

Estimation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Cell-Free Extracts of *Bifidobacterium* Species Against Methicillin-Resistant *Staphylococcus aureus* *in vitro*

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Abstract: The aim of this study was to examine if extract of bifidobacteria, a major species of the human colonic microflora participates in the barrier effect against enteropathogens by developing antimicrobial activity against virulent bacteria. Six human bifidobacteria strains were isolated from infant stools. They were characterized and identified through physiological, biochemical tests and API 20 A test system. The isolates belonged to the three species: *B. breve*, *B. longum* and *B. infantis*. The cell extracts of the isolates were examined for antimicrobial activity by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For this purpose, the methicillin-resistant *S. aureus* (MRSA) was chosen as an indicator. MRSA treated with cell-free supernatants (CFS) from bifidobacteria were examined. All the *Bifidobacterium* isolates used have been identified as novel probiotics with a greater ability to survive at low pH and high concentrations of bile salt *in vitro*. 0.5 McFarland standard (10^8 CFU/ml) of a confirmed MRSA strain was challenged with the CFS strains by employing the tube dilution method and subculture on MRS agar assays. The cell-free supernatants of the 6 LAB strains exhibited MIC values between 50 μ l/ml and 200 μ l/ml. Only two CFS of bifidobacteria (b3 and b4) had no MIC and MBC values with the concentrations under the current study. The b1, BL and BI strains showed highest antibacterial activity by MIC value with 100 conc. and by MBC value with 150 conc. Increased concentration levels of the cell-free extract (CFE) correlated with a decrease in MRSA viability. MRS broth medium (control) showed a high growth rate of MRSA without CFE. These results may provide a basis for alternative therapies for the treatment of MRSA superbug.

Keywords: *Bifidobacterium Spp.*, MBC, MIC, MRSA

1. Introduction

The probiotic micro-organisms are single-celled, non-pathogenic organisms which do not promote or cause disease. Most commonly used probiotic supplements contain the species of *Lactobacillus* and *Bifidobacterium* and they are part of normal human intestinal microbiota [1]. The World Health Organization deemed probiotics to be the next-most important immune defense system when commonly prescribed antibiotics are rendered useless by antibiotic resistance [2].

Bifidobacterium breve is primarily located in human breast

milk and the gastrointestinal tract of infant and adult humans, where they are among the first microbial colonizers, passed from the mother to her offspring. *B. breve* exhibits a symbiotic relationship with their host by exploiting their unique metabolic capabilities in order to catabolize certain carbohydrates, such as the oligosaccharides present in human breast milk, that are indigestible by their host [3]. *B. breve* has not been recorded as the cause of human disease and has been in commercial use as a probiotic since 1976 [4].

Staphylococcus aureus is one of the most important bacterial opportunistic pathogens in humans and the principal nosocomial pathogen worldwide. *S. aureus* is known for its rapid development of resistance to different antimicrobial

agents especially its resistance to beta-lactam antibiotics (so-called methicillin-resistant *S.aureus*, MRSA, a multidrug-resistant microorganism). *S.aureus* can become MRSA by the acquisition of the *mecA* gene, which encodes the penicillin binding protein (PBP2a) with a low affinity for b-lactams [5]. The PBP2a-producing MRSA strain is resistant not only to methicillin, oxacillin and nafcillin but also to all other b-lactam antibiotics including cephalosporins [6].

Probiotics must survive gastric and bile acids in order to reach the intestinal tract. Once there, they must be capable of adhering to human epithelial cells. Lastly, they prevent colonization by pathogenic bacteria, either by competitive exclusion or synthesis of antimicrobial substances [6]. Beneficial effects conferred by *B. breve*, including the inhibition of gram-negative and gram-positive pathogenic bacteria, were described by [7] and reported that *B.breve* had a specific antagonistic effect against MRSA.

Probiotics have been shown to suppress pathogen growth through the release of a variety of antimicrobial factors like defensins, bacteriocins, hydrogen peroxide, nitric oxide, and short-chain fatty acids (SCFA), such as lactic and acetic acids, which reduce the pH of the lumen [8].

A high percentage of hospital-acquired infections are caused by highly resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA). People with MRSA are estimated to be 64 percent more likely to die than individuals with a non-resistant form of the infection [9].

So that, the purpose of the present study was to determine the antagonistic activity of cell-free extract of *B. breve* against Methicillin-resistant *S.aureus* (MRSA) superbug and the estimation of the minimum inhibitory concentration and minimum bactericidal concentration.

