

Isolation, Molecular Identification and Characterization of *Bifidobacteria* Sp from Breastfed Babies

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ABSTRACT

Background: Seventy samples were collected from the feces of healthy newborn babies aged (2-4) weeks and from the arrivals to vaccination centers feed by breast milk and did not take an antibiotic in Wasit province, Iraq. The newborn babies were assessed clinically by the doctors in the hospital, placed directly in the sterile liquid MRS medium in test tubes and transferred to the laboratory within (1-2) hours to complete the isolation processes.

Methods: *Bifidobacterium* diagnosis was confirmed based on the morphological and cultural characteristics, including size of colonies, color, then studying the characteristics of cells under microscope after staining them with gram stain, additionally biochemical diagnosis of isolates of lactic acid bacteria included catalase test, production of ammonia from arginine hydrolysis, sugars fermentation test, grow in MRS broth, growth at different temperatures, growth at different concentrations of NaCl, resistance test of bacteria to bile salts, determine an ability to lower pH.

Results: that molecular study was done according to bacterial DNA extraction protocol of bacteria using specific primers. The intact DNA bands were confirmed through gel electrophoresis, proving that these isolates are members of the *Bifidobacterium* genus. The percentage of *Bifidobacterium* isolates were positive in 6 (12%) and negative in 44 (88%) in collected samples.

Conclusion:

Bifidobacterium is a major genera of bacteria that make up the gastrointestinal tract microbiota in babies, the prevalence of this bacteria is still unknown in babies as the using of antibiotics during pregnancy may affects their number.

1-Introduction

Bifidobacterium is a genus of gram-positive, nonmotile, often branched anaerobic bacterium, *Bifidobacteria* are one of the major genera of bacteria that make up the gastrointestinal tract microbiota in mammals, some *bifidobacteria* are used as probiotics [1]. Infants gut is quite sterile until their birth, where it taking bacteria from mother and the surrounding environments [2]. The microbiota bacteria makes the infants gut differ from an adult gut. Infants reach the adult stages of the microbiome at about 3 year age, when the microbiome increase and the infants takes solid foods. Breast feed infant usually colonizing by *Bifidobacterium* [3]. *Bifidobacterium* is commonest bacteria in infants gut [4]. In genotypes there is high variability more in infant over time, making them less stability when compared with adult. Children and infant under three age were showed low diversity regarding *Bifidobacterium*, where there was high diversity among individuals compared to adult [5]. *Bifidobacterium* spp bacteria contribute to the compilation of the commercial probiotic, as well as the genus is the most secure [6].

The rise of infections by antibiotic-resistant bacterial pathogens is alarming. Among these, *Klebsiella pneumoniae* is a leading cause of death by hospital-acquired infections, and its multidrug-resistant strains are flagged as a global threat to human health, which necessitates finding novel antibiotics or alternative therapies to overcome potential resistance regard multiresistant *K. pneumoniae* especially biofilm producing *klebsiella pneumonia* in clinical isolates [1, 7].

different researches were showed high count operation regards *Bifidobacterium* spp bacteria, and lower case of intestinal and stomach inflammations in babies, breast fed babies than bottles feed babies [8]. Various doctors recommended to use this bacteria as probiotic either as capsule or pill in addition to cosmetic lyophilized, therefore probiotic choice as alternative treatment had been proved a significant roles in many studies [9]. The existence of *Bifidobacterium* in maternal fecal and vaginal microbiotaa is one of the evolutionary traits that allow these organism to colonizer intestinal tract of babies, probiotic strain had been developed in term of supplement in premature baby, to countering ailment and to confer ranges of health benefit [3, 10, 11].

Acquisitions of healthy gut bacteria during early stage of life may play an important roles in an individual health during another stages of life. It was proposed, but universally not established [12, 13], that microbe begins to colonizing new born in uterus of his mother [14]. Until now, roles of the bacterial species that acquired from the habitat in infants development remain not well understood [15, 16].

In order to overcomes these challenges, molecular based method like using of "real time polymerase chain reaction "(RT-PCR)," was developed, using of genus and species specific polymerase chain reaction and the 16S rRNA in addition to different molecular marker (*clpC*, *dnaA*, *xfp*, *dnaJ* and *rpoc*), these techniques were showed more reliable in identification of bacterial species [17]. Using of 16S rRNA was considered as one of the best marker in microbial diffrenttiation [18, 19]. So using of primer pair were designed to targeted this regions in *Bifidobacterium* doesnot produced sufficient amplicon in exact determinations of species [20].

