Journal of Advances in Biology & Biotechnology
10(2): 1-9, 2016; Article no.JABB.28724
ISSN: 2394-1081

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In vitro Antagonistic Activity of *Bifidobacterium breve* Isolated from Breast-fed Infants Human Gastrointestinal Microflora against Two Clinical Strains of *Staphylococcus aureus* (MRSA and MSSA)

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/JABB/2016/28724
Editor(s):
(1) Joana Chiang, Department of medical laboratory Science and Biotechnology, China Medical University, Taiwan.
(2) Afroz Alam, Department of Bioscience & Biotechnology, Banasthali University, Rajasthan, India.
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(3) Teresita Sainz Espuñes, Universidad Autónoma Metropolitana-Xochimilco, Mexico.
Complete Peer review History: http://www.sciencedomain.org/review-history/16901

Original Research Article

Received 2nd August 2016
Accepted 7th November 2016
Published 12th November 2016

ABSTRACT

The antibacterial activity of *Bifidobacterium breve* against methicillin-resistant *Staphylococcus aureus* MRSA from human middle ear infection (otitis media) as well as methicillin-susceptible *Staphylococcus aureus* (MSSA) strain was tested *in vitro*. MRSAis a multidrug-resistant microorganism and the principal nosocomial pathogen worldwide. The *Bifidobacterium breve* (b1, b2, b3 and b4) strains which isolated from healthy human infants were employed. All the *Bifidobacterium* isolates mentioned above have been identified as novel probiotics with a greater ability to survive at low pH and high concentrations of bile salt *in vitro*, and with a growth rate of 3.5-4.5 and 5.5% in NaCl solution. The growth inhibitory effect produced by the antagonistic activity of the lactic acid bacteria on the MRSA and MSSA strains was tested on solid medium using agar spot test.

All bifidobacteria Cells (b1, b2 and b4) except (b3) showed high antibacterial activity after 24 h of

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their incubation anaerobically at 37℃ with significa nce differences (P≤ 0.05). MRS media (control) showed no activity. No significant differences appeared between MRSA and MSSA strains with antagonistic activity of bifidobacterial cells against both of them. In contrast the antibacterial activity of lactic acid bacteria (LAB) under the current study was more potent than the activity of ofloxacin (positive control).

Keywords: Antagonistic activity; Bifidobacterium breve; Methicillin-resistant Staphylococcus aureus; Methicillin-susceptible Staphylococcus aureus.

1. INTRODUCTION

Today, the term "probiotic" refers to "live microorganisms which when administered in adequate amounts, confer a beneficial physiological effect on the host," according to the Food and Agriculture Organization and World Health Organization [1]. The normal flora of the human gastrointestinal tract contains many diverse populations of bacteria which play an essential role in the development and well-being of the host. In particular, the intestinal microflora exerts a protective role against pathogens [2]. Most commonly used probiotic supplements contain the species of *Lactobacillus* and *Bifidobacterium* and they are the part of normal human intestinal microbiota [3]. The antagonism of lactic acid bacteria is exerted by competition for nutrients and for physical location, but also through the production of antimicrobial substances. These include the production of low molecular compounds (<1,000 Da), such as organic acids, and the production of antibacterial substances termed bacteriocins (>1,000 Da) hydrogen peroxide, diacetyl and acetaldehyde [4]. These compounds are able to inhibit the growth of harmful microorganisms and the most important LAB are *lactobacillus spp* and *Bifidobacterium spp.* [5]. The eventual lowering of the intracellular pH or the intracellular accumulation of the ionized form of the organic acid can lead to the death of the pathogen [6] .Beneficial effects conferred by *Bifidobacterium breve*, includes inhibition of gram- negative and gram- positive pathogenic bacteria, were described by [7,8].

Methicillin- resistant *S. aureus* (MRSA) is any strain of *S. aureus* that is resistant to all β-lactams antibiotics due to the acquisition of a transpeptidase, Penicillin Binding Protein 2a (PBP2a), involved in bacterial cell wall synthesis. PBP2a has low affinity for β-lactam antibiotics and is encoded by mec A complex gene which contains insertion sites for plasmids and transposons that facilitate the acquisition of resistance to other antibiotics (multidrug resistance: MDR), hence there are restricted treatment options for infections caused by MRSA [9].