2. Materials and Methods

2.1. Samples Collection

2.1.1. Source of the Pathogenic Isolates

The target pathogenic bacteria were clinically isolated including (4) isolates of *Staphylococcus aureus* which were obtained from the microbiology laboratory of AL-Karama teaching hospital in Wasit province. The isolates were diagnosed by using VITEK system (Healthcare, biomerieux) and the growth of the *S. aureus* was confirmed after incubation by observing the colony characteristics under a microscope and by biochemical tests.

2.1.2. Lactic Acid Bacteria

Ten fecal samples were collected from infants aged (2-4) weeks and who relied on feeding on breast milk with no trace of any kind of antibiotics during the collection of samples. Models were developed directly in the middle of the sterile liquid MRS-broth [10].

2.2. Isolation of Bacteria

The LAB bacteria were isolated as described by [10]. Stool samples from 10 infants in good health aged 2-4

weeks and with certified breastfeeding. 1 gram of stool sample was taken and placed in the middle of 9 ml of MRS - broth (pH 6.2) and was transported to the laboratory in less than 6 hours for the purpose of bacterial examination. A direct smear was performed and incubation was done for 24 hours under anaerobic conditions at a temperature of 37°C. Subculture was prepared by taking 1 ml from each test tube and added to 9 mL of MRS-broth and then placed in peptone water and incubated for 24 hours under anaerobic conditions and a temperature of 37°C. This step was repeated three times for the purpose of purification and activation. 1 ml of each dilution was taken and spread in the middle of MRS-NNL-Agar (nalidixic acid 15 µg/mL, neomycin sulphate 100 µg/mL and lithium chloride 3000 µg/mL) a special development of *Bifidobacterium spp.* and then incubated in the presence of CO₂ and a temperature of 37 ° m for 48 hours.

2.3. Diagnosis of Lactic Acid Bacteria Isolates

The isolates underwent biochemical tests that included: Catalase test, Gelatin liquefaction test, Carbohydrate fermentation test and API 20-A system (biomerieux) for diagnosis of anaerobic bacteria in order to contain the number of confirmatory biochemical tests. Characteristics of isolates were compared with what exists in the [11].

2.4. Characteristics of *Bifidobacterium sp.*

2.4.1. Growth in the Bottom of the MRS- Broth

Tubes with MRS broth media included with bacterial colonies were incubated anaerobically at a temperature of 37°C for 24-48 hours, and growth was observed in the sediment at the bottom of the test tube [10].

2.4.2. Growth at Different Temperatures

By [12], inoculum in MRS broth tubes containing 1% newly isolated bacteria were incubated under anaerobic conditions and at different temperatures (15°, 37° and 45°C) for 3-5 days. The result was considered positive if turbidity was found. [10].

2.4.3. Test for the Resistance of Bacteria to Bile Salts

This test was done according to the procedure described by [13]. Lactic acid bacteria (LAB) grown in MRS broth were centrifuged at a rate of (2000) rpm for (10) minutes and suspended in normal saline; then (1) ml of the suspension was incubated for (3) hours transported into (9) ml of phosphate buffered saline containing 1% bile and incubated for (72) hrs and subcultured on MRS agar and incubated in a candle jar at 37°C.

2.4.4. Determination of the Ability of *Bifidobacterium* to Tolerate Low pH (pH = 3)

This test was done according to [14] and [10]. The *bifidobacterium* was grown in MRS broth was centrifuged at a rate of (2000) rpm for (10) minutes and suspended in normal saline; then (1) ml of the suspension was transported into (9) ml of normal saline (pH=6.7) and (9) ml of

phosphate buffered saline (pH = 3) and incubated for (3) h, then subcultured on MRS agar and incubated in a candle jar at 37°C for (48) hrs.

2.5. Phenotypic Screening for Methicillin-Resistant *Staphylococci*

Detection of MRSA was carried out using oxacillin screen agar and cefoxitin disc diffusion test as in our previous study to be published and according to [15].

2.5.1. Oxacillin Agar Screening Test

Mueller-Hinton agar (MHA) plates containing 4% NaCl and 6µg/ml of oxacillin (Sigma, USA) were obtained from microbiology laboratory, college of science, Wasit University. Plates were inoculated with 10 µL of 0.5 McFarland suspension of the isolate by streaking and incubating at 35°C for 24 hrs. Petri dishes were observed carefully for any growth. Any growth after 24 h was considered an oxacillin resistant strain.