2-Materials and methods:

Samples Collection

Seventy samples were collected from the feces of healthy newborn babies at the age of (2-4) weeks and from the arrivals to vaccination centers who depended on breast milk for their nutrition and did not take any kind of antibiotics or medicines, samples were collected from Wasit province, Iraq from November 2022 to February 2023, in the bacteriology Unit. The patients were first assessed clinically by the doctors in the hospital and then referred for sample collection. The samples were placed directly in the sterile liquid MRS medium in Test tubes were kept in an ice box and transferred to the laboratory within (1-2) hours to complete the isolation processes [11].

Isolation and identification of bacterial isolates

Initial bacteriological diagnosis:

After the colonies has been grew on the surface of the culture medium of the primary culture, they were initially diagnosed based on the morphological and cultural characteristics, which included the size of the colonies, their color, then studying the characteristics of the cells under the microscope after staining them with a gram stain and finding out the type of their response to the dye [21].

Biochemical diagnosis of isolates of lactic acid bacteria

One pure colony is taken from the isolated bacteria, whose shape and the nature of their staining [21].

Catalase test

It is investigated by mixing a drop of H₂O₂ (3%) solution with a part of a pure colony using a glass rod on a clean glass slide [22].

Gelatin liquification test

The medium was prepared by adding 10-15% of gelatin to the nutrient broth and adjusting the pH according to the requirements of the bacteria to be diagnosed at a temperature of 37 °C for 7 days [23].

Production of ammonia from arginine hydrolysis

One ml of the bacterial culture was taken and placed in a test tube, and 1 ml of the reagent was added to it. The color change from orange to brown was observed to indicate the ability of the bacteria to decompose arginine and produce ammonia [24].

Sugars fermentation test

The positive results were observed by changing the color of the detector to yellow in the case of a decrease in the pH, noting the amount of gas formed [25].

Growth in bottoms of MRS broth:

Tube containing liquid MRS medium were inoculated by newly growing bacterial colony and incubated at 37°C of temperature anaerobically about 24-48 hours [26].

Growth aerobically on nutrient agar

Lactic acid bacteria were planted on the nutrient agar medium and incubated in an airy atmosphere at 37°C for 24 hours and the presence or absence of growth was observed [27].

Growth at different temperatures

MRS broth tubes were inoculated with 1% of modern bacterial isolates and incubate anaerobically at different temperature (5°C, 15°C, 37°C, 45°C) in a period 3-5 day, and the results were positive. In the event that it becomes cloudy [28, 29].

Growth test with different concentrations of NaCl

The test was carried out using different concentrations of sodium chloride (3.5, 4.5, 5.5)% each separately in liquid cultures of bacterial isolates aged (18-24) hours, then incubated for 24 hours. Under anaerobic conditions, growth was observed through the formation of turbidity [30].

Resistance test of bacteria to bile salts

"Lactic acid bacteria (LAB)" Has been grown in MRSbroth, centrifuged

(2000 Round Per Minute) 10 minute and then were suspended, the final suspension were incubate (72 houts). , and sub cultured on "MRS" agar and incubated using the candle-jar in 37C° [31].

Determining of Bifidobacterium to tolerating low pH

The grown bifidobacterium 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) with 9 ml of phosphate buffered saline (pH = 3) and incubated for (3) hrs. The sub cultured on "MRS agar" then incubated at 37C° for about 48 hours [32].

Molecular study.

It was done according to Bacterial DNA extraction protocol of bacteria.

Estimation of DNA quality:

The extracted DNA was estimated by using Nanodrop instrument that measured DNA concentration (ng/μL), then DNA purity checked by reading the absorbance at 260 /280 nm. The method has been proceeded according to the manufacturer instructions.

Preparing of the Primers Suspension

The primers were re-suspended by dissolving the lyophilized product, after spinning down concisely, about (295 -349 μl) PCR water (free nuclease) according to manufacturer instructions.

Bifidobacterium spp. primers

In this study all primers were provided from Integrated DNA Technologies (IDT)company Canada, were shown in the table (1).

Table (1): Specific primers of this study

gene	Primer sequences (5° - 3°)		Product size (bp)
<i>16SrRNA</i>	F	GGGTGGTAATGCCGGATG	517
	R	CCACCGTTACACCGGGAA	

3- Results

Isolation and Identification of *Bifidobacterium* from feces

Cultural and Microscopic Characteristics

Bifidobacterium seemed spherical, white, and cream in color on MRS agar with cysteine supplement. (Figure 1), (Cysteine, an amino acid with sulfur, may reduce the redox potential and give amino nitrogen as a cofactor, each of which may favor the growth of anaerobic *Bifidobacterium spp.* [16], with a "Y" and "V" shape, which were gram-positive coccobacilli upon microscopic examination. These appearances are determined by the selective medium, morphological, and physical properties (Figure 2). These were also shown to be catalase-negative.

