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Full Length Research Article

DISTRIBUTION OF BIFIDOBACTERIA IN SALIVA OF INFANTS ATTENDING POSTNATAL CLINIC IN THREE PUBLIC HEALTH FACILITIES IN KADUNA METROPOLIS, KADUNA STATE, NIGERIA

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ABSTRACT
This research is aimed at evaluating the distribution of *Bifidobacterium* species in the saliva of infants from three selected public health facilities providing postnatal services in Kaduna Metropolis, Kaduna State. Samples were randomly collected by oral swabs method from 96 male and female infants taken for postnatal clinic in the three hospitals. Sample population is infants within the age range of 0-2yrs attending postnatal clinic. Demographic information on each infant was obtained through questionnaire that was completed by the mothers. Initial identification of Bifidobacteria was carried out by subjecting sampled saliva to biochemical test of Catalase, indole and fructose-6-phosphate phosphoketolase. Based on the biochemical characteristics, the isolated bacteria were reduced to 12 isolates. Using genus specific primers for *Bifidobacterium,* molecular characterization was carried out on the 12 isolates obtained by biochemical test for *Biofidobacterium.* Out of the 12 isolates subjected to molecular analysis, five were confirmed to be *Bifidobacterium* while sequence analysis of two of the samples carried out identified the species as *Bifidobacterium longum*. All the five isolates of *Bifidobacterium longum* were from saliva samples collected from male infants aged between 1 – 6 months. In conclusion, *Bifidobacterium longum* appears to be present in male infants within the age group 1 – 6 months than females within the same age group.

Keywords: Bifidobacteria, Isolates, Infant, Saliva.

INTRODUCTION
Human gastrointestinal microbiota, otherwise known as the gut microbiota or gut flora is a collection of microorganisms living in the human intestine. The gut of many other animals also serves as habitat to numerous microorganisms (Quigley *et al.,* 2013). Typically, an adult harbor about ten times more microbial cells than human cells (Gronlund *et al*., 2007). The gut microbiota becomes more established in infants, within the first or second year after birth, during which the epithelium and abdominal mucosa becomes tolerant and supportive to the gut flora in addition to providing barrier to pathogenic organisms (Sommer and Backhed *et al.,* 2013; Faderl *et al.,* 2015). The individual microbiota load is highly variable with varying environmental conditions such as antigen exposure, infections or medications, genetic, age or hygienic factors which strongly affect the bacterial community. Also, the abundance and makeup of the gut microbiota depends on the diet of the host, as members of the microbiota have mechanisms by which they utilize available nutrients (Ju-hoon O'Sullivan *et al.,* 2010 and Turroni *et al*., 2012).
Due to their abundance in the intestine, 60% of the dry mass of feces is made up of microorganisms (bacteria, fungi, protists, archea and viruses) available in the gut flora, but little is known about their activities (Lozupone *et al.,* 2012). The intestinal microbiota composition is comparatively simple during breast feeding and becomes complicated after weaning. According to (Hopkins *et al,* 2001; Satokari *et al*.,2003) the composition becomes stable on old age and every individual is provided with a unique gut microbiota that plays specific functions in host nutrients metabolism, maintenance of structural integrity of mucosal barrier, immune modulation and protection against pathogens (Leahy *et al.,* 2005).
The microflora of the human gastrointestinal tract is well significant to the proper functioning of the digestive system which is an important organ in the human body. The functions of gut bacteria include: repair and maintenance of mucosal homeostasis, regulation of intestine epithelial development, improved absorption of nutrients, enhancement of innate immune system (Eckburg *et al*., 2005; Sears *et al* 2005; Tsai and Walter, 2009).

Bifidobacteria are a group of important and beneficial bacteria that normally live in the intestine of humans. They are one of the earliest and most abundant bacteria that reside in the neonatal gut with myriad of benefits to the host intestinal immunity (Turroni *et al.,* 2012; Ventura *et al.,* 2014; Matsuki *et al*., 2016; Bokulich *et al*., 2016). Bifidobacteria were first isolated by (Tissier in (1900) from the feces of healthy breastfed infants and since has been isolated from different ecological niches such as Gastrointestinal Tract (GIT) and Oral cavity of various mammals. (Klijn *et al*., 2005; Ventura *et al*., 2014; Laureys *et al.,* 2016).