2.5.2. Cefoxitin Disc Diffusion Test

Four isolates were subjected to cefoxitin disc diffusion test using a 30 µg disc (Oxoid). The isolate was adjusted by using 0.5 McFarland standard suspension and lawn culture done on Mueller-Hinton agar plate. Plates were incubated at 37°C for 18 h and the zone diameters were measured. An inhibition zone diameter of ≤ 21 mm was reported as oxacillin resistant bacteria and a diameter of ≥ 22 mm was considered as oxacillin sensitive bacteria.

2.6. Antagonism Activity Assay

2.6.1. Cell-Free Extract (CFE) Preparation

Cell-free extract of all lactic acid bacteria used in this study was prepared according to [10] as follows: *Bifidobacterium spp.* include (6) strains which were inoculated separately in MRS (De Man Rogosa Sharp) broth as 2 % of broth volume and incubated under anaerobic condition at 37°C for 72 h. The culture was then centrifuged at 5000 rpm for 30 min. The Supernatants were sterilized by filtration through (0.22µm) membranes (Millipore filter paper-Swinnex-25).

2.6.2. Determination of Minimum Inhibitory Concentration of CFEs

The MIC of CFEs for the test strains was determined according to [15]. One isolate of MRSA (1%) of 10⁸ cfu/ml of fresh culture was inoculated in 10 ml nutrient broth containing filter concentrates of the culture supernatant (50,100,150,200) µl and incubated aerobically at 37°C for 24 h for MIC determination

2.6.3. Determination of Minimum Bactericidal Concentration of CFEs

After the serial dilution for every treatment was done, then the bactericidal activity of CFE against MRSA was determined by plating (0.1 ml) for each treatment into Mueller Hinton sterilized petri dishes and then incubated at 37°C for 24 h, after incubation, results were recorded and

compared with the control treatment (0% of CFE), [15][16]

3. Results

3.1. Isolation and Determination of Lactic Acid Bacteria

Ten (10) fresh fecal samples were collected in the morning from healthy breastfeeding infants aged (2-4) weeks with good health and with no trace of antibiotic during sample collection time.

The *Bifidobacterium* appeared under light microscope as gram-positive cocci or rod pleomorphic, non-spore forming, non-filamentous and non-motile. *Bifidobacterium* colonies appear on MRS agar as entire opaque with or without irregular convex edges. Isolates showed negative results for catalase and gas production tests. They also showed negative results for aerobic growth on nutrient agar. The results of the growth at different temperatures for *Bifidobacterium* was positive at (35°C – 45°C) and the result was negative for growth at thermal grades of 15°C and 25°C. All isolates (b1, b2, b3, b4, BL and BI) showed growth at the bottom of the test tube as deposits in the MRS-broth (Table 1). The API 20 biochemical confirmatory diagnosis for the isolates provides evidence for the purity of isolation. Four (4) of the total isolates belong to *B. breve* species while 2 of them belong to *B. longum* (BL) and *B. infantis* (BI) with (76 and 88.6)% identification percentages respectively as shown in Table 2.

Table 1. Result of Biochemical and physiological properties of *B. breve*.

Test	Result
Gram staining	+ ve
Catalase	- ve
Growth on nutrient agar aerobically	- ve
Growth on bottom on MRS broth	+ ve
Growth on 5 and 15C	- ve
Growth on 35 and 45 C	+ ve
Gas production	-ve

Table 2. Identification of examined strains with API 20A system.

Strain	Identification with API 20A	
	Identification /other possibility	Percentages of identification
(b1)	<i>B. breve</i>	97.3%
(b2)	<i>B. breve</i>	99%
(b3)	<i>B. breve</i>	96.9%
(b4)	<i>B. breve</i>	99.9%
(BL)	<i>Bifidobacterium longum/ B.infant</i>	76%
(BI)	<i>Bifidobacterium infant/B.longum</i>	88.6%

3.2. Characteristics of *Bifidobacterium Spp.* Strains as Probiotics

Ability to tolerate bile salts and low pH (pH=3)

All bifidobacteria isolates under the current study showed the ability to resist low (pH=3). After exposure for 3 hours, and growth was observed after incubation, the isolates showed high tolerance to bile salts in media containing these salts (Table 3).

Table 3. Ability of *Bifidobacterium* isolates to tolerate low (pH) and bile salts.

bile salts%1	pH=3	<i>B.breve</i>
+	+	b1
+	+	b2
+	+	b3
+	+	b4
+	+	BL
+	+	BI

3.3. Determination MIC and MBC Values in Vitro

The minimum inhibitory concentrations of CFE of LAB against MRSA at (50, 100,150 and 200) v/v of CFE as are shown in Table4. The inhibition of staphylococcal growth increases as the concentration of b1,b2, BL and BI extract increases. Whereas b3 and b4 isolates have no MIC effect against methicillin-resistant *Staphylococcus aureus* was tested in the present study.

