A Study the Characteristics of Bifidobacterium ssp Isolated from Breast Milk as a Probiotic in Vitro

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ABSTRACT

Probiotics as live supplements or living microorganisms that, when consumed, can provide health advantages beyond basic nutrition and can also improve the host's gut microbial balance. The objective of the present study was to recognize Bifidobacterium isolates from breast milk as a potential probiotic. A total of 60 out of 90 samples (66.67%) reveal positive results collected from human breast milk at Al-Zahra teaching and Al-kut Hospital, Wasit province in Iraq by biochemical test. 30 positive samples for Bifidobacterium isolates were identified by PCR (16S rRNA sequencing), study the tolerance of Bifidobacterium isolates in simulated gastric juice, and antimicrobial activity against pathogenic bacteria (Escherichia coli and Staphylococcus aureus) by using the well diffusion method. Bifidobacterium isolates showed the ability to tolerance of simulated gastric juice. There were significant differences ($P \leq 0.05$) among the average of viable cells count of Bifidobacterium in thirty samples, where it decreased from 5.430 log CFU / mL at zero time (before the incubation time) at pH 2.3 to 4.908 log CFU / mL after an incubation period (after 3 hours) at 37º C and pH (2.3). Moreover, the isolated bacteria have antimicrobial effects against S. aureus and E. coli. Bifidobacterium isolates showed a higher of antimicrobial activity against Gram positive bacteria (S. aureus) than Gram negative bacteria (E. coli). Bifidobacterium isolated exhibited support properties as probiotic, consequently, the importance of breast-feeding compared to bottle-feeding is shown.

Keywords: Bifidobacterium, low pH, antimicrobial activity, probiotic, 16S rRNA sequencing

Introduction

Probiotics are microbial food supplements that improve the health of the host. They are used to treat altered bowel micro flora and improved gut permeability, which are frequently seen in children with acute rotavirus diarrhea, individuals with food allergies or colonic problems, and patients receiving pelvic radiation [1].

Some of the probiotics chosen may be used to diminish the risk of or treat gastrointestinal infections because it has been demonstrated that they have considerable health advantages for people. There are well-characterized strains of that can be used by people [2][3]. It must be capable of performing its function in the digestive ecology without deteriorating [4].

One of the key selection criteria for novel probiotic strains is adherence to the intestinal

mucosa, a crucial requirement for colonization through binding sites, nutritional competition, steric hindrance, or immunological modulation, mucosal surface adherence and colonization may serve as a protective mechanism against infections [5] [2].

Low pH and bile tolerance are two other significant characteristics of prospective probiotics [6][3]. Consuming probiotics has been linked to a variety of health advantages, but the prevention of diarrheal disease development, incidence, and recurrence has consistently been shown to be one of them. Because they can produce organic acids with antibacterial qualities, such lactic and acetic acids, and because they can activate the host's immune system, probiotics have been shown to be able to prevent gastrointestinal illnesses [7].

Probiotics can inhibit the growth of a number of illnesses, including Salmonella typhimurium and Escherichia coli. Bifidobacterium ssp. is one of the most commercially available probiotic strains [8] [9] [7].

Bifidobacterium are a well-recognized gut commensals with probiotic properties [10]. This implies that it confers positive implications on the health of the host. This includes aiding in the development and the development of the immune system, providence of floats and reduction in the duration and severity of diarrheal diseases [11]. The last property consequently reduces the rate of infant mortality rates from infectious diseases, to poses serious health risks and a problem in number of developing countries [12].

Lactic acid bacteria (LAB) are microorganisms which produce lactic acid throughout metabolic activity. These microorganisms are important in several food applications. LAB are generally used as probiotics with beneficial properties for human health.

According to [13] "Probiotics are the living microorganisms that are administered/consumed in an adequate amount which produces beneficial effects on the host". They are parts of the microbiota of foods like fermented vegetables and fruits , fermented meats ,and dairy products, also are present within the digestive tract of humans and animals [14].

Genera: Bifidobacterium, Lactobacillus and Streptococcus are the most common strains of enteric bacteria and have been used as probiotic [15].

