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Antimicrobial Activity Of Bifidobacterial Cells And Supernatant Against *B. Cereus* ATCC 9884

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Abstract
The aim of this work was to test three experimental strains (isolated from the faeces of exclusively breast-fed babies: Bbv1, Bbv2 and BLE) and a reference strain (BLR) of bifidobacteria for their antagonistic capacity towards *Bacillus cereus* ATCC 9884. The antagonism of these strains was studied using two methods: one consisted of co-cultivating them at 37°C with *B. cereus* and the other of bringing the concentrated supernatant of their culture into contact with that of *B. cereus* at 37 and 6°C. The effect of a series of organic solvents on the inhibitory power of bifidobacteria strains towards *B. cereus* was also explored. The results obtained indicate that at 37°C, the concentrated supernatants from bifidobacteria cultures exert an antagonistic effect towards *B. cereus* that is much greater than that observed when bifidobacteria strains are co-cultured with the pathogen strain under study. At low temperatures (6°C), the inhibitory power of the concentrated supernatants of the bifidobacteria cultures on *B. cereus* is greatly reduced and spread out over time, since 48h of contact allows only 60% inhibition on average. The speed of inhibition of *B. cereus* by bifidobacteria supernatants also highlights this latency of action at 6°C. Organic solvents such as formaldehyde, chloroform, acetone, hexane, isobutanol, methanol and diethyl ether had no effect on the antagonism of the concentrated supernatants from the bifidobacteria cultures tested against this pathogenic germ. This suggests that these solvents do not denature protein substances with antagonistic (i.e. bacteriocin-like) activity.

Key words: Bifidobacteria, *Bacillus cereus*, Antagonistic capacity, Concentrated supernatant, Organic solvents, Bacteriocin-like activity

1-Introduction

Human or animal poisoning can result from drinking water or ingesting foods not properly inspected or treated for the presence of bacteria or toxins (Naimushin et *al.,* 2002). The contamination of food products with endosporeforming bacteria is a common problem in the food industry, because endospores require more intense treatments for inactivation than vegetative cells. This results in higher processing costs and less preserved product quality. Risks associated to endospore survival during processing are especially significant in canned foods, and acidification is often used as an additional hurdle to prevent microbial growth and spoilage (Garcia et *al.,* 2003).

Bacillus cereus is an opportunistic human pathogen found to be the infectious agent of endophthalmitis and food poisoning, sometimes lethal (Drobniewski, 1993, Granum, 1997). The *Bacillus cereus* organisms are common in nature, and due to their resistant endospores, they may survive different stresses during food production, e.g. drying and heat treatment (Rosenquist et *al.,* 2005). *B. cereus* psychrotrophic strains are able to germinate and to grow during refrigerated storage; and may limit the keeping quality of pasteurized milk (Svensson et *al.,* 2006).

They elaborate several toxins, including a necrotizing enterotoxin, an emetic toxin, phospholipases, proteases, and hemolysins (Drobniewski, 1993). The emetic syndrome is caused by the ingestion of the heat-stable emetic toxin (cereulide, a cyclic peptide of 1.2 kDa) produced in foods. The diarrheal syndrome is mainly due to the ingestion of *B. cereus* cells in the foods, followed by toxin production in the small intestine (Kramer and Gilbert, 1989; Granum and Lund, 1997). A wide range of food products has been implicated in diarrhoeal food poisoning, e.g., meat, soups, milk, vegetables, and puddings (Svensson et *al.,* 2006).