Table 5. Staphylococcal growth on subculture with three concentrations of LAB extraction.

LAB strains	b 1			b 2			BL			BI			Control*
Concentrations of	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0	1.0	1.5	2.0	0
CFE % of clear tubes	+	-	-	/	-	-	+	-	-	+	-	-	++

++ moderate growth + light growth -No growth / not tested

*tube inoculated with only MRSA cells (without CFE)

The MIC and MBC values obtained for extracts against the bacterial strains are summarized in table 6. The strains b1, BL and BI observed equal values, whereas only b2 strain had no MIC value.

Table 6. Minimum inhibitory and minimum bactericidal concentration values of selected LAB extract on MRSA growth.

LAB extract	b 1		b 2		BL		BI	
MRSA growth	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	1.0	1.5	ND	1.5	1.0	1.5	1.0	1.5

ND= not detected

4. Discussion

After a series of isolation stages, pick 10 isolates of *Bifidobacterium spp.* from developing colonies on the MRS-NNL medium isolating the bacteria of lactic acid which is characterized by its colonies on this medium [10],[17]

Sometimes, *Bifidobacterium* appear as a rod-like shape that tends to be clubbed with a branch to form a 'Y' shape or irregular and this was documented by [18]. [17]

Since gram-positive strains were also catalase-negative, and unable to produce indole from tryptophan and gas from glucose as well as to reduce nitrogen, we assigned them to the *Bifidobacterium* genus. Identification of the isolated bifidobacterial strains to species was performed using API 20 A system applied specifically to anaerobic bacteria. The strains belong to *B. breve*, *B. infantis* and *B. longum*. [11, 19-21].

For biochemical test, sugar fermentation (some variation exist between BI and BL), growth at different temperatures,

Table 4. Staphylococcal growth in different concentrations of cell-free extract of LAB.

LAB strains	CFE addition volume(μl) to 10 ml of broth				
	0 control	50	100	150	200
b 1	+	+	-	-	-
b 2	+	+	+	-	-
b 3	+	+	+	+	+
b 4	+	+	+	+	+
BL	+	+	-	-	-
BI	+	+	-	-	-

(+) visible growth appearance (turbid tube)

(-) no visible growth appearance (clear tube)

The results summarized in table (5) show that after 24 h at 37 °C incubation of MRSA with b1, BL and BI extracts, more than 99% of the MRSA bacteria were eliminated at concentration (1.5 %) of bifidobacterial CFE.

ability to tolerate low pH and bile salts and finally forming deposit in bottom of broth media, the results of the current study matched with those of our previous study to be published, and these results are in agreement with other researchers [20-23].

Superbug" is a term invented by the media to describe bacteria that cannot be killed using multiple antibiotics. These bacteria are "antibiotic resistant" and have proven particularly problematic in healthcare settings where they increase the risk of worse clinical outcomes and death, a high percentage of hospital-acquired infections are caused by highly resistant bacteria, such as methicillin-resistant *staphylococcus aureus* (MRSA). People with MRSA are estimated to be 64 percent more likely to die than individuals with a non-resistant form of the infection [9].

A Lactic acid bacteria can produce antagonistic materials that vary in their spectra of activity. The antimicrobial agents from strains b1, BL, BI and b2 demonstrated a wide range and strong antimicrobial activity against MRSA. According to the study of [24] and our results, lactic acid bacteria can produce antimicrobials compounds and these bacteria exist in the gut of human infants.

Co-cultures of lactic acid strains with MRSA are used to demonstrate the potential of various probiotic bacteria to decrease the number and virulence of MRSA [6]. The inoculum concentration of the test strain extracts were 50, 100, 150 and 200 μl(v/v), and the inoculum concentration of MRSA was (1%) of 10⁸ cfu/ml. After intervals of culture incubation, the tubes were observed, turbid tubes indicated a negative result and the first clear tube was considered as MIC value. LAB produces many antimicrobial substances like organic acids, hydrogen peroxide and bacteriocins that inhibit

other bacteria and fungi [25]. The CFSs of *B.breve* b1, b2, b3 and b4, *B. infantis* (BI) or *B. longum* (BL) were 4-fold concentrated, which were then used to determine the MICs of the test strains against MRSA.

