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ISOLATION AND IDENTIFICATION OF *LACTOBACILLUS REUTERI* FROM HUMAN BREAST MILK COLLECTED IN BAGHDAD

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ABSTRACT
For many decades, breast milk has been regarded as a sterile body fluid which exerts its influence on the infant's microbiota environment via presenting only some growth factors and optimal conditions for helping the growth of bacteria. However, in recent years, breast milk has been hypothesized to be a source of commensal bacteria for the infant gut Breast milk is an important nutrient source for neonates. Lots of studies showed that this fluid has beneficial effects on the health of neonates. One reason of being beneficial is explaining by the microflora of human breast milk including beneficial lactic acid bacteria. Samples of breast milk were obtained from 31 lactating women were used in this study to isolate and identify the lactobacillus and to find out the incidence of Lactobacillus in Iraqian human breast milk. Biochemical tests and API® 50 CH were used for identification of isolates of Lactobacillus upto the genus level Results: Culture of the 31 breast milk specimens showed growth of different species of *Lactobacillus* in the specimens. Conclusion: breast milk can be a source of *Lactobacillus* for the infants Lb. reuteri were isolated from Iraqian human breast milk in high percentages (35.71)% (n=15) out of lactobacillus isolates, while Lb. acidophilus constituted (33.33)% (n=14), Lb. plantarum achieved (19.04)% (n=8), Lb . casei formed (9.52)% (n=4), and Lb.oris accomplished (2.38)% (n=1) out of actobacillus isolates.

KEYWORDS: Lb. reuteri, Isolation, Breast milk.

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, non-spore forming, catalase-negative bacteria that are devoid of cytochromes and are of nonaerobic habit but are aero-tolerant, fastidious, acid tolerant and strictly fermentative; lactic acid is the major end-product of sugar fermentation.[1] Strains of the favourable lactic acid bacteria (LAB), such as *Lactobacillus spp., Bifidobacterium spp., Enterococcus spp. and Lactococcus spp.* etc., have been used for probiotic preparation.Among antimicrobial materials produced by *Pediococcus pentosaceus*, bacteriocin have gained the greatest interest in food preservation as a natural substance .[2] The health effects of probiotics on host may include: modulating the immune system; increasing the protective effect against infection by pathogens; antiallergic effects; and preventing the cancer recurrence.[3,4,5] Cell tolerancey to acidity and bile salt are important factor that affect the probiotics to remain and exert their potential functionalities in a host.[6] To protect the host animal against the infection of pathogenic bacteria, competitive exclusion of pathogens by the adherence of LAB on host intestinal epithelium, and enhancement of the immunomodulatory activity of host by LAB, are two of the major factors.[7] *Lactobacillus fermentum* selected as an alternative treatment to prevent or treat urogenital infection based on their probiotics properties and production of bacteriocins.[8] According to the World Health organization and Food and AgricultureOrganization of the United Nations (WHO/FAO).[9] live microorganism that confer a health benefit to the host when administrated in adequate amounts are referred to as probiotics. This definition is debated since heat-killed strains exert similar beneficial effects as live bacteria.[10] Many bacterial strains with health beneficial properties belong to *Lactobacillus* or *Bifidobacterium*.[11] *Lactobacilli* are Gram-positive, non-spore-forming, catalase negative, aerotolerant or anaerobic, acid tolerant organisms with a low DNA G+C content.[12] Human milk is a complex species-specific biological fluid adapted to satisfy the nutritional requirements of the rapidly growing infant; additionally, it educates the infant immune system and confers a certain degree of protection against pathogens.[13] These effects reflect the synergistic action of many bioactive molecules, present in colostrum and milk, including immunocompetent cells, immunoglobulins, fatty acids, polyamines,

oligosaccharides, lysozyme, lactoferrin and other glycoproteins, and antimicrobial peptides.[14] Which inactivate pathogens individually, additively, and synergistically.[15]

MATERIALS AND METHODS

Samples

1. Samples Collection

Samples of breast milk were obtained from 31 lactating women, Subjects were excluded from the study if there was:

(1) Infant and/or maternal perinatal problems, (2) History of antibiotic intake in the previous month or (3) breast inflammatory condition like mastitis or breast abscess.