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Bifidobacteria are Gram-positive, non-motile, and non-spore-forming bacteria. They are part of normal intestinal microbiota in humans and animals, and are generally non-pathogenic (Delcenserie et *al.*, 2007). It is claimed that a high number of bifidobacteria are beneficial for their hosts' health (Orrhage, and Nord, 2000). It is reported that these organisms can exert beneficial effects including the reduction of serum cholesterol, activation of the immune system and inhibition of the growth of potential pathogens that may cause infectious disease in the host (Garro et al., 2006). They are known to produce lactic acid, acetic acid, ethanol, diacetyl, carbon dioxide, hydrogen peroxide, reuterin, derivatives of lactic acid such as hydroxy lactic acid, and also small peptides designated bacteriocins (Millette et *al.*, 2007).They are also reportedly antagonistic towards different pathogens (Kanbe, 1991). The *Bifidobacterium* strains showed strong antibacterial activity against Gram-negative bacteria. This activity was due to the production of organic acids, in particular, acetic acid and lactic acid. The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm. Nevertheless, it was found that several *Bifidobacterium* strains produced antibacterial substances, different from organic acids. These compounds, present in concentrated Cell-free culture supernatants of bifidobacteria, were mostly active against a narrow range of Gram-positive bacteria (Makras and De Vuyst 2006). Bifidobatreria appear to reduce the incidence of rotaviral infection, traveller's diarrhea and antibiotic associated diarrhea (McNaught and MacFie, 2001).

The major aim of this study is to exhibit high probiotic potential of the bifidobacterial strains isolated of healthy infant feaces to evaluate the antagonistic activity against *B. cereus*.

2-Materials and Methods

2.1- Strains origin

The strains used in this study were from various sources. *Bacillus cereus* ATCC 9884 (American Type Culture Collection; Rockville, MD, USA), *Bifidobacterium longum* (BLR) was generously provided by Anna Maria Ferrari (Diplomento di Sienz E Technologie alimentaire Microgioologich Sezione, Microbiologia Agaria Alimentare Ecologica Universita Degliz Studidi Milano : Italia) and three experimental strains isolated in our laboratory (Bb_vE1, Bb_vE2 and BLE) confirmed by the F6PPK activity which described by Scardovi, (1986) and carbohydrates fermentation.

2.2-Culture media

All bifidobacteria strains was incubated anaerobically at 37°C for 48h in De Man Rogosa and Sharpe medium (MRS 1.10661.0500 Merck, "Germany") containing 0.05% cysteine-HCl (Sigma, "St-Louis"), we use also MRS agar (Cat:104300, Pronadisa laboratoire conda Espane) for agar spot cellular contact.
The *B. cereus* ATCC 9884 was activated through two consecutive incubations of 18h in 9ml of Brain heart Infusion broth (BHI 5100.9 Bio-merieux, "France") and incubated at 37°C. After contact we use Ringer medium (1.155.25.01 Merck, "Germany") for different dilution and Mannitol Agar with sodium chlorur and red phenol (1.05404 Merck, "Germany") for counting.

2.3-Preparation of bifidobacteria supernatants

Supernatants of bifidobacteria were obtained from 48h old cultures in MRSc broth. Cultures were centrifuged at 7000g at 4°C for 20min and filter sterilized (0.45µm). These supernatants were concentrated at 1/10.

2.4-Antagonistic effect on *Bacillus cereus*

This study Brings to light the *in vitro* bifidobacterial antibacterial activity against *B. cereus* ATCC 9884 (by cellular and supernatant contact).

2.4.1-Cellular contact

On broth

The principle of this method is the cultivation of the bifidobacterial culture with *B. cereus* culture, the report of contact *Bifidobaterium* with *B. cereus* is 2:1; for control we cultivate pathogenic culture with MRSc alone. Ringer medium is used for different dilution and Mannitol Agar with sodium chlorur and red phenol for counting of viable cells. The times of contact are 2, 4, 6, 12, 24, 36 and 48h (Bielecka *et al*., 1998).

On Agar

For detection of antimicrobial activity of *Bifidobacterium* strains against *B. cereus* a modification of the agar spot test described by Fleming et al., (1975) was used. One colony of each strain *Bifidobacterium* was taken and spotted in the MRS agar, dried and incubated anaerobically at 37°C for 24h and we sink 10ml of Mannitol Agar with sodium chlorur and red phenol agar containing 10^8 CFU/ml of *B. cereus* and we incubate at 37°C for 24h.