Bifidobacterium are rod-shaped, non-spore forming, non-gas forming bacteria [16], have a high G+C DNA content accounting 55-67 mol-%, aiding in the stabilization of their DNA [18].

As a verified microbe with probiotic properties, Bifidobacterium has been incorporated in a number of food products, including fermented dairy products such as yoghurt, frozen ice cream and cheese, fruit juices and sold as supplements in tablet, capsule or powdered form [19].

Additionally, it has been noted that Bifidobacterium has the capacity to hydrolyze indigestible complex carbohydrates like lactulose into acetic and lactic acids, preserving the gut microbial balance by inhibiting the development of potential infections. Additionally connected to Bifidobacterium to the regulation of the large intestinal tract's acidity [20].

One of the mechanisms employed by Bifidobacterium is the production of antimicrobial substances called bacteriocins, which aid in delaying growth by potentially pathogenic organisms (PPOs) [21]. A marked abundance of Bifidobacterium is detected combined with a signification reduction in the coliform in healthy yoghurt consuming adults [22]. Another supporting study the reduced Bifidobacterium population associated with advanced aging and increased presence of PPOs [23].

Bacteriocins from Bifidobacterium have not received much research focus. The inhibitory action of bacteriocins from this genus was mainly attributed to the production of organic acids, such as lactic acid and acetic acid. These acids reduced the pH level in the colon, therefore creating an unfavourable environmental habitat for a number of pathogens and ultimately suppressing their survival in this niche. However, in recent years, further studies have attributed the antagonistic

activity of Bifidobacterium associated bacteriocins against pathogens [21] [24].

The objective of this study is testing the viability of Bifidobacterium in simulated gastric juice (pH. 2.3) and study the antimicrobial activity of Bifidobacterium isolated breast milk against pathogenic bacteria (E. coli and S. aureus).

Materials and Methods

Bifidobacterium ssp. Isolation

Bifidobacterium was successfully isolated from breast milk using a modified version of [25] procedure. The milk samples were triple-plated onto Man-Rogosa-Sharpe plates agar after being diluted in peptone water. (MRS; Liofilchem, Italy) medium with supplemented L-cysteine (0.5%), sodium propenate (0.3%), and lithium chloride (0.2%) as a selective medium and then incubated anaerobically by using gas pak (Oxoid, Basingstoke, United Kingdom) in an anaerobic workstation (Becton, Dickinson and company, USA) at 37°C for 48-72 h. From each sample, 5 to 7 typical colonies were chosen, grown in MRS-Cys broth for 48 hours, then steeped on MRS agar for another 48 hours, incubated anaerobically at 37°C, and then cultured in MRS-Cys broth and kept at -80°C in the presence of glycerol (20%, vol/vol).

Catalase Test

To establish their morphology and the outcomes of the Gram staining, the chosen isolates were examined using an optical microscope. They were also examined for catalase test.

Many microorganisms contain catalase, an enzyme that converts hydrogen peroxide into water and oxygen and produces gas bubbles. The presence of the catalase enzyme is shown by the appearance of gas bubbles $2H_2O_2 \rightarrow 2\ H_2O + O_2$.

To observe the catalase reactions of the isolates, catalase tests were conducted. Isolate overnight cultures were established on MRS agar under the right circumstances for this purpose. A 3% hydrogen peroxide solution was poured onto a randomly selected colony after 24 hours, after comparative with positive reaction for catalase test, appearance of bubble indicates to positive result.

Molecular Identification:

By sequencing a 685-bp segment of the 16S rRNA gene with primers and PCR (Table 1), all of the Bifidobacterium isolates with distinguishing gram-positive and catalase-negative morphologies were recognized to the species level.

Table(1) Primer used in this study

Sequence	Specificity	Length of sequence to amplify (bp)
F / ACT GAG ATA CGG CCCAGA CT R / CGT ATC TCT ACG GCT GTC GG	Bifidobacterim 16SrRNA	685

Preparation of Pathogenic Bacteria

The pathogenic bacteria, which involve E. coli and S. aureus, were obtained from the Laboratory of Microbiology at Al-Zahraa Teaching Hospital, the standard culture collection was kept at 4°C in 20% glycerol. They were sub-cultured three times previous to use in an suitable medium.

