS	CFS	LF
Listeria monocytogenes				
99/287s	INEI–ANLIS[a]	+++	+++	+++
99/267	INEI–ANLIS	+++	+++	+++
01/155	INEI–ANLIS	+++	+++	+++
00/110	INEI–ANLIS	+++	+++	−
99/320	INEI–ANLIS	++	+++	+++
00-3/364	INEI–ANLIS	++	++	+++
99/373	INEI–ANLIS	++	+++	+++
01/198	INEI–ANLIS	++	++	+++
01/200	INEI–ANLIS	+++	+++	−
Staphylococcus aureus				
29213	ATCC[b]	++	+++	+++
Bacillus cereus				
MBC1	INEI–ANLIS	−	−	++
MBC7	INEI–ANLIS	−	−	+
BAC1	MM[c]	−	−	+

[a] *INEI–ANLIS* Instituto de Microbiología "Dr. Carlos G. Malbrán" (Bs. As., Argentina)

[b] *ATCC* American Type Culture Collection

[c] *MM* Dr. María Morea (Italy)

[d] Inhibition halo

+ weak inhibition (<1.2 cm), ++ moderate inhibition (1.2–1.8 cm), +++ strong inhibition (>1.8 cm), − without inhibition

gyrA gene fragments. In the first case, the nucleotide single universal strand primers S-D-Bact-0008-a-S-20 (AGAGT TTGATCCTGGCTCAG) and S-D-Bact-1495-a-A-20 (CT ACGGCTACCTTGTTACGA) were used (Daffonchio et al. 1998); and for the *gyrA* gene evaluation, *gyrA*-f (5-CAGTCA GGAAATGCGTACGTCCTT-3) and *gyrA*-r (5-CAAGGT AATGCTCCAGGCATTGCT-3) primers were employed (de Clerck et al. 2004). The extracted genomic DNA, for the analysis of the subunit 16S of rRNA and the *gyrA* gene, was amplified according to Sabaté et al. (2009) and Sabaté and Audisio (2013), respectively. Gel patterns were visualized with UV by staining with "SYBR safe DNA gel stain (INVITROGEN)". On-line search for similarity was carried out at GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov).

Lipopeptides fraction recovery

The lipopeptides synthesized by *Bacillus* CBMDC3f sp. were obtained by acidic precipitation from the cell free supernatant and a subsequent recovery with methanol according to Sabaté et al. (2009). The solvent was then evaporated and the precipitate, containing different lipopeptides, was dissolved in sterile distilled water (pH 9) to obtain two samples (0.5 and 2.0 mg/mL) which were called the lipopeptides fraction (LF).

Antibacterial activity of CBMDC3f by the well diffusion assay

The inhibitory effect of *Bacillus* CBMDC3f cell suspension (CS), cell-free supernatant (CFS) and LF on the different indicator microorganisms were studied using the well diffusion assay as described by Audisio et al. (2005). To obtain CS, 1 % (v/v) Mueller–Hinton broth (MH) was inoculated with a pure culture of *Bacillus* CBMDC3f and incubated for 24 h at 37 °C. After this period, concentrations of about 1×10^7 cells per mL and 1×10^5 spores per mL were prepared. CFS was obtained by centrifugation (10,000 *g* for 10 min at 4 °C) which was filter-sterilized (0.22 µm pore-size cellulose acetate membranes). After that, 22 µL of either, CS, CFS or LF were sieved in 5 mm wells made in BHI agar plates inoculated with ca 10^7 CFU/mL of the indicator strain. Plates were incubated at 30 °C for 12–24 h and examined for inhibition halos.

The minimal inhibitory concentration (MIC) of LF from *Bacillus* CBMDC3f sp. against seven *L. monocytogenes* strains (99/287s, 01/198, 99/320, 01/155, 00-3/364, 99/373, 99/267), *S. aureus* ATCC29213 and *B. cereus* MBC1 was also evaluated.

Study of the indicator strains viability by the microplate direct contact

The following indicator strains were chosen to evaluate the effect of the CFS and the LF of *Bacillus* CBMDC3f sp. on their viable cell numbers: *L. monocytogenes* 99/287, 01/155 and 00-3/364, *S. aureus* ATCC29213, *B. cereus* MBC1, *B. cereus* MBC7 and *B. cereus* BAC1. Cells from overnight cultures grown in BHI broth were diluted in peptone water in order to obtain a suspension ca. 10^5 CFU/mL. The effect of the CFS and two concentrations of the LF (0.5 and 2.0 mg/mL) of CBMDC3f on each indicator strain were compared with two controls: sterile MH broth and distilled water at pH 9. For these studies, ninety-six-well microplates were used: the different samples were put in direct contact with the indicator strain suspensions at a 1/10 ratio and incubated at 37 °C. At prefixed times of 0, 1, 2, 4 and 24 h, an aliquot of each mixture was taken and viable cells were determined by plating on BHI (1.5 %, w/v) agar; the plates were incubated at 37 °C for 24 h (Audisio et al. 2005). The zero time (t_0) corresponds to the moment just after adding the tested solutions to the cells of the indicator strains. The assays were carried out in triplicate and the statistical analyses of the data were done according to analysis of variance (ANOVA) using InfoStat statistical software. Every contact time of the indicator pathogenic strains with the different treatments (BHI, H_2O at pH 9, CFS, and 0.5 and 2 mg/mL LF) was indicated as mean ± standard deviation (SD).

UV-MALDI-MS analysis

Materials

HPLC grade acetonitrile (J. T. Baker, USA) was used without further purification. Norharmane (nHo: 9H-pirido[3,4-b]indol) and β-cyclodextrin (Cyclomaltoheptaose) were purchased from Sigma-Aldrich Chemical Co., USA. Water of very low conductivity (Milli Q grade) was used.

Analysis

Both, the CFS and the LF of CBMDC3f were analyzed by ultraviolet matrix assisted laser desorption-ionization mass spectrometry (UV–MALDI MS) performed on the Bruker Ultraflex Daltonics Time-of-Flight/Time-of-Flight (TOF/TOF) mass spectrometer (Leipzig, Germany). Mass spectra were acquired in linear positive and negative ion modes and with the LIFT device in the MS/MS mode [fragmentation method: Laser Induced Decomposition, (LID)]. Stock solutions of samples were prepared in water at pH = 8. External mass calibration was made using β-cyclodextrin (MW 1134) with nHo as matrix in positive and negative ion mode. The matrix signal was used as an additional standard for calibration in both ion modes. Sample solutions were spotted on a MTP 384 target plate polished steel from Bruker Daltonics (Leipzig, Germany). For UV-

MALDI-MS matrix solutions were prepared by dissolving nHo in acetonitrile/water (1:1, v/v) solution. For UV-MALDI-MS experiments dry droplet sample preparation (sandwich method) was used according to Nonami et al. (1997), loading successively 0.5 µl of matrix solution, analyte solution and matrix solution after drying each layer at normal atmosphere and room temperature. The matrix to analyte ratio was 3:1 (v/v) and the matrix and analyte solution loading sequence was: (i) matrix, (ii) analyte, (iii) matrix, (iv) matrix. Desorption/Ionization was obtained by using the frequency-tripled Nd:YAG laser (355 nm). Experiments were performed using firstly the full range setting for laser firing position in order to select the optimal position for data collection, and secondly fixing the laser firing position in the sample sweet spots. The laser power was adjusted to obtain high signal-to-noise ratio (S/N) while ensuring minimal fragmentation of the parent ions and each mass spectrum was generated by averaging 100 lasers pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively.

