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ANTIMICROBIAL EFFECTS OF PROBIOTICS IN HUMAN BREAST MILK

Dr. Ezenwa C. M.[1]*, Prof. Nwoke B. E. B.[2], Dr. Emukah E.[3] , Nnagbo P. A.[1], Obasi C. C.[4], Ohabughiro N.B[1], Nwagbaraocha M. A.[5], Dr. Nwachukwu I. O.[1] and Dr. Orjiakor V. U.[2]

[1]Department of Microbiology, Imo State University, P. M. B. 2000, Owerri, Nigeria.
[2]Department of Animal and Environmental Biology, Imo State University, P. M. B. 2000 Owerri, Nigeria.
[3]Primary Health Care Development Agency, Imo State, Nigeria.
[4]Department of Public Health, Imo State University, P.M.B. 2000, Owerri, Nigeria.
[5]Department of Medical Laboratory Science, Imo State University, P.M.B. 2000, Owerri, Nigeria.

***Corresponding Author: Dr. Ezenwa C. M.**

Department of Microbiology, Imo State University, P. M. B. 2000, Owerri, Nigeria.

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ABSTRACT

Lactic acid bacteria, *Lactobacillus plantarum* and *Lactobacillus pentosus*, were isolated from the breast milk of six healthy breast feeding mothers using MRS agar media and API50CHL kit and the total Lactic Acid Bacteria (LAB) count determined. These bacteria were facultative anaerobic, gram positive, catalase negative and non-endospore forming. The LAB isolates *Lactobacillus plantarum* and *Lactobacillus pentosus* showed a great antimicrobial effect on all the indicator organisms, namely *Salmonella sp., Escherichia coli, Shigella* sp. and *Shigella* sp. *Klebsiella* sp. had the highest susceptibility of 16 mm and 14 mm on *Lactobacillus plantaru* and *Lactobacillus pentosus* respectively, while *Escherichia coli* had the least zone of inhibition of 11 mm and 6 mm on *Lactobacillus plantaru* and *Lactobacillus pentosus* respectively. It was found that human milk is a source of potential probiotic organisms which addition to infant formulas could be an alternative to the functional effects of human milk.

KEYWORDS: Probiotics, Antimicrobial, Human, Breast, Milk.

INTRODUCTION

The term probiotics is currently used for ingested microorganisms with beneficial effects to humans and animals. The introduction of the concept is generally attributed to Élie Metchnikoff, who in 1908 suggested that the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes. The most common types of microbes which are used as probiotics are lactic acid bacteria (LAB) and Bifidobacteria. A number of genera within Firmicutes phylum like *Lactobacillus, Lactococcus, Lactosphaera, Carnobacterium, Enterococcus, Streptococcus, Tetragenococcus, Oenococcus, Pediococcus, Weissella, Melissococcus, Vagococcus* constitute lactic acid bacteria.[1,2,3] LABs are Gram-positive bacteria capable to ferment carbohydrates into lactic acid and energy.[2] Some LAB differ in their metabolic pathway, for example, homofermentative bacteria like *Lactococcus* and *Streptococcus* produce two lactate molecules from one glucose molecule while heterofermentative bacteria like *Leuconostoc* is able to convert one molecule of glucose into ethanol, lactate and carbon dioxide.[4,2,5] Furthermore, lactic acid bacteria yield some organic compounds that contribute to the aroma as well as flavor of the fermented products.[4]

Human milk is a complex biological fluid that is species-specific and completely fulfills both nutritional and microbiological requirements of the new born. Breast milk boosts up immune system and builds body defense against various infectious diseases which makes it superior to other food supplements for infants. Various bioactive compounds like immunoglobulins, lysozyme, antimicrobial acids, oligosaccharides, glycoproteins for example lactoferrin, polyamines, immune cells and bioactive peptides present in breast milk are responsible for its anti-infective effect.[6,7] These bioactive compounds of human milk play a major role in the regulation of the anti-inflammatory system. Due to immunomodulatory action of human milk, the incidence as well as severity of various infectious diseases like tetanus, poliomyelitis and diphtheria is lesser in breast-fed infants than those fed with other food formulae.[8]

During the first few days after delivery, the mother produces colostrum, a thin yellowish fluid that is rich in protein and antibodies that provide passive immunity to the baby. Breast milk isn't sterile, but contains as many

as 600 different species of various bacteria, including beneficial *Bifidobacterium breve, B. adolescentis, B. longum, B. bifidum,* and *B. dentium*.[9]

Commonly claimed benefits of probiotics include the decrease of potentially pathogenic gastrointestinal microorganisms, reduction of gastrointestinal discomfort, strengthening of the immune system, improvement of the skin's function, improvement of bowel regularity and strengthening of resistance to cedar pollen allergens. Other benefits include decrease in body pathogens, reduction of flatulence and bloating, protection of DNA, protection of proteins and lipids from oxidative damage, and maintenance of individual intestinal micro biota in subjects receiving antibiotic treatment.

