Inhibition of *Bacillus cereus* Strains by Antimicrobial Metabolites from *Lactobacillus johnsonii* CRL1647 and *Enterococcus faecium* SM21

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Abstract

Bacillus cereus is an endospore-forming, Gram-positive bacterium able to cause foodborne diseases. Lactic acid bacteria (LAB) are known for their ability to synthesize organic acids and bacteriocins, but the potential of these compounds against *B. cereus* has been scarcely documented in food models. The present study has examined the effect of the metabolites produced by *Lactobacillus johnsonii* CRL1647 and *Enterococcus faecium* SM21 on the viability of select *B. cereus* strains. Furthermore, the effect of *E. faecium* SM21 metabolites against *B. cereus* strains has also been investigated on a rice food model. *L. johnsonii* CRL1647 produced 128 mmol/L of lactic acid, 38 mmol/L of acetic acid and 0.3 mmol/L of phenyl-lactic acid. These organic acids reduced the number of vegetative cells and spores of the *B. cereus* strains tested. However, the antagonistic effect disappeared at pH 6.5. On the other hand, *E. faecium* SM21 produced only lactic and acetic acid (24.5 and 12.2 mmol/L, respectively) and was able to inhibit both vegetative cells and spores of the *B. cereus*

strains, at a final fermentation pH of 5.0 and at pH 6.5. This would indicate the action of other metabolites, different from organic acids, present in the cell-free supernatant. On cooked rice grains, the *E. faecium* SM21 bacteriocin(s) were tested against two *B. cereus* strains. Both of them were significantly affected within the first 4 h of contact; whereas *B. cereus* BAC1 cells recovered after 24 h, the effect on *B. cereus* 1 remained up to the end of the assay. The LAB studied may thus be considered to define future strategies for biological control of *B. cereus*.

AQ1

AQ2

Keywords

Lactic acid bacteria
Lactobacillus johnsoni
Enterococcus faecium
Antimicrobial compounds
White rice grains
Bacillus cereus

Introduction

Bacillus cereus is an ubiquitous, facultative anaerobic, spore-forming, Gram-positive, rod-shaped bacterium commonly found in soil [1]. It has the ability to withstand harsh environmental conditions because it forms spores that are resistant to heat, dehydration and other physical stress factors [2]. Spores frequently contaminate a variety of foods, including meat, eggs and dairy products [1]. The presence of *Bacillus* spp. spores in food is a problem for the food-processing industry, as they can result in food spoilage or, more seriously, outbreaks of foodborne diseases [3]. Food poisoning produced by *B. cereus* has two manifestations: (1) the emetic illness caused by ingestion of a preformed toxin, cereulide; (2) diarrheal illness caused by enterotoxin(s) [4]. Emetic *B. cereus* foodborne intoxications are mainly linked to starchy foods such as rice and pasta.

The raw grains of rice are usually stored with a moisture content ranging

from 12 to 14 %. At this level of moisture, it is not possible for the vegetative forms of *Bacillus* spp. and other closely related species to proliferate, although the spores are able to survive these conditions as well as normal cooking procedures [5]. In particular, cooking rice and keeping it unrefrigerated for several hours before frying or re-heating lead to emetic intoxication outbreaks. This occurs because the emetic toxin cereulide can be produced during storage of the cooked rice, and it is not destroyed by frying or re-heating [6]. The prevalence of *B. cereus* in cooked rice from Chinese-style restaurants in Korea was determined to be 37.5 % [7]; indeed, rice dishes are commonly implicated in *B. cereus* outbreaks [8]. According to the Governmental Agency of Control of Buenos Aires (Argentina), *B. cereus* is in third place in the ranking of the most common bacteria associated with foodborne illness registered in the city [9].

Among the control strategies currently being used or proposed for food preservation, those based on living organisms and/or their antimicrobial products (biocontrol or biopreservation) have become increasingly popular. These preservation methods are regarded as health friendly by consumers, and they are expected to have a lower impact on the nutritional and sensory properties of foods. Most studies have focused on antimicrobial substances produced by lactic acid bacteria (LAB) [10]. The LAB produce arrays of antimicrobial substances such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides and bacteriocins [11]. In particular, organic acids create an acidic environment unfavorable for the growth of many pathogenic and spoilage microorganisms. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of the cell membrane potential, reducing intracellular pH, inhibiting active transport and a variety of metabolic functions [12]. On the other hand, bacteriocins are ribosomally synthesized peptides or proteins that have a bactericidal or bacteriostatic effect on other bacteria [13]. Many bacteriocins have shown antimicrobial activity against endospore-forming bacterial cells and in vitro spore germination [14–18]. However, in the particular case of *B. cereus* inhibition by bacteriocins or bacteriocin-like compounds in food systems, the scientific references are scarce [19–22].