Results

Phylogenetic characterization

According to the BLAST analysis, the rRNA of the 16S subunit revealed that the strain presents 100 % homology with known *Bacillus subtilis* strains such as *B. subtilis* BCRC 17435, taken from the GenBank sequence library. The complete characterization of CBMDC3f was achieved by analysis of the gyrase DNA sequence. According to this study, *B. subtilis* CBMDC3f presented 98 % homology with the gyrase DNA corresponding to *B. subtilis* subsp. *subtilis* At2 from the GenBank database. Both nucleotide sequences were deposited at GenBank under the following accession numbers: JX120508 and JX120516, respectively. The genetic homology of *B. subtilis* subsp. *subtilis* CBMDC3f with related species can be observed in the phylogenetic tree constructed using the neighbor-joining method (Fig. 1).

Antibacterial activity of CBMDC3f

Using the agar diffusion assay, the antibacterial effects of the CS and CFS from *B. subtilis* subsp. *subtilis* CBMDC3f revealed that none of the *B. cereus* strains was sensitive to them. However, both the CS and the CFS of CBMDC3f had an important biologic activity against *L. monocytogenes* and *S. aureus* ATCC29213 (Table 1). The LF also exhibited a strong inhibitory activity against the *Listeria* strains assayed, with the exception of strains 00/110 and 01/200, and against *S. aureus* ATCC29213, whereas a weak inhibition was determined against the *B. cereus* strains (Table 1).

It was also determined that the minimal inhibitory concentration (MIC) of the LF depended on the indicator strain under study. It was equal to 0.5 mg/mL against five different *L. monocytogenes* strains (99/287s, 01/198, 99/320, 01/155 and 00-3/364), and 0.1 mg/mL against *L. monocytogenes* 99/373 and 99/267. However, greater concentrations were necessary to inhibit *B. cereus* MBC1 (1 mg/mL) and *S. aureus* ATCC29213 (2 mg/mL).

Effect of the metabolites detected in the CFS and the LF on the viable-cell number of different pathogenic bacteria

Listeria monocytogenes 00-3/364 was the most sensitive of all the *Listeria* strains studied: the LF, at a concentration of 2.0 mg/mL, generated a reduction in viability of 2 log orders at t_0. In turn, both the CFS and the LF, at a concentration of 0.5 mg/mL, exerted a similar inhibitory action against this indicator strain: after 2 h of contact with the *L. monocytogenes* 00-3/364 cells, their viability diminished rapidly, reaching a reduction of 5 log orders after 24 h. Finally, a LF concentration of 2.0 mg/mL showed a reduction of 6 log orders after 24 h (Fig. 2a). Meanwhile, *L. monocytogenes* 01/155 was sensitive to both the CFS and the LF, the latter at 2.0 mg/mL: in both cases, the viability of the listeria cells decreased one log order at time t_0. The CFS caused a further reduction of 2 log orders, as compared with the control, after 24 h of contact. The lipopeptides concentration was key to the biological effect observed: at a concentration of 0.5 mg/mL, the LF showed a significant anti-*Listeria* effect after the first 4 h of contact, whereas a lipopeptides concentration of 2.0 mg/mL showed a strong inhibitory effect after only 1 h of contact, reducing the viability of *L. monocytogenes* 01/155 in 6 log orders after 24 h (see Fig. S1a in supplementary data). *L. monocytogenes* 99/287s cells also revealed a response similar to that of the 01/155 strain against both the CFS and the LF (data no shown).

On the other hand, the number of viable cells of *S. aureus* ATCC29213 was reduced 2 orders in the presence of 2.0 mg/mL of the LF just at the beginning of the assay, effect that was maintained for 1 h, and after 24 h a decrease of 5 log orders was registered. When the LF was tested at a concentration of 0.5 mg/mL, an inhibitory action was also measured, showing 3 log orders less in the number of viable cells after 4 h of contact, situation that kept without changes until the end of the assay. The CFS also produced a decrease in the *S. aureus* ATCC29213 viability, but this effect became only significant after 24 h of contact (see Fig. 2b).

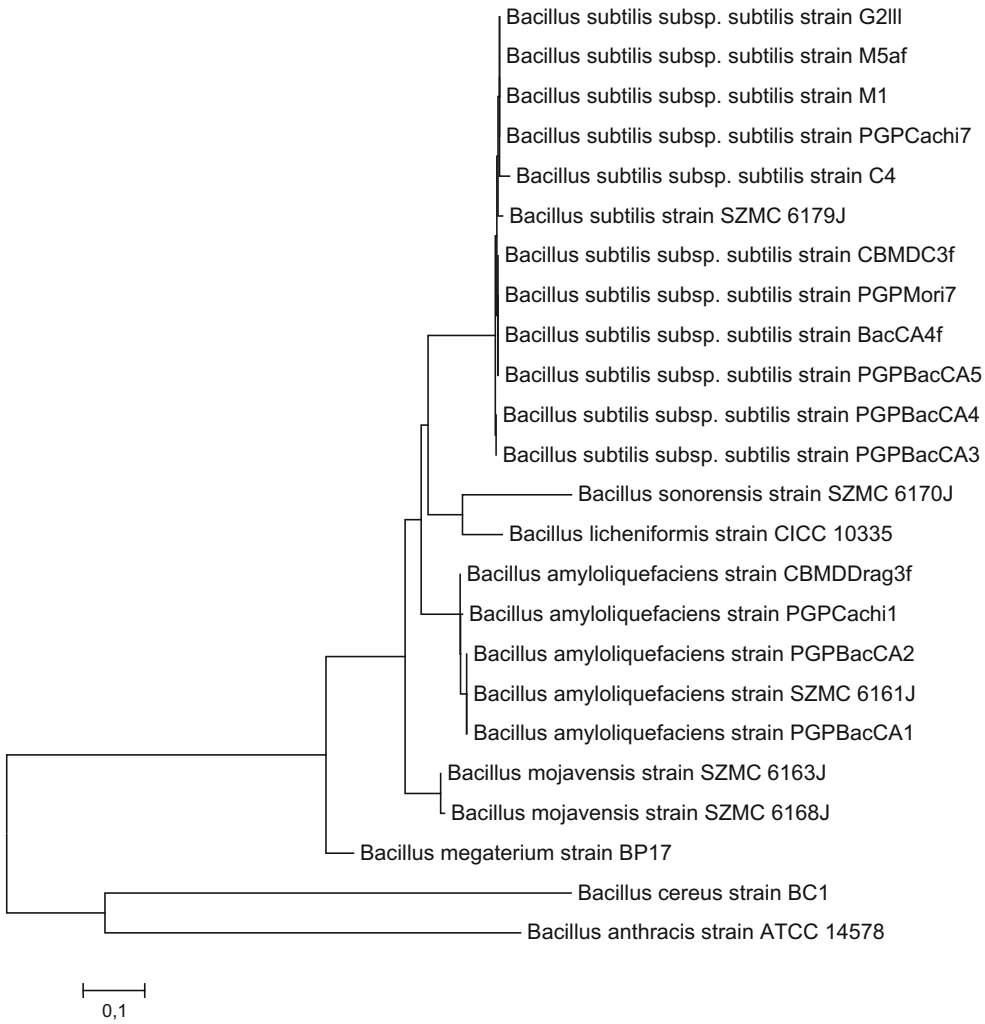

Fig. 1 Phylogenetic tree based on the sequences of the *gyr*A gene of *Bacillus subtilis* subsp. *subtilis* CBMDC3f, software constructed using MEGA version 5 (Tamura et al. 2011)