MATERIALS AND METHOD

Materials

The laboratory materials that were used in this work include; Test Tubes, Conical Flasks, measuring cylinder, petri dish, wire loop, glass slide, filter paper, disposable hand gloves, polythene bags, petri dishes, forceps, scalpel, autoclave, light microscope, incubator, weighing balance, Bunsen burner, refrigerator, distilled water, 75% Ethanol, Crystal violet, safranine, iodine, bent glass rod, forceps, NA, MSR and hydrogen peroxide.

Specimen Collection

Specimen was collected from six (6) different mothers who voluntarily donated their breast milk in Owerri under aseptic conditions. Mothers who declared to be in good, healthy health condition, having had normal and full-term pregnancy without infant or maternal problems were enrolled in the study. The mammary areola and breast skin were carefully cleaned with soap and rinsed several times with sterile water. First 500 µl of breast milk were discarded followed by the release of 500-700 µl, collected in sterile containers. Specimen were immediately cooled to 5°C and stored at -20°C.

Media Used

The culture media that were used for this work include; Nutrient agar and MRS Agar.

Sterilization

All glass wares used were sterilized after washing with detergent using hot air oven. Nutrient agar and MRS Agar, were sterilized by autoclaving at 121^0c for 15psi. Wire loops were sterilized by flaming to red hot using Bunsen burner and all laboratory benches were cleaned before and after work with 75% alcohol. Bunsen burner was lit during the work, to keep the working environment sterile.

Isolation of Lactic Acid Bacteria

Specimen were serially diluted up to 10^{-6} dilutions using sterile peptone water. 1ml aliquots of dilutions were placed into Man, Rogosa and Sharpe agar (MRSA) (pH 6.2 and pH5.5), agar (pH 6.5). The plates were incubated anaerobically for 72hrs. The media were specialized to isolate and enumerate the lactobacilli species. One to three bacterial colonies were randomly selected and inoculated with streak plate technique on duplicate MRSA agar. They were sub-cultured in MRS broth and again streaked onto MRSA to get pure colonies.

Morphological Identification

Isolates were tested for Gram-staining reaction, catalase activity and cell morphology. All Gram positive and catalase negative rods were tested for growth in MRS broth at 15^0C and production of gas from glucose. The tests were done according to the instructions of the manufacturer and the results were read after incubation of strains at 37°C for 3 days.

Gram Staining

Cells from fresh cultures were used for Gram staining. The Gram reaction of the isolates was determined by light microscopy. *Lactobacilli* are Gram positive. It means that they give purple-blue colour by Gram staining.

Catalase Test

This is a test used to differentiate catalase producing bacteria from non-catalase producing ones. The catalase produced acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. A drop of 3% hydrogen peroxide was placed on each end of a microscope slide into the use of a sterile wire loop, colonies of the test organisms are transferred on one end of the microscope slide, and the other end was not inoculated and served as a control. The presence of gas bubbles indicates a positive catalase test, while absence of bubbles indicates a negative catalase test.

Identification of Lactic Acid Bacteria

It was carried out using the API50CHI kit which contains different sugar tests. The isolates were diluted in 9ml of normal saline and mixed with the already prepared bromchresol purple which came with the kit. Later the mixture was added, mineral oil was then placed to create an anaerobic condition, incubation took place within 24 to 48 hours; the result was generated from the API data base.

Antimicrobial Activity

Five Indicator pathogenic microorganisms (*Escherichia coli, Staphylococcus aureus*, *Salmonella* sp., *Shigella* sp. and *Klebsiella* sp.) were used to determine the potency of the probiotic lactobacillus species isolated. The indicator pathogens were streaked on the MRS agar plate and a disc diffusion method were used to test the lactobacillus probiotics against the pathogens. Plates were incubated to grow cultures for 24hrs at 37°C under anaerobic conditions. Inhibition zone diameters surrounding the spotted isolates were measured. Isolates, which gave an inhibition zone bigger than 1 mm, were determined to have antimicrobial activity. All assays were performed in duplicate and the results presented were the means of duplicate trials.

RESULTS

Bacteria isolated from human breast milk were identified as *Lactobacillus* spp. by observing their colony morphology, physiological as well as biochemical characteristics using the API kit The isolates which grew on MRS agar media which had small circular white-creamy colour, convex and nontransparent colonies. Microscopically they were Gram-positive, rod shaped, non-motile, catalase negative, and lacked endospores. In the study the *Lactobacillus* specieS identified were *Lactobacillus plantarum* and *Lactobacillus pentosus*. The biochemical characterization using the API50CHL kit is shown in Table 1.

Table 1: API Carbohydrate Utilization Profile.