The milk samples were obtained by manual expression using sterile gloves, and collected in two sterile containers; one of which contained 0.5 ml of thioglycolate transport medium to provide an anaerobic condition. Initially, the nipples and mammary areola were cleaned with soap and water, dried with sterile gauze then wiped with 70% alcohol. The first drops (~500µl) were discarded[16].The two specimens including the one collected on the transport medium were subjected to culture immediately after being delivered to the laboratory.

Skin sampling was performed as a control; the areola, after treatment with alcohol was gently rubbed using sterile cotton swabs then plated on blood agar media. It yielded no bacterial growth.

2. Isolation and identification of bacteria

Pour plate technique was used to isolate the organisms.[17] One ml of the milk was inoculated into nine ml of Man, Rogosa and Sharpe (MRS) Broth (Oxoid, United Kingdom), well shaked and then they were serially ten folds diluted. One ml aliquot of the samples and dilutions were plated into MRS medium agar (MRS; Oxoid, United Kingdom) for isolation of lactobacilli, MRS-Cys agar plates supplemented with 0.05% (wt/vol) L-cysteine hydrochloride Inoculated MRS and MRS- Cys plates were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) at 37°C for 48-72 h in an anaerobic jar using Oxoid anaerogen compact gas packs (Oxoid, UK).[17] Colonies of different morphologies and sizes growing on MRS and MRS- Cys agar were chosen and transferred to MRS and MRS- Cys broth for 24 to 48 h. Bacterial isolates were characterized on the basis of their colony morphology, microscopic appearance after Gram staining (Gram positive) and Slide Catalase Test was done to verify the bacteria as Catalase negative.

3. Preservation of isolates

The different isolates were preserved in MRS broth medium containing glycerol and stored at -80 °C until further testing. The glycerol stocks of samples were prepared by adding 0.5 ml of active cultures to 0.5 ml MRS medium containing 40% sterile glycerol and thioglycolate plus 0.5ml reconstituted autoclaved skimmed milk.[18]

4. Identification of the isolated organisms

Identification by using the API identification strips (bioMérieux, Marcy l'Etoile, France). API® 50 CH in combination with 50 CHL liquid media to help in the identification of lactobacilli, API® 50 CH, API® 20A and API® ZYM for the identification of anaerobic bacteria following the manufacturer protocol, then, by referring to the Analytical Profile Index and by using the identification software (Apiweb), the isolates were identified to the genus and sometimes to the species level.

RESULTS

1. Isolation and Identification of *Lactobacillus reuteri*

Mother's milk samples were obtained in order to isolate *Lactobacillus.*[42] isolates belong to Lactobacillus genus were obtained. Depending on the cultural, Micoscopic examination and biochemical tests and API 50CHLsystem, as shown in table (1,2,3) and figure (1).