About 80% of bacteria in the intestine are *Bifidobacterium* sp. The genus *Bifidobacterium* in line with Taxonomic outline of the prokaryotes, belongs to the family *bifidobacteriaceae,* order *Bifidobacteriales,* class *Actinobacteria* and phylum *Actinobacteridae.* It comprises a gut commensal clade of Gram positive, polymorphic rod shaped, high G+C bacteria that constitute about 10% of the typical human adult intestinal microbiota (Ventura *et al*., 2007; Tojo *et al*., 2014).
Generally, bifidobacteria are more predominant in the intestine within the first week after birth, and slightly decrease after weaning and keep on decreasing with age (Yatsunenko *et al.,* 2012). It has therefore, become apparent that bifidobacteria species are passed

on from the mother to the child and it colonise the intestine of the infant at the very early stages of life (Makino *et al.,* 2019).
Members of the genus *Bifidobacterium* have significant importance with purported health promoting effects in humans across their life span (O'callaghan and Van Sinderen *et al*., 2016).
They play a major protective role to the host against pathogenic bacteria (Lewis *et al*., 2017). They are useful in priming the mucosal immunity and consequently providing adaptively and resistivity to diverse diseases later in life (Turroni *et al*., 2008). Also, Bifidobacteria play major role in most complex and diverse ecosystem of the intestinal tract of warm-blooded animals and honeybees (Marteau *et al*., 2001, Sheil *et al*., 2006; Tanabe *et al*., 2008; Preising *et al*., 2010; Chichlowski *et al*., 2011). It has however been established that bifidobacteria have several beneficial effect on human health including alleviation of constipation vitamins, prevention of diarrhea and intestinal infections, elimination of procarcinogens, production of antimicrobials against pathogenic bacteria by protecting the mucosal epithelium against invasion through immunomodulation and reduction of inflammation.

Some species of bifidobacteria such as *Bifidobacterium longum subs. longum, Bifidobacterium adolescentis, bifidobacterium bifidum, Bifidobacterium catenalum,* and *bifidobacterium breve* are reported strictly to inhabit human gut (Lamendella *et al*., 2008), others such as *B.gallinalum, B.angulatum* and *B.cuniculi*, appear to be exclusively associated with animals other than humans (Lamendella *et al.,* 2008).
Despite their documented significance in health promotion, the precise succession ontogeny of bifidobacterial species at infancy and early childhood is yet to be comprehended (Elahi *et al*., 2013). More so, there is no documented report on the bifidobacterial composition of salivary fluid of infants in health centers in Kaduna, Kaduna State.
This research work therefore, is aimed at evaluating the distribution of *Bifidobacterium* sp. in the saliva of infants (0-2years) obtained from three selected public health facilities providing postnatal services within Kaduna Metropolis, Kaduna State. This research is restricted to infants between the ages of 0-2years.

MATERIALS AND METHODS

Description of Study Area
The study was carried out within Kaduna Metropolis, Kaduna State. The State has a land mass of 48473.25km^2 and is located West Geographical Zone of Nigeria. It lies between longitude 6^o and 9^{o}E and latitude 9^o and 11^{o}N. The state has distinct Wet Season (April – October) and Dry Season November –March). Vegetation type is the Guinea Savannah.

Selection of Public Health Facilities
Random sampling technique was used in selecting three public health facilities providing postnatal service within Kaduna Metropolis. The three Public Health Centers were Yusuf Dantsoho Memorial Hospital, Nigerian Defence Academy Medical Center and Barau Dikko Public Health Care Center.

Sample Population
The study consisted of Male and Female infants 0-2years of age taken for postnatal clinic in the three selected public health facilities within Kaduna Metropolis.

Ethical Approval
Ethical permission was obtained from the ethical committee of Kaduna State Ministry of Health and the consent of the infants selected for the study was sought from the Mothers of the infants.