In this sense, we studied the antimicrobial potential of different LAB against pathogenic microorganisms [23–25]. *Lactobacillus johnsonii* CRL1647 and

Enterococcus faecium SM21 were isolated from the gut of bees reared in the Northwest of Argentina. These strains were preselected due to their lactic acid production and enterocin-like compounds synthesis with antagonistic activity mainly against *Listeria monocytogenes* [25, 26]. In this work, we evaluated the effects of these LAB on the viability of *B. cereus* vegetative cells and spores. Also, the action of *E. faecium* SM21 metabolites was assayed against vegetative cells of *B. cereus* strains on cooked white rice grains.

Materials and Methods

Lactic Acid Bacteria Strains and Growth Conditions

Lactobacillus johnsonii CRL1647 (GenBank access code EU428007) was grown in de Man Rogosa and Sharpe broth (MRS, Britania, Buenos Aires, Argentina) at 37 °C for 24 h under microaerophilic conditions, obtained with a candle extinction jar. *E. faecium* SM21 (GenBank access code EU428012) was grown in Brain Heart Infusion broth (BHI, Britania, Buenos Aires, Argentina) at 37 °C for 24 h without atmospheric control. Stock cultures of LAB were kept at −20 °C in MRS or BHI broth with 10 % (v/v) glycerol.

Indicator Strains

Seven *B. cereus* strains were used as indicator bacteria for antimicrobial activity. *B. cereus* BAC1 was kindly provided by Dr. Morea (ISPA, Bari, Italy, isolated from a dairy product, [30]). While *B. cereus* 1, 2 and 3 (clinical isolates) and *B. cereus* 4, 5 and 7 (food isolates) were provided by Instituto Nacional de Enfermedades Infecciosas (INEI-ANLIS Dr. C. Malbrán, Buenos Aires, Argentina). In particular, *B. cereus* 1, 2, 4 and 5 were phylogenetically characterized and deposited in the NCBI database under the following accession numbers: JQ322638, JQ322639, JQ322640 and JQ322637, respectively. Before each assay, all *B. cereus* strains were grown on BHI for 12 h at 37 °C.

Preparation of Cell-Free Supernatants

To obtain cell-free supernatant (CFS), *L. johnsonii* CRL1647 and *E. faecium* SM21 were grown in MRS and BHI, respectively. Culture conditions were as

mentioned above. The CFS of the two strains was obtained by centrifugation (10,000×*g*, 10 min at 4 °C) and filtration with cellulose acetate membrane filters (0.22 μm). In both cases, one CFS fraction of each culture was adjusted to pH 6.5 with NaOH (0.05 N) and another fraction was conserved at the final fermentation pH (3.5 for *L. johnsonii* CRL1647 and 5.0 for *E. faecium* SM21). The CFS fractions were kept at 4 °C until use in subsequent assays.

Qualitative Analyses of Antimicrobial Activity: Spot-on-the-Lawn Method and Well-Diffusion Assay

An active 24-h culture (100 μL) of each *B. cereus* strain was inoculated into 10 mL (prepared in tubes) of Mueller–Hinton supplemented with 1.5 % w/v agar (MH, Britania, Buenos Aires, Argentina). The mixture was poured into an empty and sterile Petri dish to obtain a lawn. For the *spot-on-the-lawn* method, 10 μL of an active culture of each LAB strains was spread on the lawn, according to Lewus and Montville [27]. The well-diffusion assay was carried out according to the method by Tagg and McGiven [28] modified by Audisio et al. [29]. Briefly, 23 μL of each of the CFS at final fermentation pH (3.5 for *L. johnsonii* CRL1647; 5.0 for *E. faecium* SM21), and the CFS at pH 6.5, was poured into wells cut into the MH agar previously seeded with the indicator strains. In both cases, plates were incubated for 24 h at 37 °C. Bacteriocin and acid antagonistic activities were determined by inhibition halos around the inoculation spot of the LAB strains (*spot-on-the-lawn* method) or around the wells seeded with CFS (well-diffusion assay) [21, 25].

Quantitative Analysis of the Antimicrobial Activity After Direct Contact with Vegetative *B. cereus* Cells

Twelve-well microplates (BD Falcon™) were filled with 100 μL of a 24-h active culture of each *B. cereus* strain with a concentration of 10^8 colony-forming units (CFU)/mL and supplemented with 3 mL of each sample: the CFS of each LAB at the final fermentation pH (3.5 or 5.0) or the CFS adjusted to pH 6.5. The plates were incubated at 37 °C, and samples were taken at different times during cell growth. Samples were serially diluted in 0.1 % (w/v) meat peptone to evaluate cell viability of *B. cereus* (expressed as CFU/mL). Controls of *B. cereus* growth in MRS and BHI broths were

included, and viable cell counts were done on agar plates of both media. *B. cereus* cells showed a similar growth in all of the culture media tested (namely BHI and MRS).

Effect of the Antimicrobial Compounds After Direct Contact with *B. cereus* Spores

In order to assay the effect of the LAB strains on the *B. cereus* spores, *B. cereus* BAC1 and *B. cereus* 3 were selected and cultured in BHI for 24 h at 37 °C. These cultures were spread out as a lawn on nutrient agar supplemented with 0.05 g/L of manganese sulfate (NAMS) and incubated at 37 °C for 4 days according to Jaquette and Beuchat (1998) [29]. Then, sporulation was observed by microscopic examination. Spores were recovered in sterile peptone water (0.1 % (w/v) meat peptone). A heating–cooling treatment was applied to activate the spores: First, the spores suspension was warmed up for 10 min at 80 °C and then kept for 1 h at 8 °C. The number of spores was determined by the counts of serial dilutions in 0.1 % (w/v) meat peptone on BHI agar plates.