Finally, the effect of the CFS and the LF were tested against three *B. cereus* strains. Each *B. cereus* had a particular response to the action of the CFS and the two LF concentrations. The viability of *B. cereus* BAC1, after 1 h of contact with the LF at 2 mg/mL, reduced 4 log orders of magnitude, reaching 5 log orders after 24 h. Meanwhile, both the LF at a concentration of 0.5 mg/mL, and the CFS produced a similar effect on the number of that pathogen cells, this reduction being lower than that registered with 2 mg/mL (Fig. 2c).

On the other hand, the assays with the CFS and the LF (2 mg/mL) against *B. cereus* MBC1 revealed a decrease in viability of 1 log order of magnitude to t_0. However, after 24 h of contact with CFS the reduction in viable cells was only about 0.5 log orders, compared with the control (MH). Similarly, the LF at a concentration of 0.5 mg/mL produced a fast decrease of about 2 log orders in viable cells during the first two h of contact, but after that the antibacterial activity decreased and, in consequence, the MBC1 viability increased approximately 1 log order. In contrast, the LF at a concentration of 2.0 mg/mL remained active throughout the experiment (24 h), reducing MBC1 viability about 2 log orders compared with the control (distilled water at pH 9) (see Fig. S2a in supplementary data).

B. cereus MBC7 viability was also affected by the presence of the CFS and the LF (both 0.5 and 2.0 mg/mL): at t_0 all the assays showed a decrease in the number of viable cells of more than 1 log order. However, after that initial response, cell viability in contact with the CFS increased, and at the end of the trial the cell number was similar to the control. On the other hand, both 0.5 and 2.0 mg/mL LF produced a reduction in MBC7 viability between 1.5 and 2 log orders, respectively, compared with the control of water at pH 9 (see Fig. S2b in supplementary data).

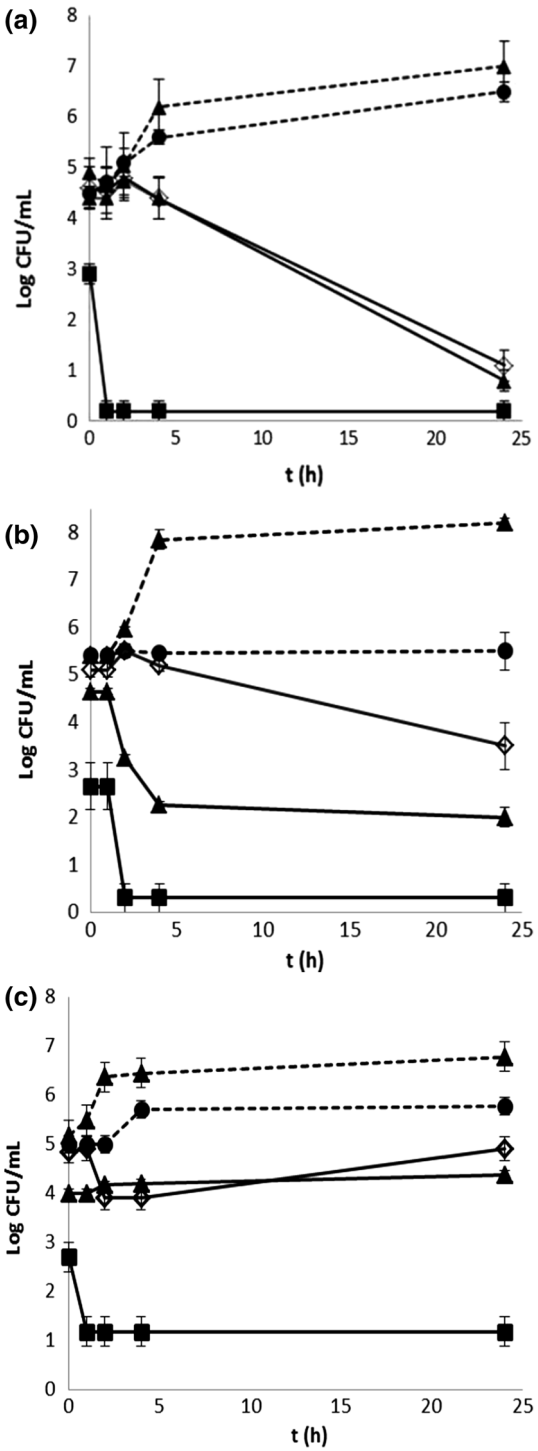

Fig. 2 Viable cells counts of **a** *L. monocytogenes* 00-3/364, **b** *S. aureus* ATCC29213 and **c** *Bacillus cereus* BAC1 strains after direct contact with CFS and LF of *B. subtilis* subsp. *subtilis* CBMDC3f. Culture medium control (*triangle, dashed line*); H_2O at pH9 (*circle, dashed line*); CFS (*open rhombus, continuous line*); LF 0.5 mg/mL (*triangle, continuous line*); LF 2.0 mg/mL (*square, continuous line*)

UV-MALDI-MS analysis

Cell-free supernatant and lipopeptides fraction of *B. subtilis* subsp *subtilis* CBMDC3f were analyzed by UV-MALDI-TOF MS. The compounds found fell into two m/z ranges 1000–1100 (see Fig. S3 in supplementary data), which includes surfactins and iturins and m/z 1400–1600 [Table 2; (see Fig. S4 in supplementary data)], which includes fengycins. Several surfactin homologues were observed as adduct of sodium at m/z 1015.04, 1030.96, 1044.92 and 1058.95 (Table 2). These peaks revealed differences of 14 m/z unities, suggesting a series of homologous molecules having different lengths of fatty acid chains (i.e., $CH_2 = 14$ Da). Additionally, surfactins at m/z 1074.86 and 1088.99 were observed as adducts of potassium (Table 2). Interestingly, in the CFS two homologous of iturin were detected at m/z = 1080.92 and m/z = 1102.86. Similar surfactin homologues patterns were obtained for m/z range between 1000 and 1100 from either CFS or LF (see Fig. S3 in supplementary data).

The UV-MALDI spectrum of CFS showed signals in the m/z range 1400–1700 (see Fig. S4 in supplementary data). Protonated forms of fengycins were detected at m/z 1449.53, 1461.82, 1477.63 and 1505.67 (Table 2). Additionally, sodiated adducts were detected at m/z 1499.54 and 1527.57. On the other hand, when LF was used total intensity of the spectrum was lower and only one of those homologues was detected (Table 2).

