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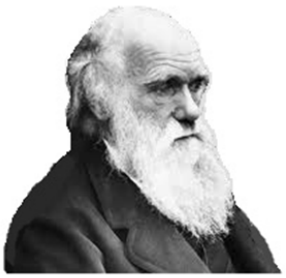

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Antibacterial properties of probiotics bacterial isolated from human breast milk

Ekeoma Nmesoma Blessing[1], Ihenetu Stanley Chukwuemeka[2,*], Ukeachu Chigozie David[3], Ubah Gerald Onuawuchi[4]

[1]Department of Medicine, Gregory University Uturu, Abia State, Nigeria

[2]Department of Chemistry, Imo State University Owerri, Nigeria

[3]Department of Microbiology, Imo State University, Nigeria

[4]Department of Microbiology, University of Port Harcourt, Nigeria

*E-mail address: Iihenetustanley@yahoo.com

ABSTRACT

This research work assessed the antibacterial properties of probiotic bacterial which was isolated from the breast milk. The breast milk has a distinct amalgamation of minerals, proteins, lipids, carbohydrates and various vitamins that endorse the proper development, growth and immunity of the children. That's the reason behind it's consideration to be a comprehensive and inclusive food for new born babies. Furthermore, it is also abundant in various bioactive compounds which encourage the maturation of the immune system over and above developed body's defense against infections. This research used a standard methodology to isolate the bacteria. In the midst of these bioactive agents, probiotic bacteria were properly isolated from human milk in this research work by means of selective MRS media. Five *Lactobacillus* spp. were isolated from every one of the three breast milk samples and two *Enterococcus species* were observed as the potential probiotics, and identified using morphological and biochemical tests which include *L. rhamnosus, L. plantarum, L. casei, L. fermentum, L. acidophilus, En. faecalis* and *En. faecium.* The isolated bacteria were facultative anaerobic, catalase negative, gram positive and non-endospore forming. Sugar fermentation arrangements of equally isolated bacteria were also significantly different. The adding up of breast milk probiotics to the children formulae possibly will be an innovative substitute to mimic some of the purposeful consequences of human milk in children who are not breastfed.

***Keywords*:** probiotic, breast milk, micro-organism, human breast milk, *L. rhamnosus*, *L. plantarum, L. casei, L. fermentum, L. acidophilus, Enterococcus faecalis, Enterococcus faecium*

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1. INTRODUCTION

Probiotics are microorganisms that are believed to provide health benefits when consumed (Rijkers *et al*., 2011). The term probiotic is currently used to name ingested microorganisms associated with beneficial effects to humans and animals (Magdalena *et al*., 2006).

The term came into more common use after 1980. The introduction of the concept is generally attributed to recipient Élie Metchnikoff, who in 1907 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" (Magdalena *et al*., 2006).

A meaningful development of the potential market for probiotics has resulted to higher prerequisites for scientific authentication of putative beneficial effects conferred by the microorganisms (Rijkers *et al.,* 2011; Allen *et al.*, 2010; Gueimonde *et al*., 2007). Findings on the medical reimbursements of probiotics have yet to expose a cause-effect relationship, and their medical usefulness has yet to be conclusively confirmed for utmost of the studies conducted consequently.

The probiotic contender must be a taxonomically distinct microbe or combination of microbes (genus, strain level and species). It is normally admitted that most outcomes of probiotic are strain-specific and cannot be stretched to other probiotics of the same species or genus.

This requests for a precise identification of the strain, i.e. phenotypic and genotypic characterization of the verified microorganism (Reid *et al*., 2010; Zacarias *et al*., 2011). Probiotics must be safe for their proposed use.

Once it comes to the microbiological fact of breast milk, human milk is certainly an important factor in the beginning and development and of progress composition of the neonatal gut microflora given that it comprises basis of microorganisms to the infant gut for numerous weeks after birth.

The constitution of the gut microflora is comprehensively prejudiced by the diet of the infant. As a result, the manifestation of a limited predominant Gram-positive species in breast milk might be a cause explaining why microbiota of breast-fed infants is constituted of a slender spectrum of species, and a further dissimilar microbiota develops after deterring (Reid *et al*., 2010; Daniel *et al*., 2014).

The findings on the microbiology of human milk are constrained to the identification of possible pathogenic bacteria in medical cases of mastitis or infant infections. However, it is clear that the impediment of infant from contagious diseases owing to the biological flora of human milk.

Even though, there is incomplete knowledge in the region of the commensal or probiotic bacteria that breast milk comprehend, bacteria frequently isolated from this biological liquid comprise *staphylococci, lactobacilli, streptococci, enterococci* and *micrococci.*

Bacteria from these genera can be effortlessly isolated from the fresh milk of different healthy women.