For the direct contact assays, 100 µL of a *B. cereus* BAC1 spores suspension with 10^6 CFU/mL was poured in twelve-well microplates (BD Falcon™). Three milliliters of the CFS of the producer strain was added to the plates and incubated at 37 °C. Samples were taken at different times during cell growth until 24 h of incubation. Serial dilutions were made with 0.1 % (w/v) meat peptone, and the number of spores was calculated by counts on BHI agar plates. Controls were included in each assay by inoculating the *B. cereus* BAC1 spores in 3 mL of MRS or BHI sterile medium.

Preliminary food model: Analysis of the effect of the CFS (pH 6.5) of *E. faecium* SM21 in cooked rice contaminated with *B. cereus* vegetative cells.

Three grams of long-grain white rice (*Oriza sativa* L.) was autoclaved in glass bottles (50 mL) for 15 min at 121 °C. Then, 50 µL of active cultures (10^4 CFU/mL, BHI) of vegetative cells of *B. cereus* BAC1 or *B. cereus* 1 was inoculated on rice. Following, 20 mL of the CFS from *E. faecium* SM21 (pH 6.5) was added. These *B. cereus*-CFS-rice systems were incubated 24 h at 37 °C. Samples were taken at different times (0, 4 and 24 h) to evaluate *B.*

cereus cell viability (expressed as CFU/mL) by cell counts on BHI agar plates. Control of each *B. cereus* strain was grown in cooked rice plus sterile BHI broth. All viable cells were determined in BHI agar at 37 °C.

Production of Organic Acids and HPLC Quantification

The organic acid profile in the CFS of the two LAB strains was determined by HPLC. Prior to the analysis, the CFS from MRS (lactobacilli) and BHI (enterococci) cultures was deproteinized [25]. The HPLC analysis was carried out on a Gilson modular HPLC system with an autoinjector (model 234, Gilson Inc., USA), a piston pump (model 307, Gilson Inc., USA) and an UV detector (model 118, Gilson Inc., USA). A BioRad Aminex HPX-87H ion exclusion column with a diameter of 7.8 mm and a length of 300 mm was used. Running conditions were as follows: the column temperature was 30 °C, and the flow rate of the mobile phase (5 mmol/L H_2SO_4) was 0.6 mL/min. The injection volume was 20 μL. Detection was carried out with at 210 nm. Peak profiles, integration and quantification were obtained with a CR601 Shimadzu chromatopac integrator (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan). All assays were carried out in duplicate.

Statistical Analysis

Software GraphPad Prism 5.0 (2009, GraphPad Software Inc., USA) and Excel (2007, Microsoft Office, USA) were used for statistical analysis of the data obtained. Differences were statistically significant at $P < 0.05$. All assays were carried out in duplicate.

Results

Qualitative Analysis of the Antimicrobial Spectrum

The *spot-on-the-lawn* technique revealed that *L. johnsonii* CRL1647 cells were able to inhibit six *B. cereus* strains: BAC1, 1, 2, 3, 5 and 7, whereas *E. faecium* SM21 only inhibited two indicator strains: *B. cereus* 1 and 5. From these results, we selected *B. cereus* 1, 5 and BAC1 in order to evaluate the inhibitory activity by the well-diffusion assay. By this method, the CFS of *E. faecium* SM21 inhibited two *B. cereus* strains at pH 5.0 and this effect was maintained at pH 6.5 (Table 1). On the other hand, *L. johnsonii* CRL1647

showed inhibitory effect at pH 3.5, whereas no inhibition was observed at pH 6.5.

Table 1

Qualitative analysis of the inhibitory activity of *Lactobacillus johnsonii* CRL 1647 and *Enterococcus faecium* SM21 against *B. cereus* strains by well-diffusion assay

Strain	**CFS pH value**	**Well-diffusion assay**		
		***B. cereus* indicator strain**		
		BAC1	**1**	**5**
E. faecium SM21	5.0	+	−	+
	6.5	+	−	+
L. johnsonii CRL 1647	3.5	+	+	+
	6.5	−	−	−
CFS cell-free supernatant				
(+) Inhibition halo (≥2 mm of diameter)				
(−) No inhibition halo				

