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Antibacterial activity of Bifidobacterial cells and supernatant against *S. aureus*

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Abstract

Background: Bacteria have always been present in our food. Some of them, such as lactic acid bacteria, are useful, while others, such as pathogenic bacteria, are not, because of the many problems of food poisoning and illness that they cause, as well as the multiple spoilage of foodstuffs that they provoke. As a result, food safety is a constant concern. **Methods:** Three experiment (Bbv1, Bbv2 and BLE) confirmed by the F6PPK activity and carbohydrates fermentation and one reference bifidobacterial (BLR) strains were examined for there antimicrobial activity against *S. aureus* 49444 in broth and agar by cellular and supernatant contact. **Results:** In associated cultures, Bifidobateria strains exhibit a strong antagonistic activity towards *S. aureus* 49444 by agar spot contact (plus to 8mm for all strains) or in broth contact (total inhibitory after 24 hours of contact). The bifidobacterial supernatant contact by agar spot method shows a good inhibitory activity (the inhibitory zones exceed 8mm). The supernatant contact results in broth medium demonstrated a significant reduction of *S. aureus* level (plus to 61% after 48 hour) with bifidobacterial supernatant contact incubated at 6°C. At 37°C a great reduction of *S. aureus* population was noticed to a total destroy (after 12 hour for Bbv1, Bbv2 and 24 hour of contact for BLE and BLR). The bifidobacterial antimicrobial activity of supernatant was resistant to different organic solvents. **Conclusion:** The results of this study give us a clear idea of the effectiveness of the Bifidobateria strains tested against *S. aureus*, and constitute a promising means of biological control for future tests against pathogenic microorganisms.

Keywords: Bifidobacteria; supernatant; antibacterial activity; *Staphylococcus aureus*.

1-Introduction

The term probiotic (pro-life ; opposite of antibiotic) had previously been used to describe other growth-promoting substances but is now exclusively used in accord with the context as proposed by fuller, 1989 “a live microbial feed supplement which beneficially affect the host animal by improving its microbial balance” (O’sullivan and Kullen, 1998). Lactobacillus and Bifidobacterium spp. Are predominant members of the commensal intestinal flora (Savagodo et *al*., 2006). In terms of numbers of bacterial cells, bifidobacteria are the most obvious of the lactic acid bacteria with regard to the human intestinal microflora but lactobacilli are commonly present (Tannock, 1995), they dominate the indigenous microbiota of infants, which is established shortly after birth, but their number decreases in adults, then becoming the third more abundant genus after Eubacterium and Bacteroides (Guarner and Malagelada, 2003) and they are considered beneficial to all age groups because they help build resistance to infection in the host, it has been suggested that bifidobactérie play a role in acting as a barrier against colonisation the gastrointestinal tract and they exert the antagonistic activity exerted against pathogens (Lievin et *al*., 2000). Numerous studies report on the inhibition of a wide range of pathogenic microorganisms by bifidobacteria either in vitro or in vivo, (Servin, 2004; Makras, and De Vuyst 2006).Therefore, there exist considerable interest in the use of fermented milk product with bifidobacteria for probiotic purposes (Garro et *al*., 2004).

Staphylococcus aureus is a major bacterial pathogen responsible for a broad and divergent range of human and animal infections, including toxin-mediated food borne diseases (Ananou et *al*., 2005). The symptoms of food poisoning in humans are mainly due to the secretion of emetic and pyrogenic toxins named staphylococcal enterotoxins (Fueyo et *al*., 2005). The staphylococcal enterotoxins comprise a family of at least 10 structurally related toxins that share the ability to hyperstimulate T cell populations (Poli et *al*., 2002). The ingestion of 0.2–1.0µg of staphylococcal enterotoxins secreted by approximately 10^6/g *S. aureus* can provoke nausea, vomiting, retching, abdominal cramping, sweating, chill, prostration, weak pulse, shock, shallow respiration, and subnormal body temperature (Millette et *al*., 2007). In addition to common forms of food poisoning, staphylococcal enterotoxins may be important in several other human diseases such as sudden infant death syndrome (Newbould et al., 1989) and Kawasaki syndrome (Leung et al., 1993).