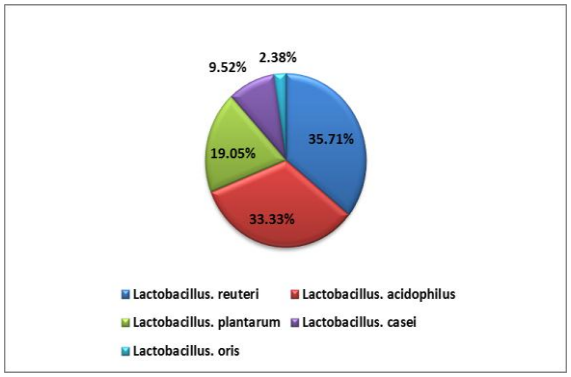

Figure (1): percentages of *Lactobacillus* isolates from human breast milk

2. Morphological Examination of colonies

Morphological examination of all *Lb.reuteri isolates* grown on MRS agar medium showed white, large, smooth, round colonies with entire margin. On SL agar medium, the colonies were large, cream- to beige-colored, little convex, little mucoid, smooth and circular with entire margin. According to.[19] when *Lactobacillus* species are the predominate flora in the source material, MRS agar can be used for their isolation. This medium supports good growth of *Lactobacillus* because it contains all the nutrients for *Lactobacillus* growth.[19] On the other hand, based on the low pH, the presence of tween 80 and acetate, SL agar is highly selective medium for *Lactobacillus* species.[21,22,23], Also they were developed small, smooth and convex with light yellow colonies on blood agar medium.[24,25]

3. Microscopic Examination

In microscopic examination, the cells of *Lb.reuteri* reacted positively with Gram stain and appeared as purple rods with rounded ends that occurred singly, in

pairs or in short chains and non-spore forming as shown in figure (2).[26]

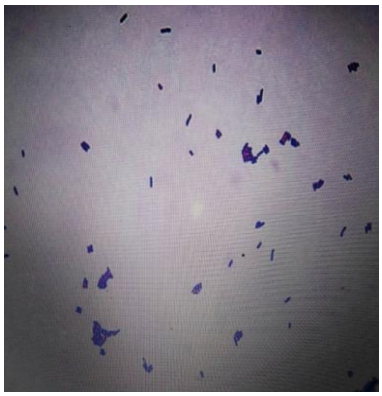

Figure (2): *Lactobacillus reuteri* isolated from human breast milk. Purple rod cells appears single, in pairs or in short chains.

4. Biochemical Tests

The biochemical characteristics of the *Lb.reuteri* isolates were similar, as shown in table (3).

All the bacterial isolates were catalase, oxidase, gelatinase negative, unable to grow at 15°C while they were able to grow at 45°C. All lactobacillus isolates were able to grow in the presence of (6.5 and 7) % NaCl whereas at 10% NaCl they were not able to grow. In the presence of 4% sodium taurocholate and at pH 3.9, all lactobacillus isolates were able to grow.

The sugars (glucose, melibiose, maltose, sucrose, lactose, raffinose, fructose, galactose) were fermented by all *Lb.reuteri* isolates while 10 carbohydrates (xylose, rhamnose, sorbitol, ribose, mannitol, mannose, salicin, trehalose, gluconate, arabinose) were not fermented by these isolates. The carbohydrates fermentation test is the most important test to identify species belong to Lactobacillus genus.[23,27] as in table (3).

According to the morphological and biochemical tests, the isolates were identified as *Lactobacillus Spp*. Depending on the results of API 50 CHL KIT (15) isolates of lactobacillus reuteri were identified, as shown in table (2).

Table (1): Numbers and percentages of *Lactobacillus* isolates from human breast milk samples

Isolates Type	No. of Lactobacillus isolates	Percentage of *Lactobacillus* isolates (%)
Lactobacillus reuteri	15	35.71
Lactobacillus acidophilus	14	33.33
Lactobacillus Plantarum	8	19.04
Lactobacillus casei	4	9.52
Lactobacillus oris	1	2.38
Total	42	%100

Table (2): Lactobacillus identification by API 50 CHL system and the results it:

Substrate	Result	Substrate	Result
Negative control	-	Esculin ferric citrate	-
Glycerol	-	Salicin	-
Erythritol	-	D-Cellobiose	-
D-Arabinose	-	D-Maltose	+
L-Arabinose	+	D-Lactose	+
D-Ribose	±	D-Melibiose	+
D-Xylose	+	D-Saccharose	+
L-Xylose	-	D-Trehalose	-
D-Adonitol	-	Inulin	-
Methyl-βD-xylopyranoside	-	D-Melezitose	-
D-Galactose	+	D-Raffinose	+
D-Glucose	+	Amidon	-
D-Fructose	-	Glycogen	-
D-Mannose	-	Xylitol	-
L-Sorbose	-	Gentibiose	-
L-Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
D-Mannitol	-	D-Fucose	-
D-Sorbitol	-	L-Fucose	-
Methyl-$\alpha D-mannopyroside$	-	D-Arabitol	-
Methyl-αD-glucopyranoside	-	L-Arabitol	-
N-Acetylglucosamine	-	Potassium gluconate	-