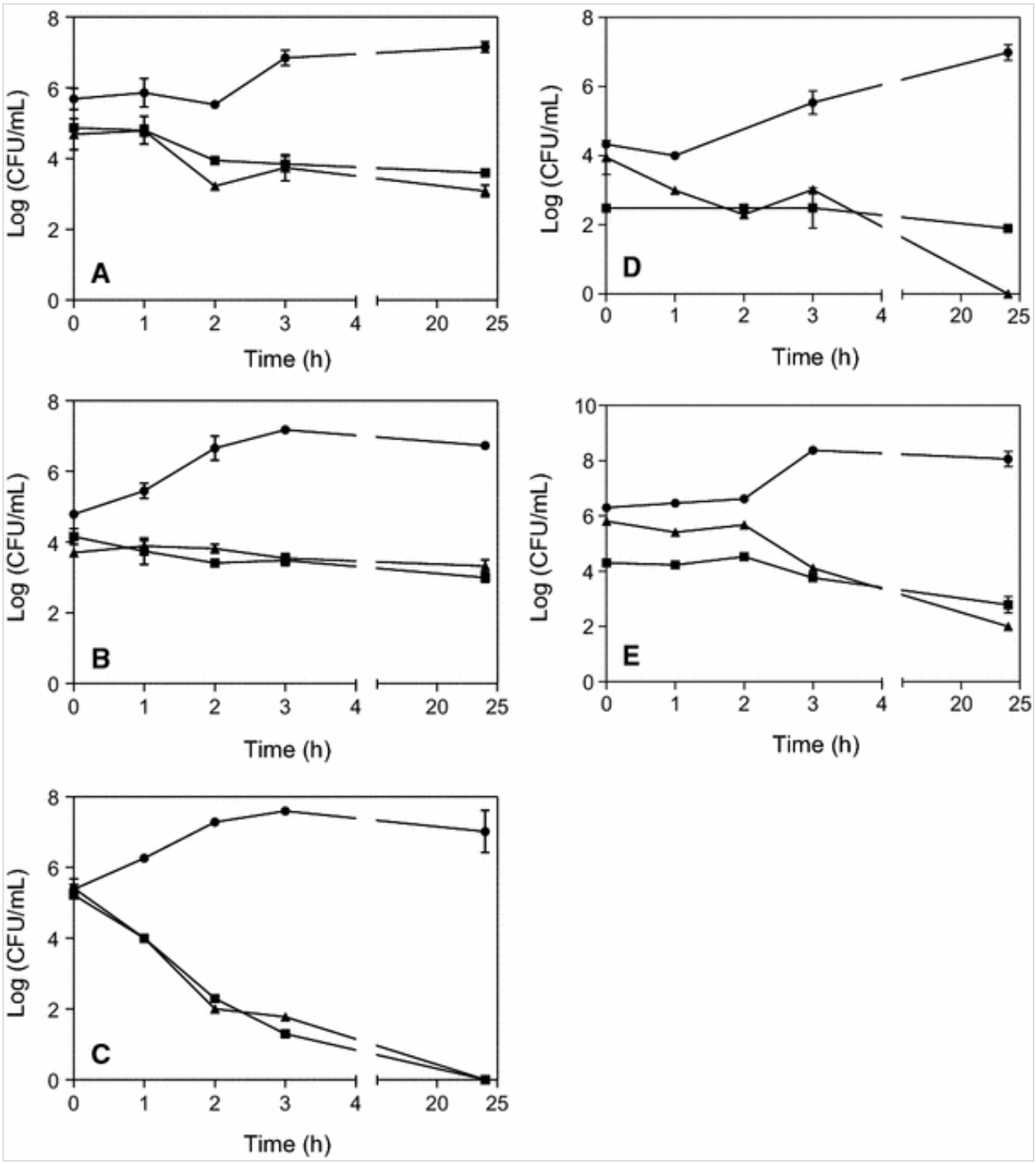
Quantitative Analysis of the Microbial Spectrum After Direct Contact

The effect of the CFS was assayed against the following *B. cereus* strains: *B. cereus* 1 (clinical origin), *B. cereus* 4, 5 and 7 (isolated from food) and BAC1 (isolated from dairy products). Quantitative analysis showed that the CFS from *E. faecium* SM21 inhibited all *B. cereus* strains tested both at pH 5.0 (final pH of the fermentation) and at pH 6.5. A significant reduction in viability of *B. cereus* 1 was observed after 24 h of contact (Fig. 1a) showing a difference of about 4 log units in the number of viable cells compared with the control. Direct contact of the CFS from *E. faecium* SM21 with *B. cereus* 4 showed a reduction of 3 log units after 24 h of incubation (Fig. 1b). When *B. cereus* 5 was exposed to the *E. faecium* SM21 CFS at pH 6.5 and pH 5.0, a strong inhibition was detected, reaching the detection limit of the technique used, after 24 h of contact (Fig. 1c). A different situation was detected with

B. cereus 7; at time zero, there was an immediate reduction in the number of viable cells (one log unit) when it was contact with the CFS at pH 5.0, while, at pH 6.5, the CFS inhibited the pathogen in 7 log units after 24 h of contact (Fig. 1d). Regarding *B. cereus* BAC1, the *E. faecium* SM21 CFS produced a gradual decrease in pathogen viability right from the beginning and reached a difference of nearly 5 log units after 24 h of contact both at pH 5.0 and at pH 6.5 (Fig. 1e).