For structural characterization of surfactins homologues, UV-MALDI-TOF MS/MS (fragmentation method: LID) was performed (Fig. 3a and b).

Figure 3a shows the MS/MS spectrum of the $[M + Na]^+$ ion detected at m/z 1015.04. Its fragmentation yielded ions at m/z 902.09 (ion 3, Fig. 3a; $[M\text{-}113 + Na]^+$, $[M\text{-}Leu + Na]^+$), 788.98 (ion 7, Fig. 3a; $[M\text{-}226 + Na]^+$, $[M\text{-}(Leu)_2 + Na]^+$), 672.92 (ion 13, Fig. 3a; $[M\text{-}341 + Na]^+$, $[M\text{-}(Leu)_2\text{-}Asp + Na]^+$), and 326.58 (ion 18, Fig. 3a; $[M\text{-}668 + H]^+$, $[M\text{-}(Leu)_2\text{-}Asp\text{-}Val\text{-}(Leu)_2 + Na]^+$) (Table 3). These fragments correspond to the sequential losses of the amino acid residues of Leu, Leu, Asp and the (Leu/Leu/Asp/Val/Leu/Leu) moiety (see the structure assigned to the numbered ions in Table 3). The common peak at m/z 685.81 (ion 12, Fig. 3a) could be assigned as an internal protonated fragment ions $[(H)Leu^2\text{-}Leu^3\text{-}Val^4\text{-}Asp^5\text{-}Leu^6\text{-}Leu/Ile^7(OH) + H]^+$. This fragment ion represents a characteristic marker ion for identification of surfactins. Besides, the common peaks at m/z 594.73, 481.65 and 267.49 observed could be assigned as sodiated internal peptide fragment ions $[Leu^3\text{-}Val^4\text{-}Asp^5\text{-}Leu^6\text{-}Leu/Ile^7(OH) + H + Na]^+$,

Table 2 Main peaks detected by UV-MALDI-TOF mass spectrometry analysis of the lipopeptides produced from *Bacillus subtilis* subsp *subtilis* CBMDC3f; Samples: cell-free supernatant (CFS) and lipopeptides fraction (LF)

Lipopeptides	Chemical formula	Calculated	Experimental	
			CFS	LF
Surfactin	$[C_{50}H_{86}N_7O_{13}Na]^+$	1015.62	1015.04	WS
Surfactin	$[C_{51}H_{88}N_7O_{13}Na]^+$	1029.63	1030.96	1030.97
Surfactin	$[C_{52}H_{90}N_7O_{13}Na]^+$	1043.65	1044.92	1045.24
Surfactin	$[C_{53}H_{92}N_7O_{13}Na]^+$	1057.66	1058.95	1059.34
Surfactin	$[C_{53}H_{92}N_7O_{13}K]^+$	1073.64	1074.86	1075.05
Surfactin	$[C_{54}H_{94}N_7O_{13}K]^+$	1087.65	WS	1088.99
Iturin	$[C_{49}H_{76}N_{11}O_{15}Na]^+$	1081.54	1080.92	1082.05
Iturin	$[C_{49}H_{76}N_{11}O_{15}K]^+$	1097.51	WS	1097.67
Iturin	$[C_{52}H_{83}N_{11}O_{15}]^+$	1101.61	1102.86	WS
Fengycin	$[C_{71}H_{108}N_{12}O_{20}H]^+$	1449.78	1449.53	WS
Fengycin	$[C_{72}H_{110}N_{12}O_{20}H]^+$	1463.80	1461.82	WS
Fengycin	$[C_{73}H_{112}N_{12}O_{20}H]^+$	1477.82	1477.63	WS
Fengycin	$[C_{73}H_{112}N_{12}O_{20}Na]^+$	1499.80	1499.54	WS
Fengycin	$[C_{75}H_{116}N_{12}O_{20}H]^+$	1505.85	1505.67	1504.58
Fengycin	$[C_{75}H_{116}N_{12}O_{20}Na]^+$	1527.83	1527.57	WS

WS without signal

$[Val^4\text{-}Asp^5\text{-}Leu^6\text{-}Leu/Ile^7(OH) + H + Na]^+$and $[Leu^6\text{-}Leu/Ile^7(OH) + H + Na]^+$. Furthermore, fragmentation of the chain side of the aminoacids was also observed. For instance loss of COOH moiety (44 Da) (ion 1, Fig. 3a), loss of CH_2CH_2COOH moiety from glutamic acid (72 Da) (ions 2 and 6, Fig. 3a) and, loss of water (18 Da) (ions 8 and 15, Fig. 3a) and $CONH_3$ moiety (46 Da) (ions 5 and 9, Fig. 3a) were also detected in the spectrum shown in Fig. 3a and detailed in Table 3. Then the sequence could be deduced based on the fragmentation profile as β-OH fatty acid-Glu^1-Leu^2-Leu^3-Val^4-Asp^5-Leu^6-Leu/Ile^7.

Figure 3b shows the MS/MS spectrum of the species observed as $[M + K]^+$ ion at m/z 1088.9. Dissociation yielded ions at m/z 974.13 (ion 4, Fig. 3b; $[M\text{-}113 + K]^+$, $[M\text{-}Leu + K]^+$), 861.01 (ion 9, Fig. 3b; $[M\text{-}226 + K]^+$, $[M\text{-}(Leu)_2 + K]^+$),746.97 (ion 12, Fig. 3b; $[M\text{-}341 + K]^+$, $[M\text{-}(Leu)_2\text{-}Asp + K]^+$), 648.74 (ion 15, Fig. 3b, $[M\text{-}442 + K]^+$, $[M\text{-}(Leu)_2\text{-}Asp\text{-}Val + K]^+$), 534.73 (ion 17, Fig. 3b; $[M\text{-}555 + K]^+$, $[M\text{-}(Leu)_2\text{-}Asp\text{-}Val\text{-}Leu + K]^+$) and 420.58 (ion 20, Fig. 3b; $[M\text{-}668 + K]^+$), $[M\text{-}(Leu)_2\text{-}Asp\text{-}Val\text{-}(Leu)_2 + K]^+$). These fragments match with the sequential losses of the amino acid residues Leu/Leu/Asp/Val/Leu/Leu from the potassiated molecular ion. The common peaks at m/z 685.79 (ion 14, Fig. 3b) and 498.72 (ion 18, Fig. 3b) could be assigned as internal fragment ions $[(H)Leu^2\text{-}Leu^3\text{-}Val^4\text{-}Asp^5\text{-}Leu^6\text{-}Leu/Ile^7(OH) + H]^+$ and $[Val^4\text{-}Asp^5\text{-}Leu^6\text{-}Leu/Ile^7(OH) + H + K]^+$, respectively.

The fragmentation of chain side of the aminoacids was also observed and the fragments structure assigned as well as calculated and experimental m/z values are listed in Table 4.