Treatment with selected probiotic strains like *B. breve* may be the ultimate answer to the decolonization of MRSA because they do not increase the risk of multi-drug resistance of this pathogen [10].

As such, the purpose of the present study was to determine the antagonistic activity of *B. breve* cell against MRSA and compared with another strain of MSSA.

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, Wasit province, Iraq. These isolates were diagnosed by using VITEK system (Healthcare, biomerieux). 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 (10) fecal samples were collected from neonates aged 2-4 weeks and who relied on feeding on breast milk with no trace of any kind of antibiotics during sample collection. Models were developed directly in the middle of the sterile liquid MRS-broth [11].

2.1.3 Isolation of bacteria

The LAB have been isolated as described by AL-Saadi [11]. Stool samples from 10 neonates in good health aged 2-4 weeks and with certified breastfeeding. 1 gram of stool sample and

placed in the middle of 9 ml of MRS - broth (pH 6.2) this was taken to the laboratory in less than 6 hours for the purpose of bacterial examination. After performing a direct smear, incubation was done for 24 hours under anaerobic conditions at a temperature of 37℃. Subculture was made by taking 1 ml from each tube mentioned above and then placed in peptone water and incubated for 24 hours under anaerobic conditions and a temperature of 37℃. This step was repeated thrice 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 µ/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 CO_2 and at a temperature of 37℃ for 48 hours.

2.2 Diagnosis of Lactic Acid Bacteria Isolates

The isolates underwent biochemical tests that included: Catalase test, Gelatin liquefaction test, Carbohydrate fermentation test and API 20A system 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 [12].

2.3 Characteristics of *Bifidobacterium breve*

2.3.1 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℃ for 24-48 hours and growth was observed in the sediment at the bottom of the test tube [11].

2.3.2 Growth at different temperatures

By Buck and Gilliland [13] where inoculum in MRS broth tubes 1% newly isolate bacterial and incubated in anaerobic conditions and different temperatures (15°, 37° and 45°) for 3-5 days the result is positive if the turbidity found [11].

2.3.3 Test for bacterial resistance to bile salts

This test was done according to procedure described by Fuller [14]. *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 suspension was transported to 9 ml of phosphate buffer saline containing 1% bile and incubated for 72 hrs and subcultured on MRS agar and incubated in a candle jar at 37℃.

2.3.4 Determination of the ability of *Bifidobacterium* to tolerate low pH (pH = 3)

This test was done according to Harrigan and McCance [15] and AL-Saadi [11] as the *Bifidobacterium* grown in MRS broth were centrifuged at rate 2000 rpm for 10 minutes and suspended in normal saline; then 1 ml of suspension was transported to 9 ml of normal saline (pH=6.7) and 9 ml of phosphate buffer saline (pH = 3) and incubated for 3 hours, then subcultured on MRS agar and incubated in candle jar at 37℃ for 48 hrs.

2.3.5 Phenotypic screening for methicillin - resistant Staphylococci

Detection of MRSA was carried out using oxacillin screen agar and cefoxitin disc diffusion test according to [16].

2.3.6 Oxacillin agar screening test

Mueller-Hinton agar (MHA) plates containing 4% NaCl and 6 µg/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℃ for 24 h. Petri dishes were observed carefully for any growth. Any growth after 24 h was considered an oxacillin resistant strain.

2.3.7 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 a Mueller-Hinton agar plate. Plates were incubated at 37℃ for 18 h and 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.

2.3.8 Antagonistic activity assay

For detection of antimicrobial activity, an agar spot test was used. The test was a modification of that described by [17]. Test cultures were spotted (2 to 3 ml) on the surface of MRS agar containing only 0.2% glucose and 1.2% agar and incubated anaerobically for 24 h at 30℃ to

develop the spots. The inhibitory effect of MRS was tested as a negative control on each plate. A 100-ml volume of an overnight culture of the indicator bacteria was mixed with 7 ml of soft agar (0.7%), using MRS agar for the lactic acid bacteria, and poured over the plate. The plates were incubated aerobically at 37℃. After 48 h of incubation, inhibition zones were read. A clear zone of more than 1 mm around a spot was scored as positive. Each test was performed in triplicate.