Fig. 1

Antimicrobial activity of cell-free supernatant (CFS) at pH 6.5 (*filled triangle*), pH 5.0 (*filled square*) of *E. faecium* SM21 against vegetative cells of *B. cereus* 1 (**a**), *B. cereus* 4 (**b**), *B. cereus* 5 (**c**), *B. cereus* 7 (**d**) and *B. cereus* BAC1 (**e**). Viable cells in controls (*filled circle*) were determined in BHI. The average data shown in *graph* are representative for duplicate assays. *Errors bars* corresponding to standard deviations are shown



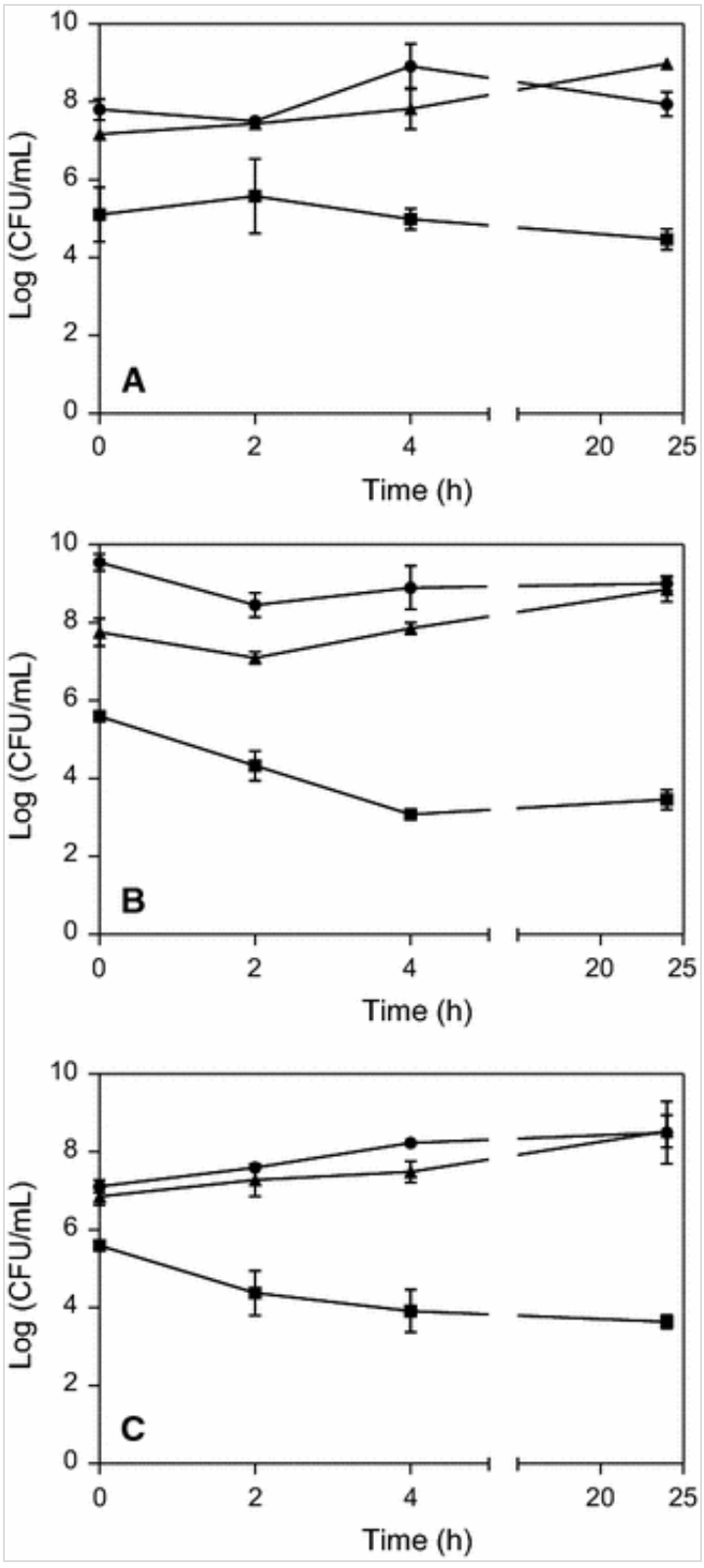
AQ3

The organic acids present in the CFS from *L. johnsonii* CRL1647 caused the immediate inhibition of *B. cereus* 1 upon contact (t_0) at pH 3.5 (final pH of fermentation), generating a pathogen reduction of nearly 3 log units compared with the control. This antagonistic effect was maintained throughout the assay with an additional decrease of 1 log unit after 24 h of contact. However, when the *L. johnsonii* CRL1647 CFS was adjusted to pH

6.5, the inhibitory effect completely disappeared (Fig. 2a). When analyzing the effect of the *L. johnsonii* CRL1647 CFS on *B. cereus* 5 at pH 3.5, the pathogen inhibition was observed at t_0 and until 24 h of contact, with a reduction in the number of viable cells of approximately 4 log units. Again, this antagonistic effect disappeared when the pH of the CFS was adjusted to 6.5, and the viability of the *Bacillus* cells was similar to the control (i.e., 10^7–10^9 CFU/mL, see Fig. 2b). Finally, the acidic CFS from *L. johnsonii* CRL1647 produced a reduction in *B. cereus* BAC1 growth of 2 log units after 2 h of contact, which disappeared after its neutralization and the viability of *B. cereus* BAC1 was similar to the control (Fig. 2c).