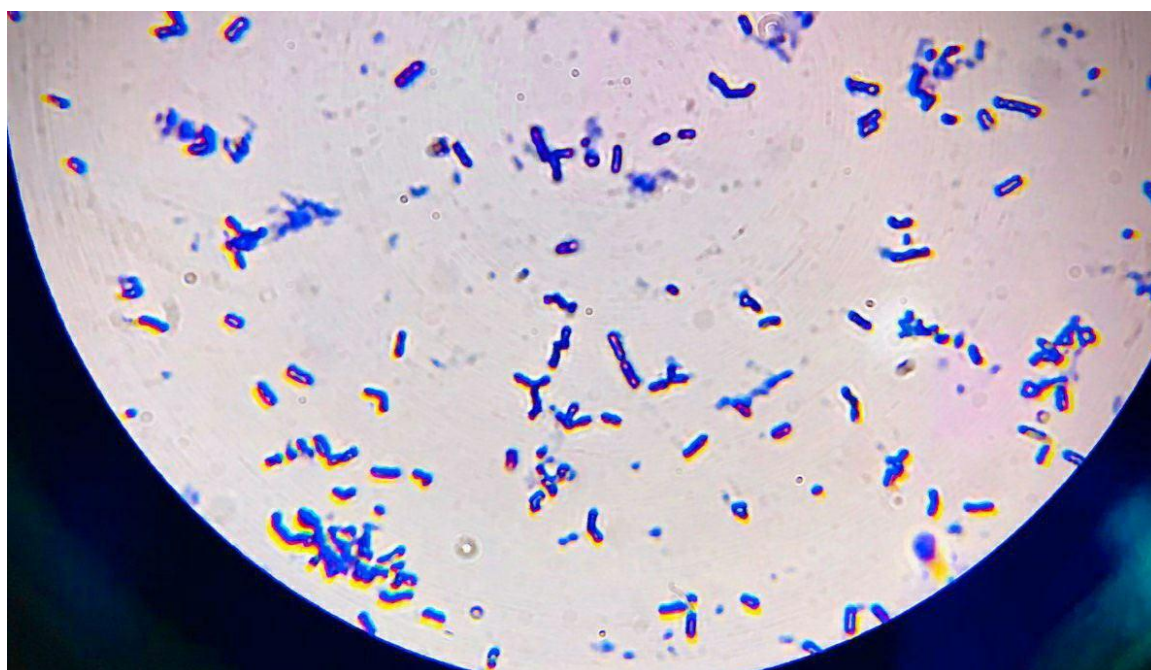


Figure 1. gram stain of *Bifidobacterium spp.* from stool of babies feeds on mother milk



Figure 2. Results of *Bifidobacterium* spp isolates

Molecular Analysis

The DNA of 70 isolates from stool of babies feeds on mother milk (breast feeding), sample was extracted. The intact DNA bands were confirmed through gel electrophoresis as shown in figure (3) and table (1). The DNA taken from them were amplified by PCR was discovered to be 511 bp, proving that these isolates are members of the *Bifidobacterium* genus.