Resistance to Low pH

To determine the transit tolerance over the simulated gastric juice, the procedure of [26] was used with slight modifications. Simulated gastric juice consisted of filter-sterilized pepsin (SIGMA-AIDRICH, Germany) at 0.3% w/v and Nacl 0.5% w/v, with pH adjustments to 2.3, Overnight culture of bifidobacterial isolates on MRS broth have been centrifuged (6000 ×g for 20 min) , the pellet were washed twice with 0.85% with sterile saline solution (pH 7.0) to disregard the media. Then re-suspended in 3 ml of the same buffer. 1 mL of the washed cell suspension were suspended in 10 mL of gastric solution at pH 2.3. Total viable counts of

Bifidobacterium were done on MRS agar, before and after an incubation period of 3 h at 37°C.

Antimicrobial Activity

Determination of antimicrobial activity by the production of bacteriocins by Bifidobacterium isolates was conducted according to [27] with minor modifications. Cell free supernatant (CFS) was obtained from Bifidobacterium strains grown for 16 hours in MRS broth. Followed by centrifugation of cell suspension for 5000 rounds per minute (rpm) for 30 min ,the pellet was discharge and the cell free supernat is used after filtrated by filters (0.2 µm-pore-size cellulose acetate filter).Agar well diffusion method is used to evaluate the antimicrobial activity of microbial extracts , the nutrient agar plate surface is inoculated by spreading a volume of pathogenic bacteria(E.coli and S.aureus) suspension was generated in 5 mL of normal saline solution with the turbidity regulated to equal that of 0.5 McFarland standards inoculum over the entire agar surface. Then, a well with a diameter of 7 mm is punched aseptically with a sterile tip, and a volume (80 µL) of the Bifidobacterium CFS is put into the wells. Then, agar plates are incubated under suitable conditions for 24 hours at 37 °C. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the wells.

Statistical Analysis

The statistical analyses of all the results were done by using the system SPSS IBMOversionO20 software, Chi-squire test. P-value ≤ 0.05 was considered statistically significant [28].

Results and Discussion

A total of 60 out of 90 samples reveal positive results collected from human breast milk at Al-Zahra teaching and Al-kut Hospital, Wasit province in Iraq by biochemical test. Bifidobacterium isolates display positive Gram stain(Fig.1), and negative for catalase.

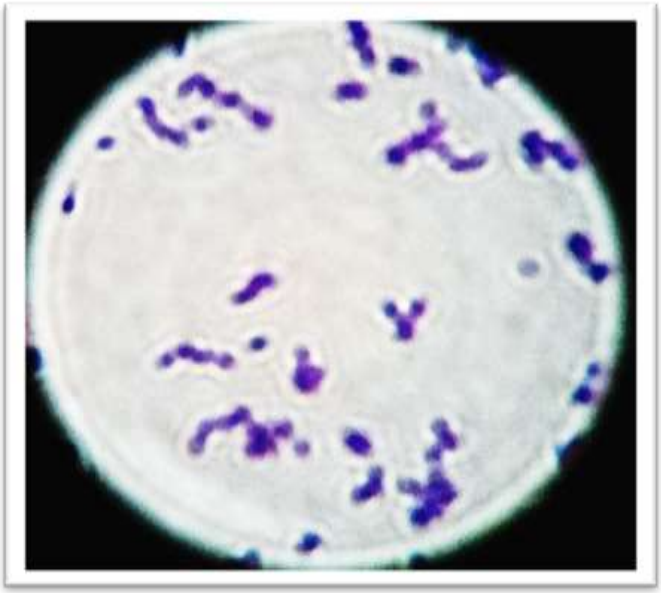

Figure 1. Gram staining results of Bifidobacterium ssp isolates

Tolerance of Bifidobacterium isolates to simulated gastric juice

Gastric acid secretion acts as the body's main line of defense against the majority of ingested bacteria there. The fact is that gastric surgery or the injection of proton pump inhibitors and other acid blockers may enable stomach microbial colonization [29].

Bifidobacterium in dietary supplements have been shown to survive gastrointestinal transit. The high survival rate enables the bacteria to exert physiological effects of potential health benefit to the host [30].