Fig. 2

Antimicrobial activity of cell-free supernatant (*CFS*) at pH 6.5 (*filled triangle*), pH 3.5 (*filled square*) of *L. johnsonii* CRL1647 against vegetative cells of *B. cereus* 1 (**a**), *B. cereus* 5 (**b**) and *B. cereus* BAC1 (**c**). Viable cells in controls (*filled circle*) were determined in MRS broth. The average data shown in *graph* are representative for duplicate assays. *Errors bars* corresponding to standard deviations are shown



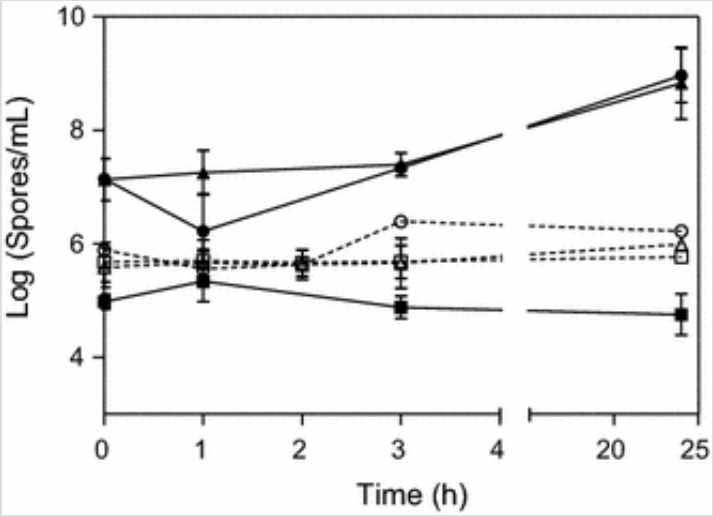
Effect of LAB Metabolites on *B. cereus* BAC1 Spores

The effect of the CFS from both LAB studied against *B. cereus* BAC1 spores is shown in Fig. 3. The *E. faecium* SM21 CFS showed a bacteriostatic effect both at pH 5.0 and at pH 6.5, while the effect of the *L. johnsonii* CRL1647

CFS was pH dependant. Thus, at pH 3.5, the CFS had an important antagonistic effect on the pathogen spores immediately after the first contact which lasted until the end of the assay, while, when the CFS was adjusted at pH 6.5, the spore viability remained similar to the control, *i.e.*, no reduction in the number of spores was detected.

Fig. 3

Antimicrobial activity of cell-free supernatant (*CFS*) of *E. faecium* SM21 (*dashed lines*, *open symbols*; *open triangle* CFS at pH 6.5; *open square* CFS at pH 5.0; ○ control) and *L. johnsonii* CRL1647 (entire *lines*, *filled symbols*: *filled triangle* CFS at pH 6.5; *filled square* CFS at 3.5; *filled circle* control) against spores of *B. cereus* BAC1. The average data shown in *graph* are representative for duplicate assays. *Errors bars* corresponding to standard deviations are shown



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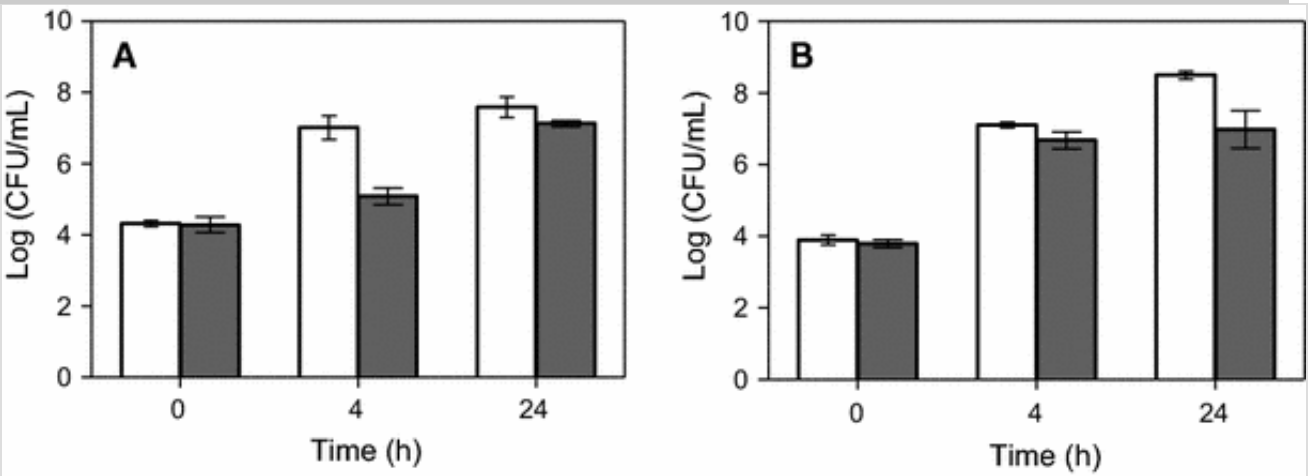
Inhibition of *B. cereus* vegetative cells by the CFS produced by *E. faecium* SM21 in a cooked rice food model.