So, these groups of bacteria should be considered the natural microbiota of human milk rather than mere contaminant bacteria (Ouwehand *et al.,* 2011; Ayad *et al*., 2011). The aim of the study is to isolate the probiotic bacteria that can be found in the breast milk.

2. EXPERIMENTAL

2. 1. Study area

Imo State of Nigeria is one of the thirty six states of the federal republic of Nigeria. It is specifically in South Eastern Nigeria. It lies between geographic co-ordinates of latitude 4º45 and 7º15' N and longitude of 6º50' E with an area of around 5,100 square km (Imo State Government, 2010). The state has a common boundary with Abia state on the East, Anambra state on the North, and Rivers state on the South (Anosike *et al.*, 2001).

2. 2. Study population

The study was carried out from May to June 2019. All the samples for this project work were collected from healthy mothers within the age of 22 to 35 years old.

2. 3. Sample Collection

Human breast milk used in this study was obtained from sixty healthy mothers within four months of given birth to healthy babies in federal medical center Owerri. The nipple and mammary areola of the breast was wiped with 70% ethanol, and about 5 mL of milk was collected in a sterile test tube using a sterile breast pump. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory.

2. 4. Serial Dilution / Culture

The serial Dilution method was used (pour plate / spread plate technology).

Procedure:

- Test tubes comprising 9 mL peptone water each were marked A-F
- By means of the sterile peptone water and distinct sterile pipettes or string, serial dilution of the samples everyone were prepared as follows:

A) 1 mL of the sample were preside over into 9ml sterile Peptone Water and homogenized $=10^1$ dilution.
B) 1 mL of A into 9 mL sterile Peptone Water of B and homogenized = 10^2 dilution
C) 1 mLof the marked B into 9 mL sterile Peptone Water of C and homogenized = 10^3 dilution
D) 1 mL of marked C into 9 mL sterile Peptone Water of D and homogenized = 10^5 dilution
E) 1 mL of marked D into 9 mL sterile Peptone Water of E and homogenized = 10^6 dilution
F) 1 mL of marked E into 9 mL sterile Peptone Water of F and homogenized = 10^6 dilution (Fawole and Oso, 1995).

After the serial dilution, 0.5 mL of the serially diluted samples each were inoculated into freshly prepared de Man, Rogosa and Sharpe (MRS) agar medium and incubated for 72 h at 37 °C in anaerobic jars containing gaspack (AnaeroGen, Oxoid, UK) (oxygen level <1%, CO_2 level between 9 and 13%). After the incubation period, colonies were randomly picked from the plates and subcultured three times on fresh MRS agar plates (Martin *et al.*, 2005; Langer, 2009).

2. 5. Identification of bacteria

After the incubation, different cultures will be observed on the Petri plates. Counting will be done for each plate and different microbes will be identified on the basis of their colour and growth pattern (Ouwehand *et al*., 2002; Ljungh and Wadstrom, (2009). Biochemial identification was carried out (biochemicl test).

3. RESULT

The results below are obtained from the microbiological analysis carried out from twelve samples obtained from healthy mothers within four months of given birth.

Table 1 below shows the bacteria count (cfu/mL). The result shows that the highest bacteria count is seen from the age range of 33-35 years (1.9×10^3).

Table 1. Total bacteria count

Ages of mothers	Total bacteria count
22 – 25	3.3×10^2
26 – 28	6.8×10^2
29 – 32	6.9×10^2
33 - 35	3.9×10^3

Table 2. Percentage Prevalence of Microorganisms

Age	Bacteria isolates				
22-25	*En. faecium*	*L. platarum*	*L. rhamnosus*	*En. faecalis*	
26-28	*En. faecium*	*L. casie*	*L. rhamnosus*		
29-32	*L. acidophilus*	*L. platarum*	*L. rhamnosus*	*En. faecalis*	
33-35	*En. faecium*	*L. acidophilus*	*L. casie*	*L. platarum*	*En. faecalis*

Bacteria isolated from human breast milk were identified as *Lactobacillus* spp. and enterococcus species by observing their colony morphology, physiological as well as biochemical characteristics (**Table 2**).

Table 3. Identification of probiotic bacteria

Bacteria strain	Gram	Cat	Sugar Fermentation						Growth at	
			Glu	Suc	Sor	Mel	Rha	Cell	15 ºC	45 ºC
L. acidophilus	+	-	-	+	+	+	-	+	-	+
L. fermentum	+	-	+	+	ND	+	-	-	-	+
L. platarum	+	-	-	+	+	+	-	+	+	-
L. casie	+	-	-	+	+	-	-	+	+	-
L. rhamnosus	+	-	+	+	+	-	+	+	+	+
En. faecium	+	-	+	+	+	-	+	+	-	+
En. faecalis	+	-	+	+	+	+	-	+	-	+

Key: gram = gram staining, cat = catalase test, glu = glucose, suc = sucrose, sor = sorbitol, mel = melibiose, rha = rhanmose, ND: no data available.