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2.4.2-Supernatant contact

On broth

After number adjustment of *B. cereus*, we put in contact with the concentrated supernatant and incubated aerobically in two different temperatures, 6 and 37°C at 2, 4, 6, 12, 24, 36 and 48h. After different dilutions in Ringer medium, we use Mannitol Agar with sodium chlorur and red phenol for counting viable cells (Topisirovic et *al.*, 2006).

On Agar

Inhibitory power of supernatant by the agar diffusion inspired to the method of Makras and De Vuyst, 2006 and was determined by a number adjustment of *B. cereus* on Mannitol Agar with sodium chlorur and red phenol Agar; the supernatants were spotted and incubated aerobically 24h at 37°C.

2.5-Treatment of the *Bifidobacterium* supernatant by solvent

100µl of different solvents (diluted at 10%) such as Formaldehyde, Chloroforme, Acetone, Hexane, Isobutanol, Methanol and Ethyl di-ether were added at 10ml of supernatants and evaporated in a centrifugal concentrator. Dried were reconstituted with sterile deionised water to 10ml (Bhunia et *al.*, 1988) and assayed for antimicrobial activity against *B. cereus*.

2.7-Statistical analysis

Each experiment was independently replicated three times in a completely randomized design. Analysis of variance (ANOVA) was performed using StatBox version 6.40 (Copyright Grimmer logiciels 1997-2002). Significant differences were accepted at $P<0.05$ level.

3-Results and discussion

3.1-Cellular contact

This study shows that the different strains of *Bifidobacterium* cultures reduced the *B. cereus* ATCC 9884 population within 24h, the differences in the degree of inhibition were observed from the diameter of inhibitory zones Fig. 1 which vary enter 7 and 9.33 mm Fig. 2 (7.67, 9.33, 9.33 and 7 mm respectively for Bbv1, Bbv2, BLE and BLR).

The results of the cellular contact in broth medium (Fig. 3) demonstrate a great antagonist effect of *Bifidobacterium* strains against *B. cereus* ATCC 9884. The slow inhibitory rate in the six first hours (0.13, 0.14, 0.23 and 0.21 LOG cfu/mL after 2 hours, 1.04, 1.07, 1.25 and 1.19 LOG cfu/mL after 4 hours and 1.51, 1.54, 1.70 and 1.73 LOG cfu/mL after 6 hours of cellular contact respectively for Bbv1, Bbv2, BLE and BLR) steady by an important decrease after 12 hours of cellular contact for all strains (6.26, 6.87, 6.79 and 7.03 LOG cfu/mL respectively Bbv1, Bbv2, BLE and BLR) to the total inhibitory after 24 hours. Noriega et *al.*, 2003 have also observed an antagonistic activity of *B. infantis* ATCC 15702 against *B. cereus* in a fermented bifidus milk, but the decrease starts after 12 hours of contact; then, the total inhibitory is observed after 14 days during the refrigerated storage and compared with the control the different is highly significant.

In the study of Rossland et *al.*, 2003 *Lactobacillus casei* 2756 exert an inhibitory effect against different strains of *B. cereus* (NVH 38, NVH 45, NVH 68 and NVH 74) to a total inhibitory after 48 hours of contact in 10% reconstituted skimmed milk at 30°C. Two years later, Rossland et *al.*, 2005 found an inhibitory effect of different strains of *Lactobacillus* and *Lactococcus* against the same strains of *B. cereus* (NVH 38, NVH 45, NVH 68 and NVH 74).

Antagonism refers to the inhibition of other (e.g. undesired or pathogenic) microorganisms, caused by competition for nutrients, and by the production of antimicrobial metabolites (Holzapfel et *al.*, 1995). However, some authors suggest that the production of organic acids is the sole factor responsible for the antagonistic activity of bifidobacteria (Fooks and Gibson, 2003; Ibrahim and Bezkorovainy, 1993). Rossland et *al.*, (2003) and (2005) related the inhibitory effect of different *Lactobacillus* and *Lactococcus* cultures to organic acids. Organic acids, in particular acetic acid and lactic acid, had a strong inhibitory effect (Makras and De Vuyst, 2006), lactic acid may reduce pH to a level where toxinogenic bacteria will either be inhibited or destroyed (Holzapfel et *al.*, 1995) and because of its higher dissociation constant, acetic acid (pK_a 4.75) shows stronger inhibition than lactic acid (pK_a 3.1) at a given molar concentration and pH (Holzapfel et *al.*, 1995). The production of other antibacterial compounds cannot be excluded (Makras and De Vuyst, 2006).