Figure 4 shows the effect of *E. faecium* SM21 CFS (pH 6.5) against the vegetative cells of two *B. cereus* strains. There was no inhibition of *B. cereus* BAC1 at the first time of contact; however, after 4 h, a difference of 2 log in the viable cells number was measured. While *B. cereus* 1 cells showed a different response to the CFS of *E. faecium* SM21 and behaved like the control during the first 4 h of contact, but after 24 h of incubation, one log unit of difference was observed.

Fig. 4

Antimicrobial activity of cell-free supernatant (*CFS*) at pH 6.5 (*gray bars*) of *E. faecium* SM21 against vegetatives cells of *B. cereus* BAC1 (**a**) and *B. cereus* 1 (**b**) in a rice food model. Controls (*white bars*) were grown in cooked rice plus sterile BHI broth. All viable cells were determined in BHI agar. The average data shown in *graph* are representative for duplicate assays. *Errors bars* corresponding to standard deviations are shown



Quantification of the Organic Acids by HPLC

The organic acid production in MRS and BHI media by *L. johnsonii* CRL1647 and *E. faecium* SM21, respectively, was analyzed by HPLC. *L. johnsonii* CRL1647 synthesized mainly lactic acid, acetic acid and phenyl-lactic acid in MRS medium with concentrations of 128, 38 and 0.3 mmol/L, respectively. *E. faecium* SM21 grown in BHI medium produced lactic acid (24.5 mmol/L), acetic acid (12.2 mmol/L), but no phenyl-lactic acid.

Discussion

The antimicrobial effect produced by LAB is generally associated with the synthesis of lactic acid, hydrogen peroxide, enzymes, bacteriocins, and/or competition for nutrients and space for growth [31–35]. However, there are scarce scientific articles about *B. cereus* inhibition by LAB metabolites. That inhibition has mainly been reported in dairy foods, such as skim milk [36] and cheese [37, 38]. It has also been determined that foods fermented by LAB at pH 4.0 or lower can often inhibit growth of *B. cereus* and other

pathogens [39]. In the current study, we analyzed the anti-*B. cereus* potential of *L. johnsonii* CRL1647 and *E. faecium* SM21, whose antimicrobial activity against different pathogenic bacteria has already been reported [25].

In this work, it was determined that *L. johnsonii* CRL1647 metabolites were active only under acidic conditions. Therefore, the antimicrobial activity of the CFS from *L. johnsonii* CRL1647 against the *B. cereus* strains is due to the synthesis of organic acids. These results agree with those observed by Røssland et al. [39], who studied the effect of lactobacilli and lactococci strains on *B. cereus* growth in reconstituted skim milk. The authors found that a reduction in CFU/mL of *B. cereus* by LAB was correlated with a reduction in pH of co-cultures. They pointed out that the initial fermentation pH reduction rate is critical to control *B. cereus* growth. Similar results were also reported by Nout et al. [40], Aryanta et al. [41] and Kingamkono et al. [42], who observed inhibition of *B. cereus* and other pathogens in LAB-fermented food products at a pH of 4.0 or less. Cho et al. [43], looking for probiotic lactobacilli strains for piglets, analyzed the antimicrobial spectrum of two LAB; however, they only found antagonistic effects against one *B. cereus* strain. To our knowledge, this is the first report of the inhibitory effect of *L. johnsonii* against *B. cereus*, together with the identification and quantification of the metabolites responsible for this inhibition.

In a previous work published, *E. faecium* SM21 has a strong anti-*L. monocytogenes* activity and the genetic determinant to produce different enterocins such as A, B and P [25]. These features are identical to other *Enterococcus* spp. strains, which have been studied mainly due to their bacteriocin synthesis [16, 21, 44, 45]. In this case, the CFS from *E. faecium* SM21 showed an important inhibition against *B. cereus* vegetative cells tested both at pH 5.0 (final fermentation pH) and at pH 6.5, which confirms that inhibition of *B. cereus* is due not only to organic acids but to also enterocins. These results also agree with those informed by Ozdemir et al. [46], who screened 57 enterococcal isolates from different origin for bacteriocin production. Two out of 34 *E. faecium* strains were able to inhibit the growth of one *B. cereus* strain, and the PCR studies showed that these strains harbored enterocins A, B and P genes. Also, it has been reported that two *E. faecium* strains, isolated from cow milk, had antagonistic properties against only one *B. cereus* strain and harbored the entA gene [47]. In this

sense, Do Nascimento et al. [17] analyzed the effect of bacteriocinogenic *E. faecium* strains on different Gram-positive pathogens. They worked with twenty-five *B. cereus* strains which were only inhibited when they were co-cultured with the *E. faecium* cells. However, those *B. cereus* strains were not sensitive to the compounds present in the CFS. Thus, the direct contact assays reported in the present work are quite remarkable, because the metabolites produced by *E. faecium* SM21 were able to inhibit the seven *B. cereus* strains tested. Furthermore, these *B. cereus* were isolated not only from food but also from clinical samples.