Table 4. Minimum inhibitory concentrations (MIC) for antibiotic susceptibility of probiotic bacteria.

Strain	Antibiotic ([MIC (µg/mL)]						
	Amp	Gen	Kan	Step	Ery	Tet	Chl
L. acidophilus	<0.06	<0.12	<0.12	<0.5	<0.06	<0.04	<0.05
L. fermentum	<0.06	<4	<8	<8	<0.5	<1	<1
L. platarum	<0.15	<8	<6	<4	<0.15	<0.25	<1
L. casie	<0.25	<8	<64	<32	<1	<1	<4
L. rhamnosus	<0.5	<4	<8	<6	<0.25	<2	<1
En. faecium	<0.8	<8	<25	<10	<0.4	<2	<8
En. faecalis	<1	<9	<16	<12	<0.25	<2	<7

Key: Amp = Ampicillin, Gen = Gentamicin, Kan = Kanamycin, Stre = Streptomycin, Ery = Erythromycin, Tet = Tetracycline, Chl = Chloramphenicol. <0.01 = Resistance, 8-15 = Moderate, <16 = Sensitive

4. DISCUSSION

Form the result obtained in Table 1, shows the bacteria count (cfu/mL). The result showed that the highest bacteria count is seen from the age range of 33-35 years (3.9×10^3). On the foundation of biochemical physiognomies (gram positive, catalase negative, endospore absence, non-motile, sugar fermentation pattern, antimicrobial activity), the isolates were classified as *Lactobacillus* spp. and *Enterococcus* species. The colonies of *Lactobacillus* isolates from all the samples are posited to be Lactobacillus acidophilus and appeared rough, dull white, 0.1-0.5 mm in diameter, and demonstrated medium to short rods as earlier studies by Jara *et al*., 2011 has found similar aforementioned characteristics in isolated *Lactobacilli*.

As of the results obtained in **Table 3** above, has revealed that organic acid production increased with the incubation time while pH of the media decreased with the increasing acid production. The capacity of substances to inhibit microbial growth is referred to as antimicrobial activity, from the result in **Table 4**, the antimicrobial agents used include streptomycin, ampicillin, erythromycin, gentamicin, kanamycin, tetracycline and chloramphenicol. *L. acidophilus* had minimum inhibition in all the antimicrobial agents between 0.04 to 0.6 µg/mL, *L. fermentum* had 0.05 to 8 µg/mL, *L. platarum* had 0.15 to 8 µg/mL, *L. casie* had 0.2 to 4 µg/mL, but 32 µg/mL in streptomycin and 64 µg/mL in kanamycin, *L. rhamnosus* had 0.25 to 8 µg/mL. *Enterococcus* species are recorded to be moderate except for *En. faecium* which is sensitive to kanamycin and *En. faecalis* which is sensitive to kanamycin. 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 would confer effective protection against infections caused by these pathogens.

All the samples have these probiotic bacteria, except for sample one that did not record *L. fermentum* and *En. faecalis*. Human breast milk composes an interesting basis to obtain new precise probiotic stains for neonates pointing at assisting an appropriate development of the gut microbiota and the immune development in infants who, for different reasons, can't be breast-fed.

The breast milk collected in this study contained strains of *L. rhamnosus*, *L. plantarum*, *L. casei*, *L. fermentum*, *L. acidophilus*, *En. faecalis* and *En. faecium*. Consequently, breast milk is considering a substantial foundation of lactic acid bacteria that appear to be of endogenous origin. Since breast milk has been suggested as a vehicle for potentially probiotic LAB, it could be considered as a natural synbiotic food that is a mixture of probiotics and prebiotics. It has correspondingly been ascertained that the breast feeding beneficially can affect infants by improving the survival and implantation of live dietary microorganisms in gastrointestinal tract.

5. CONCLUSIONS

In conclusion, the breast milk possibly will be a good and safe source for isolation of probiotic bacteria and for improve intestinal microflora of children. This report has established their use in the development of new pharmaceutical preparations and functional foods that contain milk probiotics for the betterment of health of the public. Lactic acid bacteria were isolated from human milk in pure culture and various properties of isolated bacteria were determined. All of isolates showed a tolerance to bile salt, organic acid production and antimicrobial activity against some indicator microorganisms. Phenotypic identification

effectively differentiated the isolates, especially sugar fermentation patterns. Two different isolate strains were identified and these could be used as the potential probiotic strains.

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