The aim of the present study were to isolate and characterize bifidobacterial strains exhibiting high probiotic potential and to evaluate the effectiveness of these isolates at inhibiting *S. aureus*.

2-Materials and Methods

2.1- Strains origin

The strains used in this study were from various sources. *Staphylococcus aureus* 49444 (Microbiologics CE, St cloud MN, 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).

The *S. aureus* 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 Baird Parker agar (1.05406 Merck, "Germany") additionned to egg yolk tellurite 20% emulsion (1.03785 Merck, "Germany") for counting.

2.3-Phenotypic tests

Three Algerian healthy new born faecal isolates were assigned to the genus *Bifidobacterium* through detection of fructose-6-phosphate phosphoketolase activity described by Scardovi, (1986), successed by different tests of Gram, spores formation, catalase indole production and motility. The carbohydrate fermentation is based on the use of MRSc medium with bromocresol purple and supplemented with different sugar. All incubated under anaerobic conditions (warranted by GasPak system, Oxoid, Basingstoke, Hampsphire, England) at 37°C.

2.4-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.

2.5-Antagonistic effect on Staphylococcus aureus

2.5.1-Cellular contact

On broth

The principle of this method is the cultivation of the bifidobacterial culture with *S. aureus* culture, the report of contact *Bifidobaterium* with *S. aureus* is 2:1; for control we cultivate

pathogenic culture with MRSc alone. Ringer medium is used for different dilution and Baird parker agar 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 *S. aureus* 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 Baird parker agar containing 10^8 CFU/ml of *S. aureus* and we incubate at 37°C for 24h.

2.5.2-Supernatant contact

On broth

After number adjustment of *S. aureus* we put in contact with the concentrate supernatant and incubated aerobically in two different temperature 6 and 37°C at 2, 4, 6, 12, 24, 36 and 48h. After different dilutions in Ringer medium, we use Baird Parker for counting viable cells (Topisirovic et *al*., 2006).

On Agar

Inhibitory power of supernatant by the agar diffusion was determined by a number adjustment of *S. aureus* on Baird parker Agar; the supernatants were spotted and incubated aerobically 24h at 37°C.

2.6-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 was 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 *S. aureus*.

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). A significant differences were accepted at $P<0.05$ level.

3-Results and discussion

3.1-Physiological and biochemical characteristics

All strains isolated presented a white, punctiform circular and regular contour of colony. All were Gram positive, negative for catalase, indole, motility and non-spore-forming bacteria. The F6PPK confirm the membership of the isolate strain to the genre *Bifidobacterium*.

The carbohydrate fermentation profiles of 48h incubation (Table 1) show the fermentation of glucose, lactose, fructose, inuline and galactose by all strains studied. Indeed, bifidobacteria have high affinity for these carbohydrates. On the other hand and contrary to Bbv1 and Bbv2, BLE use the arabinose the key of identification of *Bifidobacterium longum* (Tamime et *al.*, 1995) and can not ferment the salicine. The different carbohydrate fermentation indicat the membership of the Bbv1 and Bbv2 to *B. breve*.

Table1: Physiological and biochemical characteristics of isolated bifidobacteria.

Characteristics	**Bbv1**	**Bbv2**	**BLE**
Colony	White, Regular contour and punctiform	White, Regular contour and punctiform	White, Regular contour and punctiform
F6PPK	+	+	+
Gram	+	+	+
Catalase	-	-	-
Indole	-	-	-
Spores	-	-	-
Motility	-	-	-
Glucose	+	+	+
Arabinose	-	-	+
Cellobiose	+	-	-
Fructose	+	+	+
Galactose	+	+	+
Inuline	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	-	-	-
Raffinose	+	+	+
Salicine	+	+	-
Sorbitol	+	-	-
Amidon	-	-	-
Xylose	-	-	-
Glucose	+	+	+
Salicine	+	+	-