Discussion

Contaminating bacterial pathogens in the food industry have been controlled by a variety of antimicrobial agents such as sanitizers and disinfectants (Lunden et al. 2003), antibiotics (Maria-Neto et al. 2012) and bacteriocins like nisin (Imran et al. 2014), among others. However, the generalized use of these antimicrobial compounds in the elaboration of food or in the cleaning of equipment is being studied, because the past years have seen a growing resistance of foodborne pathogens against these antagonists, a fact that threatens the safety of the food industry (Gandhi and Chikindas 2007). For that reason, in this work we focused on the antibacterial potential of a *Bacillus* strain and its metabolites.

It is well known that within *Bacillus* species there are subspecies, and it is important to correctly identify a microorganism that may have a potential industrial use, besides its applications at the laboratory (Rooney et al. 2009). The identification involves analysis of genes that codify proteins with a high variability and that can be used as phylogenetic markers. In the case of *Bacillus* the *gyrA* gene is used (Porwal et al. 2009). Sequencing of the *gyrA* gene that codes for the A subunit of DNA gyrase of *Bacillus* is useful to discriminate between species closest to *B. subtilis*, as well as to distinguish between its subspecies (Nakamura et al. 1999; Porwal et al. 2009). The analysis of the nucleotide sequence of both the 16S rRNA and the *gyrA* gene allowed the complete characterization of the *Bacillus* strain studied as *B. subtilis* subsp. *subtilis* CBMDC3f.

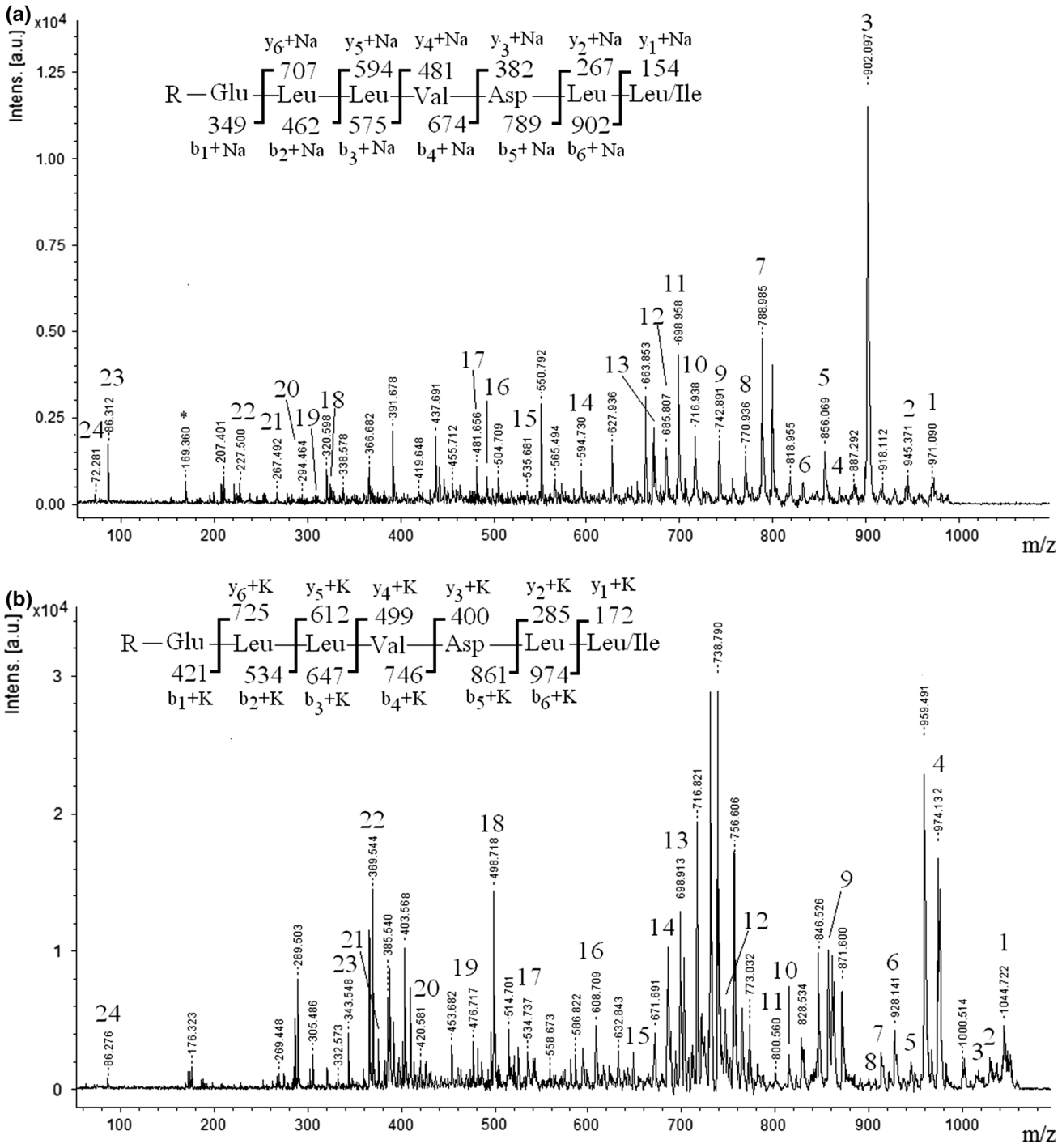

Fig. 3 UV-MALDI MS/MS spectrum of the **a** surfactin homologue with m.w. 994.26 detected as $[M + Na]^+$ at m/z 1015.0 and **b** surfactin homologue with m.w 1050.37 detected at m/z 1088.9 as $[M + K]^+$ (see 'Discussion' section). Matrix: norharmane

In this work, the agar diffusion technique was used for a rapid screening of the antibacterial spectrum of *B. subtilis* subsp. *subtilis* CBMDC3f. Nine *L. monocytogenes*, three *B. cereus* and *S. aureus* ATCC29213 were chosen as indicator strains. These pathogens were not arbitrarily selected. All of them are foodborne pathogenic bacteria which produce severe diseases and infections in humans (Gandhi and Chikindas 2007). In particular, the FDA and FAO have declared zero tolerance regarding *L. monocytogenes* in ready-to-use food. *B. subtilis* subsp. *subtilis* CBMDC3f showed an important antibacterial potential, because both the CS and CFS, inhibited growth of all the *Listeria* strains

Table 3 Main peaks observed in the UV-MALDI MS/MS spectrum of the surfactin homologue with m.w. 994.26 detected at m/z 1015.0 as $[M + Na]^+$ (Fig. 3a)