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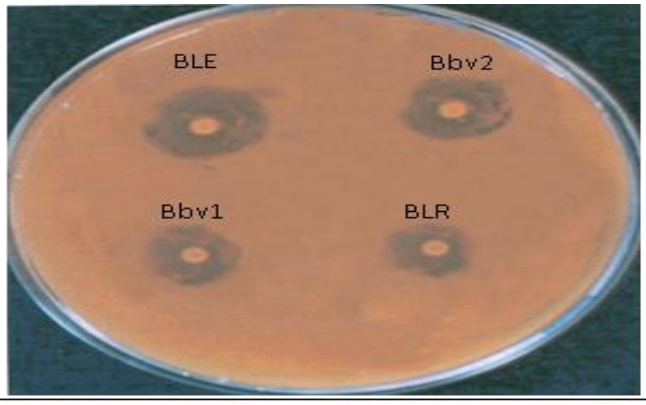

Figure 1. Antagonistic effect of different strains of *bifidobacterium* against *B. cereus* ATCC 9884 by cellular contact in agar.

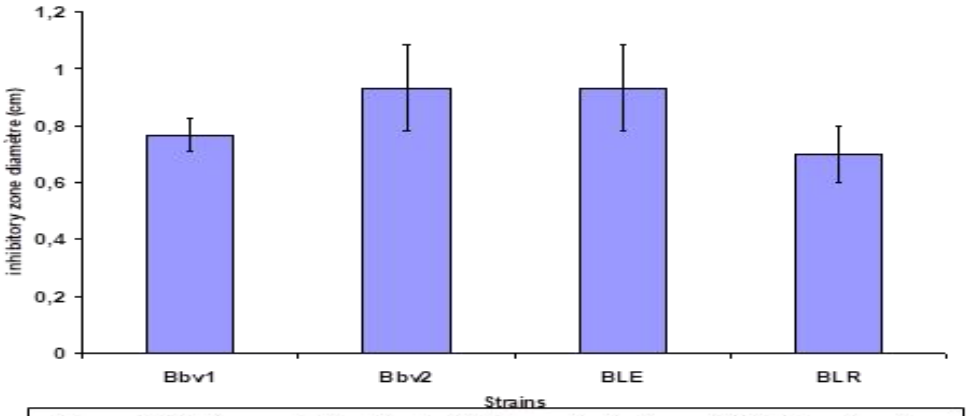

Figure 2. Antagonistic effect of different strains of *bifidobacterium* against *B. cereus* ATCC 9884 by cellular contact in agar.

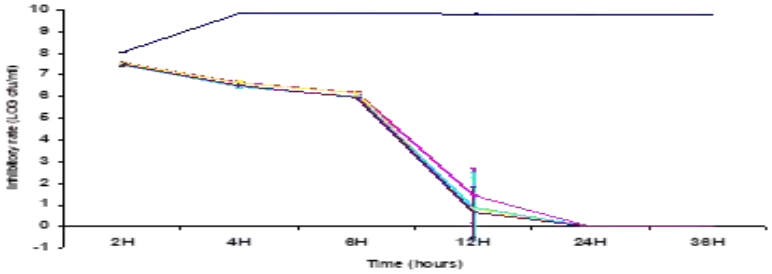

Figure 3. Antagonistic effect of different strains of *bifidobacterium* against *B. cereus* ATCC 9884 by cellular contact in broth.