3.2-Antagonistic effect on Staphylococcus aureus

3.2.1-Cellular contact

On agar

The antimicrobial potential of bifidobacteria cells against *S. aureus* is presented in Fig 1, Antagonistic activity was mesured by clear zone diameter around bifidobacteria colonies. The fig 2 indicate clearly the significant inhibitory degree ($P \leq 0.05$) of bifidobacterial strains against *S. aureus* 49444 who exceed 8 mm for all bifidobacterial strains after 24 hour of contact in solid medium.

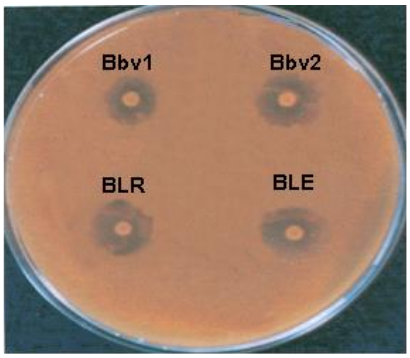

Figure 1. Antagonistic zone between bifidobacterial strains against S. aureus 49444.

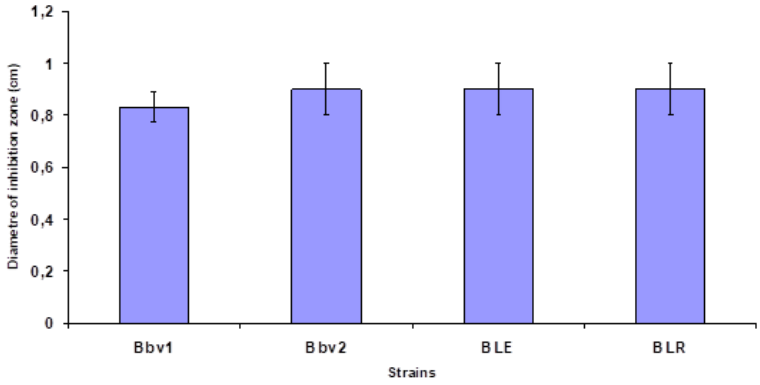

Figure 2. Antagonistic effect of different strains of *bifidobacterium* against *S. aureus* by cellular contact in agar

On broth

After a slight diminution of *S. aureus* 49444 viable cells (de l'ordre de 0.17, 0.19, 0.23 et 0.24 cfu ml^{-1} respectivly for Bbv1, Bbv2, BLE and BLR) after 2 hours (Fig 3) of contact with bifidobacterial strains, the inhibition pass of 1.07, 1.16, 1.22 and 1.12 cfu ml^{-1} after 4 hours to 1.55, 1.61, 1.78 et 1.92 cfu ml^{-1} after 6 hours of cellular contact. At 12 hours of contact the inhibitory of *S. aureus* 49444 recorded passed to plus to 50% to a total inhibitory after 24 hours of contact.

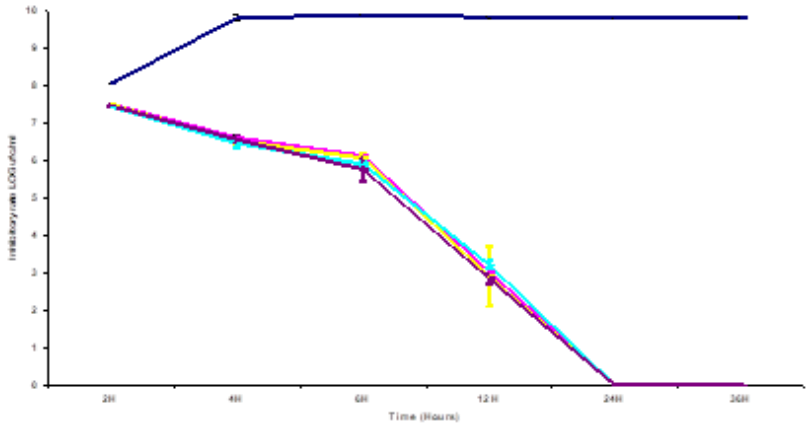

Figure 3. Antagonistic effect of different strains of *bifidobacterium* against *S. aureus* 49444 by cellular contact.