Ion	Ion structure assigned	m/z exp	m/z calc.
1	$[M\text{-}44 + Na]^+$	971.09	971.63
2	$[M\text{-}70 + Na]^+$	945.37	945.61
3	$[M\text{-Leu} + Na]^+$ $[M\text{-}113 + Na]^+$	902.09	902.55
4	$[M\text{-}113\text{-}30 + Na]^+$	872.10	872.48
5	$[M\text{-}113\text{-}46 + Na]^+$	856.07	856.52
6	$[M\text{-}113\text{-}70 + Na]^+$	832.56	832.52
7	$[M\text{-}(Leu)_2 + Na]^+$ $[M\text{-}226 + Na]^+$	788.98	789.46
8	$[M\text{-}226\text{-}18 + Na]^+$	770.94	771.44
9	$[M\text{-}226\text{-}46 + Na]^+$	742.89	743.44
10	$[M\text{-}226\text{-}72 + Na]^+$	716.94	717.43
11	$[M\text{-}226\text{-}90 + Na]^+$	698.96	699.43
12	$[(H)Leu\text{–}Leu\text{-}Val\text{-}Asp\text{-}Leu\text{–}Leu/Ile(OH) + H]^+$	685.81	685.45
13	$[M\text{-}(Leu)_2\text{-}Asp]^+$ $[M\text{-}341 + Na]^+$	672.92	674.44
14	$[(Leu\text{-}Val\text{-}Asp\text{-}Leu\text{-}Leu/Ile(OH) + H + Na]^+$	594.73	594.35
15	$[M\text{-}440\text{-}18 + H]^+$	535.68	536.33
16	$[M\text{-}440\text{-}60 + H]^+$	492.65	493.35
17	$[Val\text{-}Asp\text{-}Leu\text{–}Leu/Ile(OH) + H + Na]^+$	481.65	481.26
18	$[M\text{-}666 + H]^+$	326.58	327.20
19	$[M\text{-}666\text{-}18 + H]^+$	308.55	309.19
20	$[M\text{-}666\text{-}32 + H]^+$	294.46	295.14
21	$[(Leu\text{–}Leu/Ile(OH) + H + Na]^+$	267.49	267.17
22	$[LeuLeu + H]^+$	227.50	227.18
23	$[Leu\text{-}44]^+$	86.31	86.09
24	$[Val\text{-}44]^+$	72.28	72.08

and *S. aureus* ATCC29213. The ability of CBMDC3f to inhibit *L. monocytogenes, S. aureus* and *B. cereus*, pathogenic microorganisms highly adaptable to survive and grow under a great variety of environmental conditions, is a relevant and promising result.

The number of indicator strains assayed is an important detail, because previous studies reporting biological activity of *Bacillus* strains against *L. monocytogenes* and *B. cereus*, only tested one or two indicator strains of each type (Ouoba et al. 2007; Huang et al. 2008). Kaewklom et al. (2013) reported that a bacteriocin can be proposed as a bioprotector in the food industry due to its anti-*L. monocytogenes* activity; however they tested this compound against only one *L. monocytogenes* strain. Audisio et al. (2005) have observed and reported that the response of different *Listeria* strains to the same bacteriocin can be highly variable. Hence, using or testing a single indicator strain to determine whether a bacterium or its metabolites can be a potential antagonist will probably yield incomplete results, which can be not always reliable or reproducible.

It is worthy to note that the LF also exhibited a high antibacterial spectrum, wherever seven *Listeria* strains, three *B. cereus* strains and *S. aureus* ATCC29213 were inhibited. The minimum inhibitory concentration (MIC) of this fraction was in general 0.5 mg/mL against *Listeria* strains, 1 mg/mL against *B. cereus* MBC1 and 2 mg/mL *S. aureus* ATCC29213. These values reveal that the antibacterial activity of the LF is pathogen dependent. A similar situation has been reported by Gomaa (2013), who determined that *B. licheniformis* M104 mainly synthesized lipopeptides active against *S. aureus* ATCC25928; however these compounds were not able to inhibit *Listeria*. In this sense, Lee et al. (2010) also informed the specific antagonistic activity against *B. cereus* by an antibiotic-like lipopeptide compound produced by *B. subtilis,* which however had no inhibitory effect on *Listeria* nor on *S. aureus*.

The assays where a direct contact occurred between either the CFS or the LF, and the different indicator strains evaluated, confirmed the results obtained by the well diffusion assay; moreover, they revealed the high sensitivity to the LF of two *L. monocytogenes* strains (01/155, 00-3/364) and *S. aureus* ATCC29213. Similar results were reported by Sabaté and Audisio (2013), who assayed the antibacterial activity of another *B. subtilis* subsp *subtilis* strain; however, they had only evaluated the antilisteria effects. In this work it was determined that CBMDC3f had

Table 4 Main peaks observed in the UV-MALDI MS/MS spectrum of the surfactin homologue with m.w. 1050.37 detected at m/z 1088.9 as $[M + K]^+$ (Fig. 3b)

Ion	Ion structure assigned	m/z exp	m/z calc
1	$[M\text{-}44 + K]^+$	1044.72	1043.66
2	$[M\text{-}58 + K]^+$	1029.49	1029.64
3	$[M\text{-}70 + K]^+$	1017.30	1017.64
4	$[M\text{-Leu} + K]^+$ $[M\text{-}113 + K]^+$	974.13	974.51
5	$[M\text{-}113\text{-}30 + K]^+$	944.95	944.52
6	$[M\text{-}113\text{-}46 + K]^+$	928.14	928.56
7	$[M\text{-}113\text{-}61 + K]^+$	913.54	913.54
8	$[M\text{-}113\text{-}90 + K]^+$	884.68	884.55
9	$[M\text{-(Leu)}_2 + K]^+$ $[M\text{-}226 + K]^+$	861.01	861.43
10	$[M\text{-}226\text{-}46 + K]^+$	814.95	815.48
11	$[M\text{-}226\text{-}61 + K]^+$	800.56	800.46
12	$[M\text{-(Leu)}_2\text{-Asp} + K]^+$ $[M\text{-}341 + K]^+$	746.97	746.40
13	$[M\text{-}341\text{-}44 + K]^+$	702.88	702.47
14	[(H)Leu Leu Val Asp Leu Leu/Ile(OH) $+H]^+$	685.79	685.45
15	$[M\text{-}440 + K]^+$	648.74	647.33
16	$[M\text{-(Leu)}_2\text{-Asp-Val} + K]^+$ $[M\text{-}440 + H]^+$	608.71	609.43
17	$[M\text{-(Leu)}_2\text{-Asp-Leu} + K]^+$ $[M\text{-}553 + K]^+$	534.73	534.25
18	[(Val Asp Leu Leu/Ile(OH) $+H] + K^+$	498.72	499.26
19	$[M\text{-}553\text{-}58 + K]^+$	476.72	476.30
20	$[M\text{-(Leu)}_2\text{-Asp-(Leu)}_2 + K]^+$ $[M\text{-}666 + K]^+$	420.58	421.17
21	$[M\text{-}666\text{-}46 + K]^+$	375.65	375.21
22	$[LeuLeuAspVal\text{-}71 + H]^+$	369.54	369.25
23	$[LeuLeuAsp + H]^+$	343.55	343.21
24	$[Leu\text{-}44]^+$	86.28	86.09

the ability to always keep the number of *B. cereus* below 10^5 cells/mL; in particular, 2 mg/mL of the LF reduced to 10^1 the number viable cells. This antibacterial feature can have an important impact in the food industry, due to the fact that the consumption of foods containing $>10^5$ *B. cereus*/g may result in food poisoning (Aksu et al. 2000).