Control Bbv1 Bbv2 BLE BLR

3.1-Supernatant contact

The selectivity of MRSc appear clearly with 3 mm diameter in the supernatant contact with the pathogenic cell.

The bifidobacterial supernatant exert a large inhibitory effect (Fig. 4) against *Bacillus cereus* ATCC 9884 at 37°C and after 24 hour of contact on agar medium (with 13.67, 13.33, 15 and 16.33 mm of clear halo respectively for Bbv1, Bbv2, BLE and BLR).

The medium supernatant contact at 6°C reveals trifling levels of antagonistic activity at each contact time (Fig. 5). The inhibition level of Bbv1 recorded don't exceed 56% after 48 hours of contact (with 0.10, 0.97, 1.96, 2.65, 3.25, 3.95 and 4.30LOG cfu/ml respectively at 2, 4, 6, 12, 24, 36 and 48 hours of contact), Bbv2 also don't exceed 54% (with 0.12, 1, 1.83, 2.66, 3.28, 3.89 and 4.15 LOG cfu/ml respectively at 2, 4, 6, 12, 24, 36 and 48 hours of contact). *B. cereus* ATCC

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9884 sustain the same losses in contact with supernatant of BLE and BLR and demonstrate an maximal inhibitory respectively under 57% and 60% (with 0.12, 1.08, 2.04, 2.72, 3.43, 4.05 and 4.33 LOG cfu/ml for BLE and 0.13, 1.24, 2.40, 2.82, 3.38, 4.13 and 4.55 LOG cfu/ml for BLR respectively at 2, 4, 6, 12, 24, 36, 48 hours of contact). At 37°C the bifidobacterial supernatant behave differently and exert a great inhibitory effect. After 2 hours 0.57, 0.51, 0.63 and 0.60 LOG cfu/ml of inhibitory effect were recorded respectively for Bbv1, Bbv2 BLE and BLR, after 4 hours the inhibitory effect exceed 20% (1.56, 1.54, 1.88 and 1.84 LOG cfu/ml) and plus to 29%

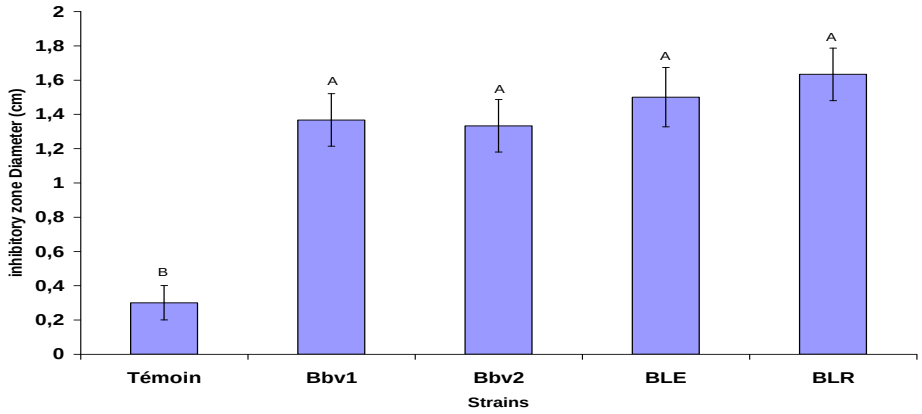

Figure 4. Antagonistic effect of different strains of *bifidobacterium* against *B. cereus* ATCC 9884 by supernatant contact in agar.

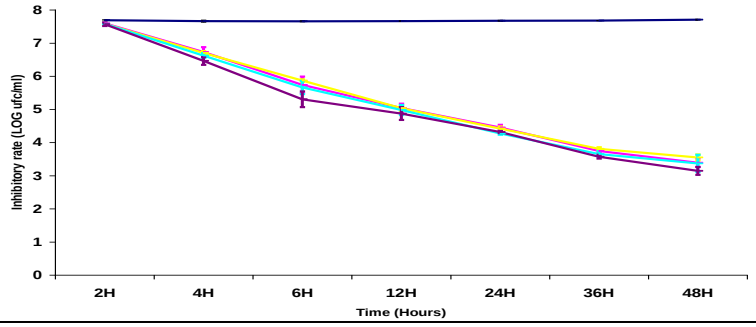

Figure 5. Antagonistic effect of different strains of *bifidobacterium* against *B. cereus* ATCC 9884 by supernatan contact in broth at 6°C.