Isolates	Glycerol	Erythritol	D-aruhinose	L-arabinose	Ribose	D-xylose	Adonital	B-metehy-xylos	Galaetose	D-glucose	D-fructose	D-mannose	L-sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	X methyl D Manocide mmanimmma	X- methyl D glucose	N acetyl glucosamine	Amygdaline	Arbutin	Esculin	Salicin	Cellobioae	Maltose	Lactose	Melibiose	Sacchrose	Trehalose	Imuline	Melezitose	D-raffinose	Amidone	Glycogene	Xylitol	B-gentibiose	D-turanose	D-lyxose	D-tagatose	D-fucose	D-arabitol	D-arabiyol	Gluconate	2 Cetogluconate	5 Cetogluconate	Homo & Hetro	Growth at 15^{O}C	Growth at 45^{O}C	Growth at 4%Hacl	Probable Identity
1	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	He	-	+	+	Lb
2	+	-	-	-	+	+	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+	He	-	+	+	Lb

Isolates: 1=*Lactobacillus plantarum*
2=*Lactobacillus pentosus*

He = hetero fermenters =*Lactobacillus plantarum* and *Lactobacillus pentosus*
All the 3 strain was able to ferment ribose, glucose, fructose, N-acetyl glucosamine, Arbutin, esculin, salicine.
The strain identified as *Lactobacillus pentosus* and *Lactobacillus plantarum* were able to ferment 28 sugars

The total Lactic Acid Bacteria (LAB) count of the 6 breast milk specimen were determined. Result showed that specimen D had the highest LAB count of 1.6 x 10^{8} cfu/ml while specimen A had the least LAB count of 1.3 x 10^{6} cfu/ml. The result is shown in Table 2 below

Table 2: Total LAB Count.

Specimen	Count	Dilution	cfu/ml
A	130	10^{-4}	1.3×10^{6}
B	150	10^{-5}	1.5×10^{7}
C	190	10^{-4}	1.9×10^{6}
D	160	10^{-6}	1.6×10^{8}
E	35	10^{-5}	3.5×10^{6}
F	190	10^{-4}	1.9×10^{6}

After the antimicrobial activity on gastrointestinal organisms which include *Salmonella* sp., *Escherichia coli*, *Shigella sp*. and *Klebsiella* sp. It was observed that all the intestinal bacteria were susceptible to the Lactobacillus sps (*Lactobacillus plantarum* and *Lactobacillus pentosus*) isolated from the breast milk. The result showed that *Klebsiella* sp. had the highest zone of inhibition of 16mm and 14mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) respectively *Shigella* sp. had 14mm and 10mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) respectively and *Salmonella* sp. had 12mm and 12mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) while *Escherichia coli* had the least zone of inhibition of 11mm and 6mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) in diameter. The zone of inhibition measured in diameter in millimeter (mm) is shown in Table 3.

Table 3: Antibiogram Of Lab Isolates Zone of Inhibition (mm).

Indicator strains	Lactobacillus plantarum	Lactobacillus pentosus
Salmonella sp.	12	12
Escherichia coli	11	6
Shigella sp.	14	10
Klebsiella sp.	16	14

DISCUSSION

On the basis of colonial, morphological and biochemical characteristics (gram positive, catalase negative, endospore absence, non-motile, sugar fermentation pattern, bile tolerance activity and antimicrobial activity) using the API 50CHL, the isolates were identified as *Lactobacillus plantarum* and *Lactobacillus pentosus*. The colonies of Lactobacillus isolate characterized to be *Lactobacillus plantarum* and *Lactobacillus pentosus* which appeared rough, dull white, 0.1-0.5 mm in diameter, and demonstrated medium to short rods. Earlier studies have found similar aforementioned characteristics in isolated lactobacilli.[10,11,12,13,14]

The total Lactic Acid Bacteria (LAB) count of the 6 breast milk specimen were determined. Result showed that specimen D had the highest LAB count of 1.6×10^{8} cfu/ml while specimen A had the least LAB count of 1.3×10^{6} cfu/ml.

The capacity of substances to inhibit microbial growth is referred to as antimicrobial activity. The result showed that *Klebsiella* sp. had the highest zone of inhibition of 16mm and 14mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) respectively *Shigella* sp. had 14mm and 10mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) respectively and *Salmonella* sp. had 12mm and 12mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) while *Escherichia coli* had the least zone of inhibition of 11mm and 6mm (*Lactobacillus plantarum* and *Lactobacillus pentosus*) in diameter. Inhibitory activity was demonstrated by some of all the test strains against *E. coli* 0157:H7, except for *S. thermophilus*. These findings are in agreement with that reported by Ibhanesebhor and Otobo[15] for inhibitory activities of human colostrum against *S. aureus* and coliform organisms. This may be due mainly to the high immunoglobulins (Igs) content of colostrum. Also, Heikila and Saris.[16] found that the breast milk exerted bactericidal activity against *E. coli, P. aeruginosa* and *S. aureus*. *Salmonella* sp. inhibition were evident with some the lactic cultures.

As the isolated lactic acid bacteria inhibited these pathogenic strains successfully, it may be expected that addition of these human milk probiotics to commercial food products for infants could confer effective protection against infections caused by these pathogens.

CONCLUSION

Lactic acid bacterial (*Lactobacillus plantarum*) was isolated from human milk in pure culture and various properties of isolated bacteria were determined using the API50CHL identification kit. The Lab count was determined and the isolate showed antimicrobial activity against all the indicator microorganisms.

Conflict of Interest: None declared.

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