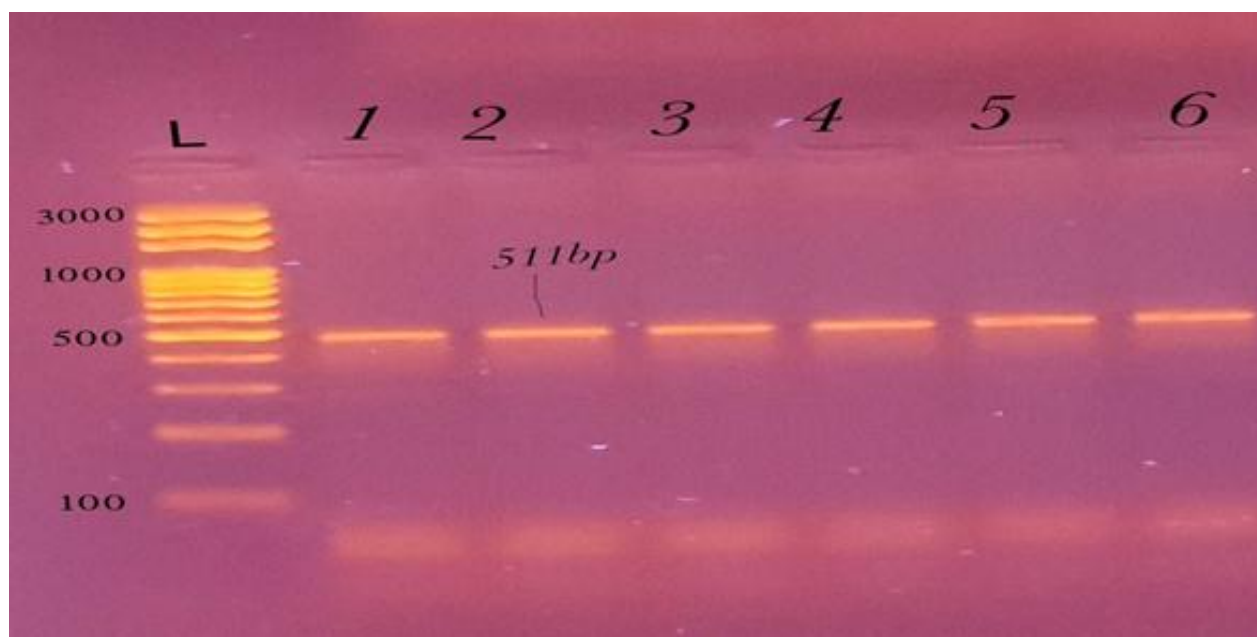


Figure (3)"Agarose gel electrophoresis image that showed the PCR product,of 16S rRNA gene of Bifidobacteria isolates at 511 bp PCR product size respectively . The Lane (L): DNA marker (100-3000bp.

Table 2: The percentage of presence of *Bifidobacterium* in samples of stool from babies feeds on mother milk (breast feeding).

Sample		
Results	Frequency	Percent
Positive	6	12.0%
Negative	64	88.0%
Total	70	100.0%

4- Discussion

In a previous study [33] it has been proposed the using of medium contain MRS added to l-vancomycin, bromocresol purple and cysteine, this will provide optimum condition in isolation and counting probiotic. It was explored the probability to improving "LcS" select medium by Sutula et al., (2012) used all MRS component, but without meat extract and glucose, instead amended it using maltose as a carbohydrate source, vancomycin, l-cysteine, bromocresol purple and Tween 80.

Another study [34] mentioned that breast milk has beneficial impacts on human health and is a significant source of nutrition for newborns. One intention is that the micro biota in this human milk contains advantage bacteria. Results: The colonies of Gram –positive, catalase –negative bacteria from particular medium of typical bifidobacterial organisms were identified at the genus level after incubation using MRS media.

Optimize method in order to be reliable and selective enumerations of member regarding *Lb. casei* group. It was assayed *Lb. casei*-groups strain initially on different agar media using various selective compound used as basal media, mixing of all MRS component except meat extract and glucose [35]. MRS media has been chosen since it was widely improved as optimal media for enumeration and growth under anaerobic and aerobic conditions, this is principally due to its acidity and nutritional composition [36].

Our findings of *Bifidobacterium* prevalence using PCR were agreed to [37] study mentioned that the PCR analysis had high specificity and sensitivity in administered bacterial types. So, in 9 of 252 contributed women were showed to have any bacterial types in their "breast milk" at time point. Although it was found statistical significant increase in existence of postpartum in probiotic group, 50 of 129 babies in probiotic groups.

In previous studies of maternal supplementations, *Lactobacillus* species were recovered in breast milk as 12%-53 of mothers after supplementation [38, 39]. Over recent five years, various studies had been used sequencing method in order to characterizing breast fed related to microbiome and its correlation to women-infant characteristic like stages of lactation [40, 41].

But our data were not in consistency with the studies of [42-44], as the statically Analysis revealed that the variations were significant. ($p \leq 0.05$) among the presence of *Bifidobacterium* (77.5%).

Current results suggested that stages of lactation, delivery mode, maternal atopy and sex might affected the existence of specific bacterial genera. Moreover, neither those characteristic, nor presence of sibling had been defined over-all compositions of breast fed-related to microbiome. Due to limits in information, it was not surely to evaluate effects of maternal antibiotics using and sign or symptom of mastitis. Information about antibiotics utilizing and the existence of mastitis would had been useful particularly with respect to an observations that probiotic supplementations may promotes stability of microbiota related to the breastfed, methodological difference in laboratory analysis may influences the observed microbiome in this study or in another studies [37].

Competition Interest

Authors of this research declared there is no conflict of interest.

Authors contribution

Hajar T. Mahdi: Material preparing, questionnaire designer, data collection, explaining of outcomes and writing on article.

Prof Dr. Zainab N. Al-Saadi: Article proposal designer, statistical analysis, editing and writing.

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