2.4 Statistical Analysis

Statistical analysis was conducted to determine the statistical differences among different treatments against tested strains. Statistical analysis was carried out using T- test. Data were analyzed using R_ bioconductor software version 3.1.1. A P- value of ≤ 0.05 was considered to indicate statistical significance.

3. RESULTS

3.1 Isolation and Diagnosis of *Bifidobacterium*

Ten fresh fecal samples were collected in the morning from healthy breastfeeding infants aged 2-4weeks with good health and did not have any type of antibiotic during sample collection time. In the laboratory, fecal samples were put into test tubes containing MRS-broth and after a series of isolation stages, Four isolates of *Bifidobacterium* were picked for developing colonies on the MRS-NNL medium.

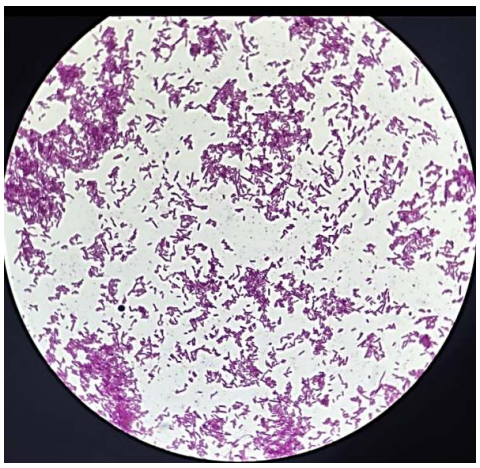

Fig. 1. *Bifidobacterium* morphology with gram stain examined under light microscope X1000 power

The *Bifidobacterium* appeared under light microscope as gram- positive rod pleomorphic, non-spore forming, non-filamentous (Fig. 1).

Bifidobacterium colonies appear on MRS agar as entirely opaque with or without irregular convex edges and light shadow around the colonies with heavy turbidity when grown in MRS broth as show in Fig. 2.

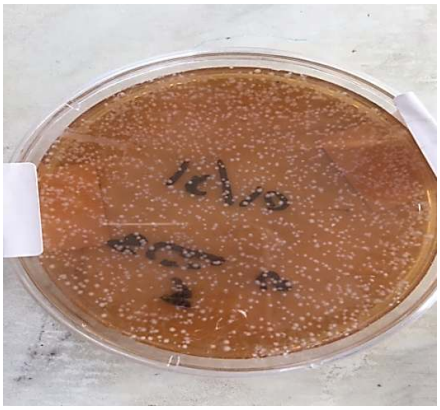

Fig. 2. *Bifidobacterium* colony on MRS-agar

Fig. 3. Sediment of *Bifidobacterium* in MRS broth

Isolates showed a negative results for both catalase test and gas production. All 4 isolates showed negative results in growth on nutrient agar in the presence of air .They showed growth in the bottom of the test tube as deposit in the MRS – broth (Fig. 3). The results of the growth in different temperature for *Bifidobacterium* was positive at 45℃ – 43℃ and no growth at thermal grades 15℃ and 5℃. The results are presented in Table 1.

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 15℃	- ve
Growth on 35℃ and 45℃	+ ve
Gas production	- ve

Table 2. Results of the sugars fermentation

	Sugar	*Bifidobacterium*
1	Glucose	+
2	Galactose	+
3	Sucrose	+
4	Ribose	-
5	Lactose	+
6	Maltose	-
7	Raffinose	-

Bifidobacterium breve fermented many sugar such as glucose, sucrose, galactose and lactose as shown in above Table 2.

The API 20A biochemical confirmatory diagnosis for isolates gives evidence for the purity of isolation.

All 4 strains had a very good identification as shown in Table 3.

Table 3. Identification of examined strains with Api 20 A system

Strain	Identification with API 20A	
	Identification	Percentages of identification
(b1)	*B. breve*	97.3%
(b2)	*B. breve*	99%
(b3)	*B. breve*	96.9%
(b4)	*B. breve*	99.9%

3.2 Characteristics of *Bifidobacterium breve* Strains as a Probiotics

3.2.1 Ability to tolerate bile salts and low pH (pH=3.0)

Results showed that four isolates of *B. breve* were highly tolerant to bile salts in media containing these salts.