Control Bbv1 Bbv2 BLE BLR

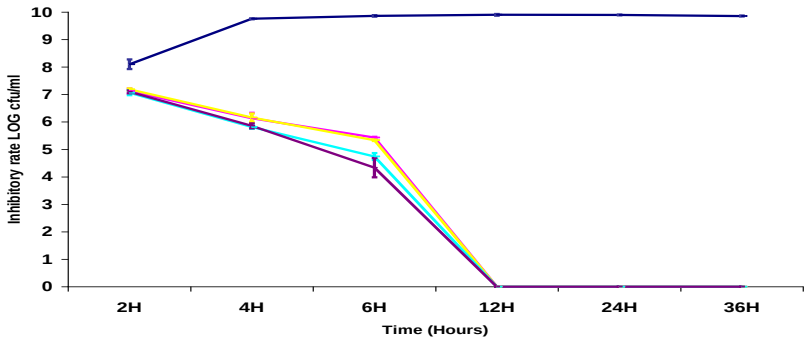

Figure 6. Antagonistic effect of different strains of *bifidobacterium* against *B. cereus* ATCC 9884 by supernatan contact in broth at 37°C.

Control Bbv1 Bbv2 BLE BLR

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after 6 hours (2.27, 2.36, 2.96 and 3.37 LOG cfu/ml) to the total inhibitory after 12 hours of contact.
In our results the bifidobacterial supernatant exert a great antimicrobial activity against *B. cereus* ATCC 9884 mostly at 37°C where that attain 100% of inhibition. Many mechanisms have been postulated by which bifidobacteria could produce antimicrobial activity (Servin, 2004). Recent studies directed by Topisirovic et *al.,* 2006 have also observed an antibacterial activity of *Lactobacillus helveticus* BGRA43 supernatant against *Bacillus cereus* ATCC 11778 by agar spot method which induce to an inhibition zone up to 4 mm.
The production of one or more antimicrobially active metabolites is part of the complex mechanism by which a culture becomes established in the presence of other competing organisms; (Holzapfel et *al.,* 1995). The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm (Makras and De Vuyst, 2006). In two different studies directed by Rossland et *al.,* (2003) and (2005) and Bengharbi et al., (2024). they find that at a slower acid production rate, produced by different strains of *Lactococcus* and *Lactobacillus*, *B. cereus* have the opportunity to sporulate.
Makras and De Vuyst, 2006 found that *B. longum* exert a good antagonistic activity compared with *B. breve* and the concentration of organic acids present in cell-free supernatant of *B. longum* is superior (60.9mM for acetic acid and 39.9mM for lactic acid) to *B. breve* (33.2mM for acetic acid and 12.5mM for lactic acid) this is in accord with our results. Noriega et *al.,* 2003 remark a great production of lactic and acetic acids who justify the inhibitory of *B. cereus* by the bifidus milk.
In numerous reports it has been suggested that other inhibitory substances may contribute to the antagonistic activity as well (Klaenhammer, 1988; Bielecka, et *al.,* 1998; Enan, 2000; Messi et *al.,* 2001; Abriouel et *al.,* 2002; Hechard et Sahl 2002; Servin, 2004; Gagnon, et *al.,* 2004; De Kwaadsteniet et *al.,* 2005; Grande et *al.,* 2006).
The study of Grande et *al.,* 2006 indicate that In controls rice gruel incubated at 37°C, dense populations of bacilli were observed after 24 h. However, in gruels supplemented with enterocin AS-48, no viable cells were detected during the whole test period. At 6°C, a bacteriocin concentration of 20 µg/ml only produced some inhibition of bacterial growth in gruels inoculated with vegetative cells, but no viable cells were detected in samples supplemented with 30 µg/ml. In another study directed by Abriouel et *al.,* 2002 found a difference in the effect of the bacteriocin AS-48 (at different concentrations 5 and 10 µg/ml) against different strains of *B. cereus* (CECT 131 and LWL1) at a different temperatures (37, 15 and 5°C). total inhibitory of *B. cereus* (after 4 hours four CECT131 and 24 hours for LWL1) at 37°C and a great decrease in the other temperature without total inhibition after 72 hours. And they explain this difference in the inhibitory by the difference in the fatty acid composition at 37°C and 5°C.