Among the test strains, b1, BL and BI showed the strongest anti- MRSA activity (MIC 100µl, MBC 150 µl), whereas b2 observed (MBC 150 µl). This result was not compared with other studies due to lack of similarity in the tested bacteria and CFE concentrations under the current study.

The difference in the anti-MRSA activity between the strains tested might be due to the nature of the antimicrobial compounds they produced [5]. The anti-MRSA activity of b1, BL, and BI may be mediated mainly by the organic acid production, i.e lactic acid and acetic acid. In fact, the decline in pH arising from the production of organic acid from LAB is a well-known factor to inhibit certain pathogenic strains. [6]

The MIC value of b2 extract was not detected with concentrations may be its MIC value located between 50 and 100 µl of CFE concentration. The MIC and MBC values obtained for extracts against the bacterial strains varied among the three extracts. The MIC values corresponded well to the MBC values in b1, BL and BI extract in comparison with control; there were low and moderate differences of growth rate respectively (Table 7).

The inhibitory effect of *Bifidobacterium in vitro* was mainly due to the high acidity that resulted from the primary metabolic products of carbohydrate fermentation which include; lactic acid, hydrogen peroxide and other products. The acids produced by LAB enter into the sensitive bacterial cells and interfere with the necessary metabolic process such as substrate translocation and oxidative phosphorylation, and leads to decrease in the internal pH of bacterial cells [26]. There is also synergistic effect between lactic acid and acetic acid in the inhibitory effect of *Bifidobacterium* against some bacteria such as salmonella [10]. The mechanisms underlying *Bifidobacterium* inhibition of test bacteria may be due to presence many fractions containing proteins with a molecular mass below 5.000 Da and the finding of *in vitro* pointed to the peptideic nature of the *Bifidobacterium* linked to bacteria inhibition [27]. However, there are also reports of compounds of proteinaceous nature with antagonistic activity against all bacteria (these proteinaceous inhibitors target the cell membrane and depolarize it, and also inhibit synthesis of the cell wall. One of these peptides characterized as Bacteriocin is called Bifidocin B [26]. Diacetyl, hydrogen peroxide {H₂O₂ can have a strong oxidizing effect on membrane lipids and cellular proteins}, organic acids such as lactic acid, acetic and propionic acids, the most documented kind of metabolites. [7] [28]. All of the reasons above give clear reasons for the high activity that was obtained in our results.

Another report showed that a mixture of bacteria isolated from the faeces of human breastfed infants containing *B. bifidum* was related to lowering of the pH level [29]. Another mechanism of action has been proposed as a *B.*

infantis strain developed broad spectrum antimicrobial properties through the production of antimicrobial compounds, unrelated to acid production, which inhibited the growth of pathogens [30]. We have recently provided evidence that selected bifidobacterium strains isolated from human neonatal stools inhibit pathogens [31], [32]. Our results presented here and related to the antimicrobial activity of *B.breve*, *B.longum* and infant bifidobacterium strains are consistent with one of the mechanisms of action dependent on the production of antimicrobial compounds, as previously hypothesized. Indeed, we have provided evidence that the bactericidal activity of the strains b1, BL, BI and b2 *in vitro* results from antimicrobial compounds present in the spent culture supernatants (in CFSs), suggesting that they are secreted.

Non-dissociated form of, for example, lactic acid triggers to lower internal pH of the bacterial cell, which causes collapse in electrochemical proton gradient in sensitive bacteria, hence having a bacteriostatic or bactericidal effect [23] and [8]

5. Conclusion

Healthy breastfeeding infants from our study sites are potent sources of bifidobacteria (Friendly bacteria) isolates and these friendly bacteria have anti- MRSA activities *in vitro* especially when they grow as co-culture (CFSs) with the pathogens. The particular inhibitory metabolites are other than organic acids.

Anyway, the performed studies are of potential importance for the treatment of MRSA *in vitro* by determination the lowest concentration (MIC) of bifidobacterial extracts and then determine the bactericidal effect (MBC) that kill the MRSA.

The studied strains of bifidobacteria of human origin (b1, b2, b3, b4, BI and BL) can be considered promising as a basis for the development of probiotics as part of the alternative treatment of these serious multi-resistant strain, by ability to synthesize extracellular antimicrobial compounds that granted unfavourable conditions for growth of *Staphylococcus aureus*.

Finally, *Bifidobacterium* isolates conferred that it is the status of a probiotic bacterium with activity against MRSA *in vitro*

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