3.2.2-Supernatant contact

Agar spot

A part from the control that the halo diameter of inhibition zone doesn't pass 3 mm. Release of antagonist substances from the bifidobacteria supernatant was confirmed by the large inhibition zone of *S. aureus* 49444 growth observed by the agar spot assay who exceed 13 mm (Fig 4).

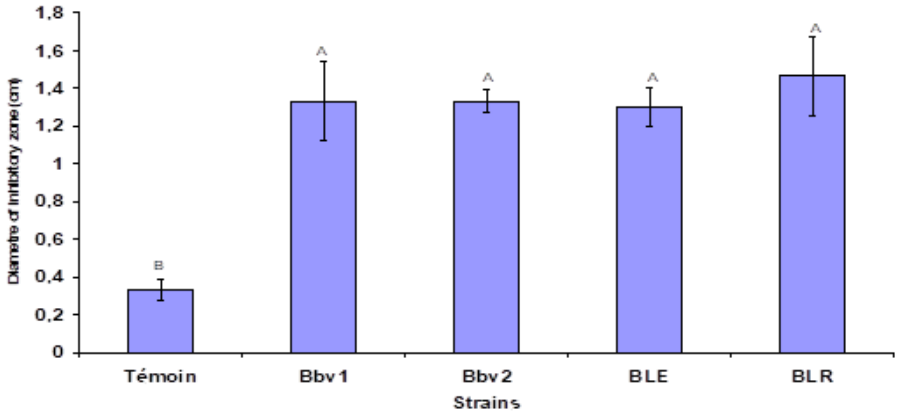

Figure 4. Antagonistic effect of different strains of *bifidobacterium* against *S. aureus* at 37°C by supernatant contact in broth.

At 6°C

The constant cell number of *S. aureus* all a long of experience but for the others the rate of decrease is trifling (Fig 5). From different strains, 0.15 and 0.96 LOG cfu/mL reductions were observed respectively After 2 hours and 4 hours of contact and after 6 hours of contact we record a variability in the antagonistic activity interstrains who vary enter 1.49 and 1.82 LOG cfu/mL, but after 12 hours of contact this difference appear clearly (2.19 and 2.69 LOG cfu/mL). 24 hours of contact provoke plus to 3.76 LOG cfu/mL of inhibitory who don't keep to increase after 36 hours of contact who reach 4.71 LOG cfu/mL and to keep it after 48 hour of contact.

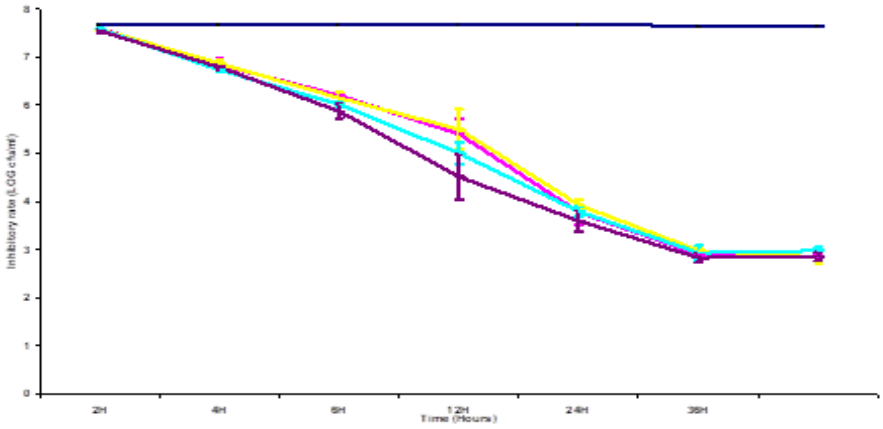

Figure 5. Antagonistic effect of different strains of *bifidobacterium* against *S. aureus* 49444 by supernatan contact in broth at 6°C.