UV-MALDI-MS is an innovative technique which is highly efficient not only for rapid typification of microorganisms through analysis of their secondary metabolite spectra (Leenders et al. 1999), but also for assaying the production of natural compounds both in solid and liquid cultures (Price et al. 2007). The current study analyzed production of lipopeptides both in the CFS and the LF from *Bacillus* CBMDC3F, using UV–MALDI-TOF MS. The following lipopeptides were identified in CFS: surfactin, iturin and fengycin with four, two and four homologues per family, respectively. Furthermore, four homologues of surfactin and only one of iturin and fengycin were identified in the LF. Besides, both in the CFS and the LF the signals proved differences in m/z 14 between them, which suggests a series of homologue molecules that contain fatty acids with variable chain lengths (i.e., $CH_2 = 14$ Da). As has been mentioned before, lipopeptides were obtained through acid precipitation and consecutive methanol extraction (LF). Mass spectrometry revealed that this recovery affected the *B. subtilis* subsp. *subtilis* CBMDC3f lipopeptide profile when compared with the CFS mass spectrum (a sample that was less manipulated). It is noteworthy than different homologues of surfactin and only one of fengycin were observed in the LF. This situation may explain the different antibacterial potential determined for the CFS and the LF in this work. Similarly, Mukherjee and Das (2005) informed that the difference in the degree of inhibitory response and microbial specificity between crude lipopeptides from *B. subtilis* DM-03 and DM-04, could be caused by the presence of several homologues in the extracted lipopeptides fraction. The results of this work show that MALDI-TOF mass spectrometry can detect the different lipopeptides with high sensitivity and precision, and without the requirement of many steps related with the isolation and chromatographic separation of the compounds, which are time-consuming.

Two surfactins isoforms, a sodium adduct at m/z 1015.04 and a potassium adduct at 1088.99, were identified by an MSMS (LID) experiment and by correlation with previous results (Sun et al. 2006; Pathak and Keharia 2014). Successive loss of aminoacids has been observed as reported previously (Oka et al. 1993; Kim et al. 2010; De

Faria et al. 2011). Moreover, common peaks ions as the internal protonated fragment ion [(H)Leu^2-Leu^3-Val^4-Asp^5-Leu^6-Leu/Ile^7(OH) +H]$^+$,were in both cases also detected (Hue et al. 2001; Tang et al. 2010; Pathak and Keharia 2014.

In conclusion, *B. subtilis* subsp *subtilis* CBMDC3f presented a broad spectrum of inhibitory activity against *L. monocytogenes, B. cereus* and *S. aureus*, all of them foodborne pathogens. Surfactin, iturin and fengycin were detected in the CFS, whereas only surfactin predominated in the LF; this suggests that the applied extraction method recovered mainly surfactin homologues. Since the LF exerted antibacterial activity against all of the indicator strains, it may be inferred that surfactin synthesized by this bacterium was responsible for the biological effect. Interestingly, the only case in which fengycin or iturin could be involved was against *L. monocytogenes* 00-3/364, because the CFS showed a significant inhibitory effect.

Acknowledgments This work had the financial support of CIUNSa (PI 1974), UBA (0055BA) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) of Argentina (PICT2011-07/67 and PICT 2012-0888) and CONICET-Argentina (PIP 0072CO and PIP 00019). M.C. Audisio, R. Erra-Balsells and G. Petroselli are Research Members of CONICET, Argentina.

Conflict of interest The authors declare not conflict of interest related to this work.

References

Aksu H, Bostan K, Ergün Ö (2000) Presence of *Bacillus cereus* in packaged some spices and herbs sold in Istanbul. Pak J Biol Sci 3:710–712

Audisio MC, Terzolo HR, Apella MC (2005) Bacteriocin from honeybee beebread *Enterococcus avium* active against *Listeria monocytogenes*. Appl Environ Microbiol 71:3373–3375

Ayed HB, Hmidet N, Béchet M, Chollet M, Chataigné G, Leclère V, Jacques P, Nasri M (2014) Identification and biochemical characteristics of lipopeptides from *Bacillus mojavensis* A21. Process Biochem 49:1699–1707

Bhunia AK (2008) *Bacillus cereus* and *Bacillus anthracis*. In: Bhunia AK (ed) Foodborne microbial pathogens: mechanisms and pathogenesis. Springer, New York, pp 135–147

Burkard M, Entian KD, Stein T (2007) Development and application of a microtiter plate-based autoinduction bioassay for detection of the lantibiotic subtilin. J Microbiol Methods 70:179–185

Compaoré CS, Nielsen DC, Ouoba LI, Berner TS, Nielsen KF, Sawadogo-Lingani H, Diawara B, Ouédraogo GA, Jakobsen M, Thorsen L (2013) Co-production of surfactin and a novel bacteriocin by *Bacillus subtilis* subsp. *subtilis* H4 isolated from Bikalga, an African alkaline *Hibiscus sabdariffa* seed fermented condiment. Int J Food Microbiol 162:297–307

Daffonchio D, Borin S, Frova G, Manachini P, Sorlini C (1998) PCR fingerprinting of whole genomes: the spacers between the 16S and 23S rRNA genes and of intergenic tRNA gene regions reveals a different intraespecific genomic variability of *Bacillus cereus* and *Bacillus licheniformis*. Int J Syst Bacteriol 48:107–116

de Clerck E, Vanhoutte T, Hebb T, Geerinck J, Devos J, De Vos P (2004) Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. Appl Environ Microbiol 70:3664–3672

De Faria AF, Teodoro-Martinez DS, de Oliveira Barbosa GN, Vaz BG, Serrano Silva Í, Simone Garcia J, Tótolac MR, Eberlinb MN, Grossmand M, Alvesb OL, Durranta LR (2011) Production and structural characterization of surfactin (C_{14}/Leu_7) produced by *Bacillus subtilis* isolate LSFM-05 grown on raw glycerol from biodiesel industry. Process Biochem 46:1951–1957

Foster TJ (2005) Immune evasion by staphylococci. Nat Rev Microbiol 3:948–958

Freyre-González JA, Manjarrez-Casas AM, Merino E, Martinez-Nuñez M, Perez-Rueda E, Gutiérrez-Ríos RM (2013) Lessons from the modular organization of the transcriptional regulatory network of *Bacillus subtilis*. BMC Syst Biol 7:127–141

Fuchs SW, Jaskolla TW, Bochmann S, Kötter P, Wichelhaus T, Karas M, Stein T, Entian KD (2011) Entianin, a novel subtilin-like lantibiotic from *Bacillus subtilis* subsp. *spizizenii* DSM 15029T with high antimicrobial activity. Appl Environ Microbiol 77:1698–1707

Gandhi M, Chikindas ML (2007) *Listeria*: a foodborne pathogen that knows how to survive. Int J Food Microbiol 113:1–15

Gomaa EZ (2013) Antimicrobial activity of a biosurfactant produced by *Bacillus licheniformis* strain M104 grown on whey. Braz Arch Biol Technol 56:259–268

Hiradate S, Yoshida S, Sugie H, Yada H, Fujii Y (2002) Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry 61:693–698

Huang X, Wei Z, Zhao G, Gao X, Yang S, Cui Y (2008) Optimization of sterilization of *Escherichia coli* in milk by surfactin and fengycin using a response surface method. Curr Microbiol 56:376–381