3.3-Treatment of the *Bifidobacterium* supernatant by organic solvent

Organic solvent don't affect the antimicrobial activity of the bifidobacterial supernatant (Table 1). Yildrim et *al.,* 1998 found the same result with bifidocin B the bacteriocin produced by *Bifidobacterium bifidum* and treated by different organic solvents.

Table 1. Activity of bifidobacterial supernatant on *B. cereus* ATCC 9884 treated by organic solvent.

	Bbv1	Bbv2	BLE	BLR
Formaldehyde	+	+	+	+
Chloroforme	+	+	+	+
Acetone	+	+	+	+
Hexane	+	+	+	+
Isobutanol	+	+	+	+
Methanol	+	+	+	+
Ethyl di-ether	+	+	+	+

4. Conclusion

This study has demonstrated in vitro the antagonistic activity of four strains of bifidobacteria, three of which come from our laboratory's collection, against the pathogenic strain *Bacillus cereus* ATCC 9884. The inhibitory action of the bifidobacteria strains at 37°C was clearly demonstrated by the development of clear halos more than 8 mm in diameter around the pathogen when cocultured with bifidobacteria cells for 24 hours, and more than 14 mm in diameter when concentrated supernatants from bifidobacteria cultures were brought into contact with *B. cereus*. The inhibition kinetics of the two pathogenic germs by the different strains of bifidobacteria at 37°C differed depending on whether they were confronted with the bifidobacteria probiotic cells themselves, or rather with the supernatants from their cultures. In fact, the concentrated supernatants from the bifidobacteria cultures proved to be more effective and faster in this antagonistic action against *B. cereus*. Cellular comparison of *B. cereus* ATCC 9884 and different strains of bifidobacteria showed that there is significant bifidobacteria antagonism, manifested by clear halos of varying diameters from 7 to 9.33 mm.

Co-culture of these protagonists at 37°C showed that after only 12 h of cohabitation, more than 80% of *B. cereus* cells were inhibited. After 24 hours of co-culture, not a single living cell of the pathogen studied remained. Still at 37°C, the concentrated supernatants from the bifidobacteria cultures had a greater and more rapid effect on the viability of *B. cereus*, which was eliminated after just 12 hours of contact. The bifidobacteria strains BLE and BLR are the most effective in this antagonistic action. Thus, the kinetics of inhibition of *B. cereus* by the supernatants of bifidobacteria cultures at 37°C is totally different from that generated by the confrontation of the cells with each other, since it does not display as great an action latency. Still at 37°C, the concentrated supernatants from the bifidobacteria cultures had a greater and more rapid effect on the viability of *B. cereus*, which was cancelled out after only 12 hours of contact. The bifidobacteria strains BLE and BLR are the most effective in this antagonistic action. Thus, the kinetics of inhibition of *B. cereus* by the supernatants of bifidobacteria cultures at 37°C is totally different from that generated by the confrontation of the cells with each other, since it does not display such a significant latency of action. It would appear that *B. cereus* is more resistant to the antagonistic action of supernatants at low temperatures, probably by adapting its fatty acid composition. Organic solvents such as formaldehyde, chloroform, acetone, hexane, isobutanol, methanol and diethyl ether had no effect on the antagonistic action of concentrated supernatants from bifidobacterial cultures against the pathogen tested. This suggests that these solvents do not denature protein substances with antagonistic (i.e. bacteriocin-like) activity.

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