Amygdalin	-	Potassium 2-ketogluconate	-
Arbutin	-	Potassium5-ketogluconate	-

Table (3): Biochemical tests of the *Lactobacillus reuteri* isolates

Test	Result
Catalase, oxidase, gelatinase test	-
Morphology	Rod
Growth at 45°C	+
Growth at 15°C	-
Gram stain	+
Fermentation of :	
Glucose	+
Mannitol	-
Maltose	+
Mannose	-
Sucrose	+
Salicin	-
Lactose	+
Ribose	-
Raffinose	+
Fructose	+
Galactose	+
Trehalose	-
Xylose	-
Rhamnose	-
Melibiose	+
Sorbitol	-
Arabinose	-
Growth at 6.5,7% NaCl	+
Growth at 10% NaCl	-
Growth at 4% sodium taurocholate	+
Growth at pH 3.9	+
Arginine hydrolysis	-

-: negative result, +: positive result, *Lactobacillus* reactions as listed in the 9th edition of Bergey's manual and systemic bacteriology.[28]

DISCUSSION

Lb. reuteri were isolated from Iraqian human breast milk in high percentages (35.71)% (n=15) out of lactobacillus isolates, while *Lb. acidophilus* constituted (33.33)% (n=14), *Lb. plantarum* achieved (19.04)% (n=8), *Lb . casei* formed (9.52)% (n=4), and *Lb.oris* accomplished (2.38)% (n=1) out of lactobacillus isolates. Bacterial colonization of the infant gut is a gradual process that exerts a strong influence on the health status of the host, since the members of the gut microbiota may contribute to the barrier effect against pathogens Human milk is a source of bacteria to the infant gut, where they may play a variety of antiinfectious, immunomodulatory, and metabolic roles. In fact, recent studies indicate that the mammary gland contains its own microbiota during late pregnancy and lactation. This bacterial community may differ depending on the individual and the health status of the lactating women. It seems that certain bacteria from the maternal gut can use mononuclear immune cells to colonize, first, the mammary gland and, later, the infant gut through breast-feeding.and to the maturation of the intestinal immune system.[29] This bacterial community may differ depending on the individual and the health status of the lactating women. It seems that certain bacteria from the maternal gut can use mononuclear immune cells to colonize, first, the mammary gland and, later, the infant gut through breast-feeding.[30] [31] Investigated that L. reuteri was found in the breast milk of mothers from around the world, and of the 220 samples collected from as many mothers, 32 were found to be positive for L. reuteri, giving an overall prevalence of 15%. Breast milk from Japanese mothers showed the highest frequency of colonization with L. reuteri (11 of 20 giving positive samples). Women from other locations showed a lower prevalence of L. reuteri. Total lactobacilli were found in higher numbers in most samples examined, with 93 of 220 samples being positive (42% prevalence) in this regard. Other study,[32] recovered lactobacilli representing 55.2% of the total isolated bacteria from 30 breast milk specimens. Lactobacilli were detected in the milk of 100% of the mothers participating in the study. The majority of the identified *lactobacilli* species belonged to the species L. *fermentum-1* (40%) and the o isolated a *L. salivarius* strain from human milk and infant feces of a mother-child pair. species *L. brevis- 2* (34%), followed by *L.*

acidophilus, L. fructivorans, L.plantarum-1, L. brevis-1 and *L. delbrueckii*. Different lactobacilli species were also recovered by[33]. They can also contribute to the reduction of the incidence and severity of infections in the breast-fed infant by different mechanisms such as production bacteriocins and biosurfactant.[34,35,36]

CONCLUSION

In this work we can be conclude that human breast milk contains lactic acid bacteria is a natural source of LAB for the newborns and may be considered a synibiotic food.

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