The results of simulated stomach juice on the capability of Bifidobacterium is displayed in Table (2). The mean viable cell counts of Bifidobacterium in thirty samples were

decreased from 5.430 ± 0.444 log CFU\ mL on zero time (before incubation period) in pH 2.3 to 4.908±0.587 log CFU\ mL after incubation period (3 h) at 37 ºC in pH 2.3(Table 3). There are significant differences (P ≤ 0.05) among the viable counts for each sample of Bifidobacterium isolate during the (incubation period 3 h) at 37ºC in pH 2.3. Based on our results, the viability of Bifidobacterium has established to be successful to encounter the minimum principle of probiotic cells per mL at pH 2.3 after contact to simulated gastric juice for 3 h. From 30 isolates of Bifidobacterium, there are significant differences (P ≤ 0.05) among available in twenty-two isolates, while there no significant differences (P ≥0.05) in eight isolates of Bifidobacterium during the incubation period at (pH 2.3) of simulated gastric acid for (3h) (37°C)(Table 2).

According to [18] demonstrated that Bifidobacterium is a strong ability to grow at low pH conditions (pH 3.2), [44] founded that all Bifidobacterial isolates displayed the ability to grow at low (pH 3.0) after exposure for 3 hours, and the study by [31] [32] concluded that the acid tolerance of Bifidobacterium is weak. Our findings are consistent with above mention studies.

Table 2: Mean values (log CFU m^{-1} ± SD) of tolerance of Bifidobacterium ssp. isolated from breast milk to simulated gastric juice for each sample.

Sample No.	Before incubation (time 0)	After incubation (3 h at 37°C)
1	5.23 ± 0.03 [a]	5.18 ± 0.06 [a]
2	5.31 ± 0.02 [a]	5.19 ± 0.06 [a]
5	6.18 ± 0.05 [a]	6.07 ± 0.06 [b]
6	5.43 ± 0.02 [a]	5.33 ± 0.02 [b]
10	5.96 ± 0.04 [a]	4.82 ± 0.10 [b]
11	5.90 ± 0.09 [a]	5.80 ± 0.09 [a]
13	5.06 ± 0.02 [a]	4.58 ± 0.10 [b]
16	5.24 ± 0.03 [a]	5.21 ± 0.03 [a]
17	5.24 ± 0.04 [a]	4.72 ± 0.07 [b]
20	5.26 ± 0.03 [a]	4.14 ± 0.03 [b]
23	5.33 ± 0.02 [a]	4.90 ± 0.11 [b]
25	5.36 ± 0.04 [a]	5.09 ± 0.06 [b]
26	4.73 ± 0.09 [a]	4.41 ± 0.03 [b]
27	5.28 ± 0.02 [a]	3.89 ± 0.07 [b]
30	4.73 ± 0.07 [a]	4.58 ± 0.09 [a]
31	5.42 ± 0.02 [a]	4.23 ± 0.03 [b]
32	4.86 ± 0.08 [a]	4.12 ± 0.04 [b]
33	5.63 ± 0.10 [a]	4.01 ± 0.03 [b]
34	5.73 ± 0.11 [a]	4.91 ± 0.07 [b]
35	5.41 ± 0.55 [a]	5.23 ± 0.61 [a]
37	5.89 ± 0.07 [a]	5.40 ± 0.01 [b]
39	5.56 ± 0.57 [a]	5.38 ± 0.57 [a]
40	5.15 ± 0.03 [a]	4.78 ± 0.10 [b]
41	6.14 ± 0.04 [a]	5.64 ± 0.13 [b]
42	5.14 ± 0.05 [a]	3.91 ± 0.07 [b]
43	5.04 ± 0.03 [a]	5.01 ± 0.03 [a]
44	6.42 ± 0.02 [a]	4.65 ± 0.14 [b]

47	6.22	± 0.03 [a]	5.80	± 0.08 [b]
68	5.05	± 0.03 [a]	4.89	± 0.02 [b]
70	5.45	± 0.02 [a]	4.93	± 0.04 [b]

Different superscript letters in the same rows represent significant differences ($p \leq 0.05$).