All isolates of *B. breve* (b1, 2, 3 and 4) showed ability to resist to low (pH=3.0) after exposure for 3 hours, then growth was observed after incubation (Table 4).

Table 4. Ability of lactic acid bacteria to tolerate the pH3.0 and bile salts 1%

Bile salts 1%	pH=3.0	*B. breve*
+	+	b1
+	+	b2
+	+	b3
+	+	b4

3.3 Phenotypic Detection of Methicillin Resistance

Three *S. aureus* isolates were oxacillin resistant (MRSA) by agar dilution test and one isolate was sensitive. The oxacillin- resistant strains SA1, SA3 and SA4 had diameter of inhibition zone equal to or more than 21 mm by disc diffusion test with cefoxitin, whereas only the strain (SA2) was sensitive to cefoxitin.

3.4 Antibacterial Activity by Agar Spot Diffusion Assay

Table number 6 shows the inhibitory effect of four *Bifidobacterium breve* isolates, the results were expressed in mm, by measuring the distance between the limited colony bacteria and the beginning of the zone of non-inhibition of the indicator strain. The highest diameter observed by b4 strain compared with others with a significant differences ($P \leq 0.05$). For all strains except b3 included in this study showed a significant activity against both MRSA and MSSA higher than ofloxacin.

Table 5. Determination MRSA and MSSA strains by Oxacillin agar screening test and Cefoxitin disc diffusion test

Isolates of *S. aureus*	OST/ growth	CST/mm
SA1*	+	≤ 21
SA2	-	≥ 22
SA3*	+	≤ 21
SA4*	+	≤ 21

OST: Oxacillin sensitivity test
CST: Cefoxitin sensitivity test
**considered as MRSA*

The strain b4 showed a stronger activity (Fig. 4) against both MRSA and MSSA strains with an inhibitory zone 45 mm followed by b1, b2 and finally b3 strain as well as the absence of significance differences between tested bacteria by agar spot assay.

Table 6. The inhibitory effect of *Bifidobacterium spp*.

Pathogenic strains / Code of *Bifidobacterium*	MRSA	MSSA
	Mean value of diameters mm	
b 1	43	42
b 2	35	32
b 3	22	20
b 4	45	45
Ofloxacin (5 µg)	22	23

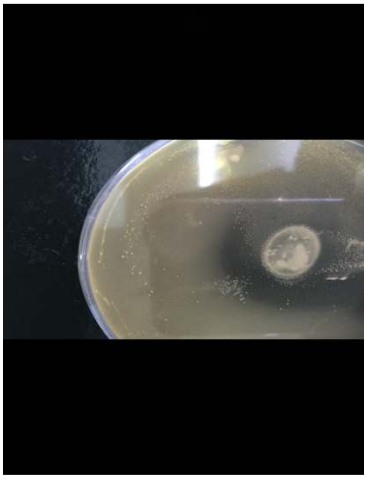

Fig. 4. Positive antagonistic effect of *Bifidobacterium* against MRSA isolated from gut of infants on pathogenic microorganisms using agar spot assay which was measured by millimeter in (MRS soft agar)

4. DISCUSSION

Important to human health, the gastrointestinal microflora contains a complex collection of microorganisms forming a biologically pivotal component of the host body [18]. This microflora is composed of different species of microorganisms. The microflora exerts properties which are potentially health- promoting for the host .Among components of the microflora ,it has been suggested that bifidobacteria play a role in acting as a barrier against colonization of the gastrointestinal tract by pathogenic bacteria [19].

Members of the genus *Bifidobacterium* are considered the most prominent colonizers of the human gastrointestinal tract subsequent to birth, representing ~25% - 80% of the cultivatable bacteria isolated from the feces of infant and adult humans [20] and [21].

The MRS-NNL medium showed good selectivity for *Bifidobacterium sp* and suppressed growth of the other intestinal microflora and fungi. Of 10 isolated anaerobic bacterial cultures (one per each medium), 4 were characterized by cellular morphology typical of the genus *Bifidobacterium* and 6 were also Gram-positive, The *Bifidobacterium* appeared under light microscope as gram- positive rod, sometimes that tends to be clubbed with a branch to form a 'Y' shape.and this was documented by [22].