Hue N, Serani L, Laprévote O (2001) Structural investigation of cyclic peptidolipids from *Bacillus subtilis* by high-energy tándem mass spectrometry. Rapid Commun Mass Spectrom 15:203–209

Imran M, Revol-Junelles AM, Paris C, Guedon E, Linder M, Desobry S (2014) Liposomal nanodelivery systems using soy and marine lecithin to encapsulate food biopreservative nisin. LWT-Food Sci Technol. doi:10.1016/j.lwt.2014.12.046

Kaewklom S, Lumlert S, Kraikulb W, Aunpada R (2013) Control of *Listeria monocytogenes* on sliced bologna sausage using a novel bacteriocin, amysin, produced by *Bacillus amyloliquefaciens* isolated from Thai shrimp paste (Kapi). Food Control 32:552–557

Khochamit N, Siripornadulsil S, Sukon P, Siripornadulsil W (2015) Antibacterial activity and genotypic-phenotypic characteristics of bacteriocin-producing *Bacillus subtilis* KKU213: potential as a probiotic strain. Microbiol Res 170:36–50

Kim P, Ryu J, Kim Y, ChI Y-T (2010) Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from *Bacillus Subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. J Microbiol Biotechnol 20:138–145

Lee NK, Yeo IC, Park JW, Kang BS, Hahm YT (2010) Isolation and characterization of a novel analyte from *Bacillus subtilis* SC-8 antagonistic to *Bacillus cereus*. J Biosci Bioeng 110:298–303

Leenders F, Stein TH, Kablitz B, Franke P, Vater J (1999) Rapid typing of *Bacillus subtilis* strains by their secondary metabolites using matrix-assisted laser desorption/ionization mass spectrometry of intact cells. Rapid Commun Mass Spectrom 13:943–949

Lunden J, Autio T, Markkula A, Hellstrom S, Korkeala H (2003) Adaptive and cross-adaptive responses of persistent and non-persistent *Listeria monocytogenes* strains to disinfectants. Int J Food Microbiol 82:265–272

Maria-Neto S, Candido Ede S, Rodrigues DR, de Sousa DA, da Silva EM, de Moraes LM, Otero-Gonzalez Ade J, Magalhães BS, Dias SC, Franco OL (2012) Deciphering the magainin resistance process of *Escherichia coli* strains in light of the cytosolic proteome. Antimicrob Agents Chemother 56:1714–1724

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory Cold Spring Harbor, New York

Mukherjee AK, Das K (2005) Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol Ecol 54:479–489

Nakamura LK, Roberts MS, Cohan FM (1999) Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. *nov*. and *Bacillus subtilis* subsp. *spizizenii* subsp. *nov*. Int J Syst Evol Microbiol 49:1211–1215

Nonami H, Fukui S, Erra-Balsells R (1997) β-Carboline alkaloids as matrices for matrix-assisted ultraviolet laser desorption time-of flight mass spectrometry of proteins and sulfated oligosaccharides: a comparative study using phenylcarbonyl compounds, carbazoles and classical matrices. J Mass Spectrom 32:287–296

Oka K, Hirano T, Homma M, Ishii H, Murakami K, Mogami S, Motizuki A, Morita H, Takeya K, Itokawa H (1993) Satisfactory separation and MS-MS spectrometry of six surfactins isolated from *Bacillus subtilis natto*. Chem Pharm Bull 41:1000–1002

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16:115–125

Ouoba LI, Diawara B, Jespersen L, Jakobsen M (2007) Antimicrobial activity of *Bacillus subtilis* and *Bacillus pumilus* during the fermentation of African locust bean (Parkia biglobosa) for Soumbala production. J Appl Microbiol 102:963–970

Pandey H, Kumar V, Roy BK (2014) Assessment of genotoxicity of some common food preservatives using *Allium cepa* L. as a test plant. Toxicol Rep 1:300–308

Patel H, Tscheka C, Edwards K, Karlsson G, Heerklotz H (2011) All-or-none membrane permeabilization by fengycin-type lipopeptides from *Bacillus subtilis* QST713. Biochim Biophys Acta 1808:2000–2008

Pathak KV, Keharia H (2014) Identification of surfactins and iturins produced by potent fungal antagonist, *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) tree using mass spectrometry. 3. Biotech 4:283–295

Pedersen PB, Bjørnvad ME, Rasmussen MD, Petersen JN (2002) Cytotoxic potential of industrial strains of *Bacillus* sp. Regul Toxicol Pharmacol 36:155–161

Porwal S, Lal S, Cheema S, Kalia VC (2009) Phylogeny in aid of the present and novel microbial lineages: diversity in *Bacillus*. PLoS One 4:e4438. doi:10.1371/journal.pone.0004438

Price NP, Rooney AP, Swezey JL, Perry E, Cohan FM (2007) Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. FEMS Microbiol Lett 271:83–89

Romero D, de Vicente A, Rakotoaly RH, Dufour SE, Veening JW, Arrebola E, Cazorla FM, Kuipers OP, Paquot M, Pérez-García A (2007) The itulin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol Plant Microbe Interact 20:430–440

Rooney AP, Price NPJ, Ehrhardt C, Swezey JL, Bannan JD (2009) Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. *nov*. Int J Syst Evol Microbiol 59:2429–2436

Sabaté DC, Audisio MC (2013) Inhibitory activity of surfactin, produced by different *Bacillus subtilis* subsp. *subtilis* strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. Microbiol Res 168:125–129

Sabaté DC, Carrillo L, Audisio MC (2009) Inhibition of *Paenibacillus larvae* and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. Res Microbiol 160:193–199

Savadogo A, Tapi A, Chollet M, Wathelet B, Traore AS, Jacques P (2011) Identification of surfactin producing strains in Soumbala and Bikalga fermented condiments using polymerase chain reaction and matrix assisted laser desorption/ionization-mass spectrometry methods. Int J Food Microbiol 151:299–306

Seydlova G, Svobodova J (2008) Review of surfactin chemical properties and the potential biomedical applications. Cent Eur J Med 3:123–133

Sun L, Lu Z, Bie X, Lu F, Yang S (2006) Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2, from *Scutellaria baicalensis* Georgi. World J Microbiol Biotechnol 22:1259–1266

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Tang J-S, Zhao F, Gao H, Dai Y, Yao Z-H, Hong K, Li J, Ye W-C, Yao X-S (2010) Characterization and online detection of surfactins isomers based on HPLC-MS analyses and their inhibitory effects on the overproduction of nitric oxide and the release of TNF-α and IL-6 in LPS-induces macrophages. Mar Drugs 8:2605–2618

Teixeira ML, Dalla Rosa A, Brandelli A (2013) Characterization of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii* showing inhibitory activity towards *Haemophilus parasuis*. Microbiology 159:980–988

Thasana N, Prapagdee B, Rangkadilok N, Sallabhan R, Aye SL, Ruchirawat S, Loprasert S (2010) *Bacillus subtilis* SSE4 produces subtulene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated beta-amino acid. FEBS Lett 584:3209–3214

Vater J, Kablitz B, Wilde C, Franke P, Mehta N, Cameotra SS (2002) Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Appl Environ Microbiol 68:6210–6219