Table 3.Tolerance of Bifidobacterium to acidity in zero time and after three hours

	log-zero time		log- After 3 hours		P-value¥
	N	Mean ± SD	N	Mean ± SD	
tolerance to low ph	**30**	**5.430 ± 0.444 [a]**	**30**	**4.908 ± 0.587 [b]**	**0.001****

Numerous defense mechanisms against invasive infections are supported by the human gastrointestinal tract (GIT). Acidity of the stomach is one of them [33]. In transient to the large intestine, Bifidobacterium have to be able to with stand the weakening effects of low acid levels in the stomach and high acids levels in the large intestines [34]. It can be considered that Bifidobacterium ssp. have a tolerance, which can survive acidic pH and which is usually detected as the sole viable Bifidobacterium sp. in fermented milks [35].

The acid tolerance of luminal bacteria looked to be related to the ability to increase synthesis of H^+ - ATPase in response to low pH. Thus, there is a possibility that the acid tolerance of Bifidobacterium is dependent on the ability to synthesize H^+ -ATPase. [36], the H^+ - ATPase activity of the non-acid-tolerant strains decreased, although that of the acid-tolerant strains increased when the environment was acidified and the action of this enzyme in several strains and species was compared [32]. Some mechanisms regulate the homeostasis of interior pH. The translocation of proton ATPase is the highest important for fermentative bacteria [37]. It appears that proton-translocations ATPase's show most important parts in moving protons out of the cells and in dropping their net permeability to protons [38].

Proton pumping via the F1Fo-ATPase is not just one of the strategies that gram-positive bacteria utilize to tolerate acidity. Other processes include modifications to the cell membrane and regulatory systems, changes to various metabolic pathways, and amino acid decarboxylation [39].

Bifidobacterium in dietary supplements have been shown to survive gastrointestinal transit. The high endurance degree enables the bacteria to exert physiological effects of potential health advantage to the host [30].

Antimicrobial Assays of Bifidobacterium

Another vital trait a potential probiotic organism is required to possess is the ability to have inhibition effects against potential pathogenic organisms (PPOs) [40].

Nevertheless, it appears clear that the normal flora of human milk aids to inhibit infant infections and this may be one of the causes that describe why the antimicrobial activity showed by fresh collected human milk is lost after pasteurization [41].

The effects of probiotics on the growing of S. aureus and E. coli are existing in (Fig.2), the thirty isolated of Bifidobacterium showed antimicrobial activity against S.aureus with rang of diameter inhibition zone (20mm-27mm) **(**Fig.3) , and these present results under study are coincidence with findings by [42][43][44] **.** The antimicrobial activity of Bifidobacterium against E.coli with rang of diameter inhibition zone for thirty samples (15mm-25mm **(**Fig.4) **.**Our results is consistent with results by [45] [46] [47] [42] [43] [18].

Bifidobacterium isolates demonstrated more antimicrobial action (AMA) against, Gram positive in comparison with Gram negative bacteria (Table 4), this observation was in consistence with previous study by [43].