All bifidobacteria are irregular rods that exhibit sedimentation growth in broth media which is one of the diagnostic phenotypic characteristics of bacteria noted by the researcher [11]. They show a negative result with catalase test, which agrees with the researchers [23].

Bifidobacteria can be distinguished from other intestinal microflora by their ability to break down glucose through the so-called "bifid shunt" this is an agreement with [24].

All isolates (b1, b2, b3 and b4) exhibited ability to resist low (pH=3) after exposure for 3 hours, and growth was observed after incubation, because of lipotheichoic acid and S-layer proteins. These results are in agreement with [25]. One of the characteristucs of a probiotic strain is its tolerance to bile salts, all isolates in this study have ability to tolerate bile salts and this is in agreement with the research by [26]. High tolerance to acid and bile salts helps probiotic bacteria survive the hard physical-chemical conditions of GI tract and thus, is a prerequisite for normal flora to be used as probiotics .Before bacteria reaches intestinal tract, it must first survive during the passage through the stomach where the pH can be as low as 1.5 to 3.0 and remain viable for 4 h or more [27,28].

Across the world [29,30,16] where the most clinically important resistance mechanism in staphylococcal isolates to β-lactam antibiotics is the acquisition of a mec A gene is intrinsically resistant to inhibition by β-lactams [29] and [9]. In this study, *S. aureus* was isolated from patient with otitis media and have a multi -drug – resistance activity. Therefore, we investigated the antagonistic activity of lactic acid bacteria as an alternative therapy against a more severe pathogenic strain.

A Lactic acid bacteria can produce antagonistic compounds that vary in their spectra of activity.

The antimicrobial agent from strain *B. breve* demonstrated a wide range and strong antimicrobial activity against both MSSA and MRSA bacteria, these results clearly demonstrate that the antibacterial activity of the bifidobacterial cells may play a significant role in favoring maximum inhibition against MRSA and MSSA strains compared with the ofloxacin activity.

[31] measured the anti-MRSA activity, which is present in mixtures containing different ratios of LAB strains, they found highest inhibition zone diameter to be 3.47 cm.

Inhibitor effect was correlated with the width of inhibition zone by spot on lawn technique which used to screen antimicrobial activity. The antimicrobial activity of *B. breve* strains is mainly due to organic acids; such as lactic acid, formic acid and acetic acids, reuterin, proteinaceous compounds, cyclic dipeptides, ethanol, acetone, hydrogen peroxide, diacetyl and bacteriocins [32,11,33].

As described, these experiments prove the bactericidal activity of *B. breve* against MRSA strain. This phenomenon can have a practical application if it can be performed *in vivo*. We believe the study will provide an opportunity to explore this natural process by letting the antagonism between cells of bifidobacteria and MRSA play out to the advantage of human health.

It is difficult to compare our results with results of other studies concerning *B. breve* with both MRSA and MSSA since the majority of information available is on the *B. breve* cells and other lactic acid bacteria with the *S. aureus* sensitivy to MSSA or its resistance to MRSA separately, [31,34] and [35] are some of these studies .Our situation is very different, the same antagonistic activity of bifidobacterial strains included in this study against two staphylococcal strains was exhibited.

5. CONCLUSION

Several bifidobacteria strains from resident infant human gastrointestinal microflora exert antimicrobial activity, suggesting that they could participate in the “barrier effect” produced by the indigenous microflora. This organism showed high tolerance to acid (PH3) and bile conditions. The strains evaluated comply with the functional and safety characteristics of probiotics. Our results were consistent with those of other researchers.

In conclusion, results of the present study illustrated that *Bifidobacterium breve* isolates have the ability to synthesize antimicrobial compounds that can inhibit the growth of the pathogenic *S. aureus* strains, proving that lactic acid bacteria can serve as a potential alternative in therapy applications. Our findings lend support to the assertion that there are components produced by cells of lactic acid bacteria (*B. breve*) that can inhibit growth and eliminate MRSA and MSSA.

ACKNOWLEDGEMENTS

I’m grateful to the college of Science, Wasit University and AL-Karama teaching hospital for supporting this research.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Peer-review history:
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