At 37°C

Results demonstrated a constant decrease of *S. aureus* concentration (Fig 6) during the first 6 hours (0.43, 0.43, 0.53 and 0.49 LOG cfu/ml at 2 hours, 1.33, 1.38, 1.54 et 1.48 LOG cfu/ml after 4 hours and 2.08, 2.17, 2.46 et 2.56 LOG cfu/ml at 6 hours of supernatant contact against *S. aureus* 49444 at 37°C respectively for Bbv1,Bbv2, BLE et BLR. The remarkable point after the 12 hours of contact is the difference between the *B. longum* strains (BLE and BLR) when we record a total inhibition and the *B. breve* strains (Bbv1 and Bbv2) who the inhibition exceed 80% for a total destroy after 24 hours of contact.

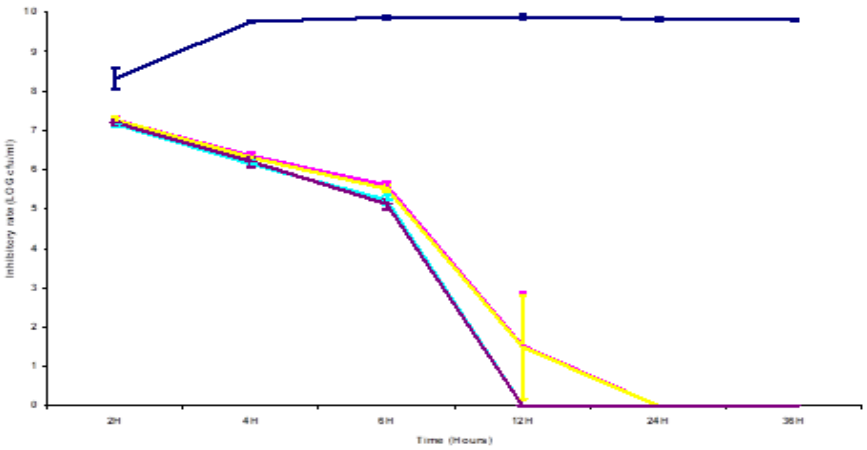

Figure 6. Antagonistic effect of different strains of *bifidobacterium* against *S. aureus* 49444 by supernatan contact in broth at 37°C.

3.3- Effect of solvent on supernatant activity

No effect has been recorded in the inhibitory effect against *S. aureus* 49444 (Table 2) for all cases of bifidobacteria supernatant treated by the organic solvents.

Table 2: antagonistic effect on S. aureus of the Bifidobacterium strains treated by organic solvents.

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

4-Discussion

Recent studies directed by Topisirovic et *al.*, (2006) have also observed an inhibitory effect of *Lactobacillus herveticus* BGRA43 and *Lactococcus lactis* subsp. *Lactis* BGSM1-19 against *S. aureus* ATCC25923 (with an inhibitory zone of respectively 8 mm and 2 mm with culture and supernatant) in the comparison with our results indicate that the bifidobacterial strains have an important inhibitory effect against *S. aureus* compared to other lactic acid bacteria. In general, Hurst and Collins-Thompson (1979) find a fairly poor competitor of *Staphylococcus aureus* to starter lactic acid bacteria at fermentation temperatures.

Results observed in bifidobacterial supernatant contact with *S. aureus* are in accord with liévin et *al.*, (2000) who record an inhibitory rate superior to 2.9 LOG cfu/ml after an incubation of 1 hour at 37 °C for the bifidobacterial supernatant against *S. aureus* and plus to 5.4 LOG cfu/ml after 3 hours contact.

The MRSc medium presents a selectivity characteristic by the inhibitory effect exerted in the agar spot method of the supernatant contact. But the antagonistic substances in the bifidobacterial supernatant were more effective against the pathogenic bacteria.