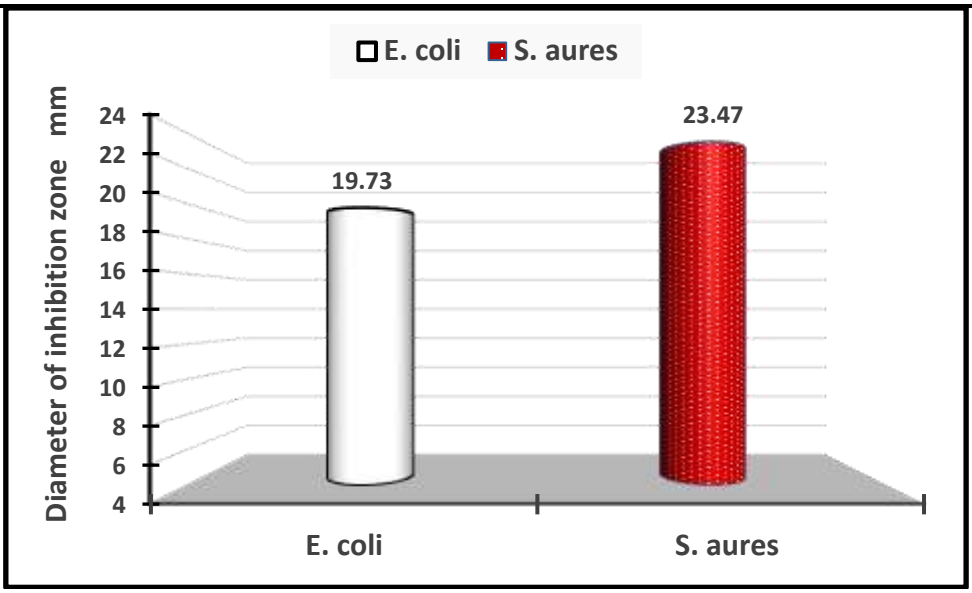

Figure 2.Antimicrobial activity of Bifidobacterium against E.coli and S.aureus for thirty samples.

Table 4. mean antimicrobial activity of Bifidobacterium against E.coli and S.aureus

	E. coli		S. aures		P-value¥
	Mean	SD	Mean	SD	
Diameter of inhibition zone	19.73	2.77	23.47	2.08	0.001**

Figure 3: Inhibition zones of Bifidobacterium ssp. isolated from breast against S.aureus

Figure 4. Inhibition zones of the Bifidobacterium ssp. isolated from breast milk against E.coli

The antimicrobial activity of Bifidobacterium due to production organic acids and bacteriocins or bacteriocin-like inhibitory substances was assessed against bacteria pathogens S.aureus and E.coli .The cell free supernat (CFS) of Bifidobacterium comprise of organic acids are compounds primarily responsible for the inhibitory effect of Bifidobacterium toward Gram negative bacteria, and bacteriocins have effect on Gram positive [48].

According to [49], the chemicals known as bacteriocins or proteinaceous substances with particular inhibitory activity against species that are closely related to each other are probably the most well researched.

This inhibitory effect against Gram positive bacteria was attributed to bacteriocins as the main inhibitory factor, thus demonstrating its effectiveness against the type of food-borne and human PPOs. On the other hand, antimicrobial effect against the Gram-negative bacteria was accounted to Bifidobacterium synthesized organic acids, consequently, the pH. is drop, and other inhibitory factors such as H_2O_2 and diacetyl [7] [43].

As expected, the wider clear ZOI observed was due to the inhibitory effects of organic acids. Bifidobacterium are reported to secrete acetic and organic acids as by-products of their metabolism. Other finding attributed the antagonistic effects of Bifidobacterium not only on organic acids, but also on proteins compounds with inhibitory factors. These are bacteriocins or bacteriocins- like inhibitory substance (BLIS) [50].

Organic acids, fatty acids, hydrogen peroxide, and diacetyl are only a few of the antimicrobial effects of the metabolic substances that lactic acid bacteria create [48].

The production of these weak organic acids results in an acidic environment which normally limits growth of both fungi and bacteria, comprising several pathogenic and spoilage microorganisms [51]. The antimicrobial properties of these acids are attached to the decline of pH to a level below the range of growth and metabolic inhibition by non-dissociated organic acid particles [52].

According to [7], the presence of more H^+ ions in the media lead to reduced pH, the pathogen would be subjected with unfavorable growth conditions, results in the reduction in colony numbers and their subsequent death.

Additionally, the antagonistic effects of the Bifidobacterium isolates cannot only be attributed to organic acids, such as lactic acid or acetic acid, but bacteriocins showed activity against the pathogen. Previous research works by [53][54][40].

Conclusions

Acid tolerance in Bifidobacterium is of particular importance, as this property is closely related to their use in human nutrient. This study has been providing indication of the capability of strains of the genus Bifidobacterium, integrates of the endogenous flora of adults and infants. Depending on these results under study, Bifidobacterium ssp isolated showed encouragement properties of probiotics and show the importance of breastfeeding compared to formula feeding. The use of Bifidobacterium synthesized organic acids or bacteriocins, may provide a biological means of preserving food products, thus ensure food safety. In the years to come, another area of research is the use of such antimicrobial factors in the form of powders or capsules, aiming at providing protection against PPOs, especially in infants and in treatment of enteropathogenic illness, as well as supplement to food adults and elderly . Since, Bifidobacterium has acidity tolerance, it is possible to use it in food preservation.

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