Stenfors Arnesen et al. [2] reported that 10^5–10^8 CFU/mL of *B. cereus* vegetative cells or spores is the infective dose for diarrheal illness. For the emetic disease, the same authors pointed out that 10^5–10^8 cells/g is often found in implicated foods, but live cells are not required for intoxication. In the present study, the viable counts of vegetative cells of the different *B. cereus* strains tested were lower than 10^5 after 24 h of treatment with the CFS from both *L. johnsonii* CRL 1647 and *E. faecium* SM21. These results show that the metabolites of both LAB strains were able to reduce the CFU/mL of the pathogen below the known infective dose.

It is well known that spores of various *Bacillus* species can survive for extended periods of time with few or no nutrients through a multitude of mechanisms that protect them from damage during the potentially long periods of dormancy [48]. Their activation can occur before the initiation of spore germination, and a number of agents can be used with this purpose but the most commonly used is a sublethal heat shock [49]. In this study, the *B. cereus* BAC1 spore suspensions were heated at 80 °C for 10 min to activate them and it was determined that they were sensitive to the CFS from *L. johnsonii* CRL1647 at pH 3.5, causing a reduction of 5 log units compared to the control culture after 24 h of contact. This reduction disappeared when *B. cereus* BAC1 spores were exposed to the same CFS but at pH 6.5. These results agree with those found by Wong and Chen [36], who showed that germination of *B. cereus* spores was inhibited at a pH below 4.2. Van Melis et al. [50] also studied the effect of a range of organic acids on the germination of *B. cereus* spores and stated that organic acids can accumulate in the inner membrane of spores, and this accumulation may interfere with the signaling cascade that is required for the germination process.

When the CFS of *E. faecium* SM21 was evaluated on *B. cereus* spores, it was observed that after the spores activation, both CFS samples (at acidic and neutral pH) generated a bacteriostatic effect, inhibiting germination. This bacteriostatic effect can help to prevent or control the outgrowth of this pathogen, reducing the risk of toxins production.

Although production of lactic acid is an essential characteristic of LAB in general and one of the main mechanisms through which these bacteria inhibit their competitors [51–53], the amount of lactic acid produced is strain dependent. Furthermore, susceptibility of pathogens to this compound or other organic acid also varies significantly. In this work, it has been determined by HPLC analyses that *L. johnsonii* and *E. faecium*, both homofermentative lactic acid bacteria, produced not only lactic acid but also acetic acid and in the case of the *Lactobacillus* cells, phenyl-lactic acid. The difference in metabolism can arise from the fact that other acids, such as acetic acid, are produced in addition to lactic acid, as end products of glucose fermentation [54, 55]. In the present assays, *L. johnsonii* CRL1647 inhibited both vegetative cells and spores of the tested *B. cereus* strains, only due to an acidic effect. On the other hand, *E. faecium* SM21 affected the viability of the *B. cereus* strains mainly by antimicrobial metabolites such as enterocins, even at near neutral pH.

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It is well known that the efficacy of bacteriocins in foods will greatly depend on a number of food-related factors (food components, precipitation, inactivation or uneven distribution of bacteriocin molecules in the food matrix), the food microbiota and/or the target bacteria. Therefore, the interactions of these factors must be understood as a dynamic process where there are changes over time [11]. Considering this, we decided to evaluate the effect of the CFS from *E. faecium* SM21 in a preliminary food model and cooked white rice grains were chosen. The rice was sterilized to avoid the presence of competitive microorganism, and only the interaction of the rice matrix and the pathogen was tested. Interestingly, in this case, the antimicrobial effect depended on the *B. cereus* strain assayed. Even though both bacilli showed similar results for the first 4 h of the trial and were inhibited, *B. cereus* BAC1 cells “recovered” and reached CFU values similar

to the control after 2 h. Meanwhile, *B. cereus* 1 was significantly affected up to the end of the assay. Grande et al. [21] found a decrease of *B. cereus* viable cells of 4.7 log units during the first 8 h of incubation of the pathogen in a rice slurry with 20 μg/mL of enterocin AS-48. Indeed, the recovery of viable cells of *B. cereus* BAC1 after 24 h of incubation would indicate that either a possible adaptation of the pathogen to the antimicrobial compounds or their concentration in the CFS was not enough. Galvez et al. [11] have pointed out that a fraction of the population may receive a lethal dose of the antimicrobial factor, leading to cell death, while the remaining fraction may survive as sublethally injured cells as well as cells with increased resistance. Therefore, further research must be done in order to explore the potential application of this CFS as a part of a hurdle technology.

Conclusions

The present study has shown that the cell-free supernatant of two LAB strains studied inhibited the growth of different *B. cereus* strains. In particular, the compounds produced by *E. faecium* SM21 showed a similar anti-*B. cereus* effects in a preliminary rice food model. Hence, these metabolites can be effective to define future strategies for biological control of *B. cereus*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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