Some studies indicate that the *Bifidobacterium* strains activity was due to the production of organic acids, in particular, acetic and lactic acid. The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm (Makras and De Vuyst, 2006). The eventual lowering of the intracellular pH or the intracellular accumulation of the ionized form of the organic acid lead to the death of the pathogen (Russell and Diez-Gonzalez, 1998; Makras and De Vuyst 2006).

Gagnon et *al.*, (2004) demonstrate by the spot test on bicarbonate buffer MRS agar a considerably reduction in the inhibition zone, but did not disappear. By This result they deduce that acid production (acetate and lactate) was not the only factor in the antagonistic activity of bifidobacteria.

In this senses, some authors have used bacteriocin to evaluate there inhibitory effect against *S. aureus* as Millette et *al.*, (2007) who used the nisin at different concentrations and stored at 4°C. This study has demonstrated that a concentration of 500 IU/mL of nisin was necessary to reduce by 65% the level of *S. aureus* on round beef steak, while 1000 IU/g led to plus to 75% of reduction after 15 days of storage at 4°C. Another study directed by De Kwaadsteniet et *al.*, (2005) find an activity zone larger than 6mm of *S. aureus* treated by a bacteriocin ST15 -produced by *Enterococcus mundtii* ST15- at 37°C in BHI medium. But Yildrim and Johnson (1998) don't found an inhibitory effect of Bifidocin B a bacteriocin produced by *B. bifidum* NCFB 1454 against *S. aureus* ATCC25923, that not be a significant explication because in the study of Topisirovic et *al.*, (2006) *Lactobacillus lactis* subsp.*lactis* BGM1-19 have demonstrate two different comportment against two different strains of *S. aureus* (inhibitory zone superior to 2mm against *S. aureus* ATCC25923 and no inhibitory zone against *S. aureus* MR427).

Laukova et *al.*, (1999) have tested the effect of enterocin CCM4231 on *Staphylococcus aureus* in milk and found a positive effect (in the sample additioned by the bacteriocin at concentration 3200 AU/ml 4hours after cultivation) after 24hours and a total inhibitory after 1 hour of cultivation *Staphylococcus aureus* SA1 before enterocin addition compared to the control sample (without

enterocin CCM 4231) who demonstrate an increase rate after 24hours. These results indicate the important role of time production of bacteriocin in the cellular contact.

5. Conclusion

This study demonstrated in Vitro the antagonistic activity of four strains of bifidobacteria against *Staphylococcus aureus* 49444. The inhibitory action of the bifidobacteria strains at 37°C was clearly demonstrated by the development of clear halos over 8 mm in diameter around *Staphylococcus aureus* cocultured with bifid cells for 24 hours, and over 14 mm in diameter when concentrated supernatants from bifid cultures were brought into contact with *S. aureus*. As regards the inhibition of *S. aureus* at 37°C by the bifides cells themselves (in the case of cocultures), there is a certain latency of 2 hours before the number of inhibited cells is multiplied by a factor of 5 to 6 at the 4^{th} hour of contact. On average, this germ is 60% inhibited at the 12^{th} hour and 100% inhibited at the 24^{th} hour of coculture by all bifid strains. The inhibitory power of concentrated supernatants from cultures of bifidobacteria on *S. aureus* at 37°C is strongly felt without latency, since more than 5% of pathogenic cells are killed in the first two hours of contact and more than 80% (for *B. breve*: Bbv1 and Bbv2) and 100% (for *B. longum*: BLE and BLR) after the 12th hour. At low temperatures, the inhibitory power of the concentrated supernatants of bifid cultures on *S. aureus* is greatly reduced and spread out over time, with 48 hours of contact achieving only 60% inhibition on average. The speed of inhibition of *S. aureus* by bifides 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 concentrated supernatants from bifidobacterial cultures towards *S. aureus*. This suggests that these solvents do not denature protein substances with antagonistic (i.e. bacteriocin-like) activity.

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