Administration of Questionnaires
Questionnaires were administered to the consenting individuals for the purpose of obtaining their demographic information such as Age, Sex, Health status, Antibiotic usage and Breastfeeding methods.

Sample Preparation and Isolation
The bacterial isolates were recovered from saliva of 0-2years old healthy breastfed infants.

Media Preparation
Garches Broth: Multiplication of bacterial isolates from saliva was carried out on (Garches broth Teraguchi *et al.,* 1982). Cultivation maintenance of bifidobacteria species were done on *Bifidobacterium* Agar (Bokulich *et al*., 2016). The liquid was distributed in flasks, Sterilized by autoclaving at 15lbs pressure (121^{o}C) for 15minutes Mixed well and about 0.2mL of the multiplied Garches broth was poured in a plate and 20mL of the prepared Bifidobacteria Agar was added and allowed to set. The preparation was inoculated at 37^{o}C anaerobically and allowed for 24h.

Identification of Isolates
The isolates obtained were identified using morphological characterization, biochemical identification and molecular characterization using PCR which was further confirmed with sequence analysis and compared with sequences in National Center for Biotechnology Information (NCBI) date base (Scardovi, 1986).

RESULTS
Table 1 shows the distribution of bacterial isolates obtained from saliva of infants 0-2years in selected Public Health Facilities in Kaduna Metropolis is presented in table1. From the 96 samples collected and cultured, bacteria were isolated from 58(60%) of the samples while no isolates were recorded in 38 (40%) of the samples. When subjected to gram staining, 52 (90%) of the 58 samples with bacteria isolated from all the 3 health facilities reacted positively to Gram Staining, while (8) 10% reacted negatively.

Table 2 shows the distribution of bacterial isolates from saliva in relation to Age and sex of 0-2years old infants in Kaduna Metropolis is shown in table 2. Out of the 58 positive samples for bacteria isolates, the highest number of isolates 36 (62%) were recorded in infants aged < 6months, followed by age groups < 12months and < 18 months, with frequencies of 13(23%), 7(12%), and 2(3%) respectively. However, salivary samples from the male infants had more isolates than females in age groups ≤ 1month and < 6 months while age groups < 12 months had fewer isolates 4 (31%)compared to female 9 (69%) of the same age group. The Males and females in the age group < 18 months had equal number of isolates 1(50%).

Table 3 shows the biochemical reaction of bacteria to indole, catalase and fructose -6- phosphate phosphoketolase test is as provided in table 3. Out of the 58 positive bacterial isolates only 12 samples were suspected to be positive for bifidobacteria in the 3 selected public health centers in Kaduna Metropolis.

Plate 1 Shows the PCR results of bifido isolates in saliva of infants in the selected public health centres with the band size of approximately 549 -563bp G-Bifid gene specific for identifying bifidobacteria. The bands shown on the plate are for the amplified region from isolates SBI3, SBI4, SBI8, SBI9 and SBI10 which tentatively identifies the isolates as bifidobacteria.

Table 4 Shows bifidobacterial sp. isolated from saliva of infants in relation to Age and Sex in selected public health facilities in Kaduna Metropolis, Kaduna State.

It was observed that all the infants which bifidobacteria were isolated from falls within the age group of < 6 months, and all were male.

Table 5 shows the sequencing results of some PCR positive samples in saliva of infants 0-2yrs from selected Public Health Centres in Kaduna Metropolis is as shown in Table 5. When this is compared with sequences in National Centre for Biotechnology Information (NCBI) data base and the identity confirmed with Basic Local Alignment Search Tool (BLAST). The sequence aligned were 99% similarity with *Bifidobacterium longum.*

Table 1. Distribution of Bacteria Isolates obtained from Saliva of Infant Aged 0-2yrs in Selected Hospitals in Kaduna Metropolis, Kaduna State

	Bacterial isolates (%)			Gram reaction of bacterial isolates (%)		
Hospital	Present	Absent	Total	+ve	-ve	Total
YDMH	20 (48)	22 (52)	42(44)	17(85)	3(15)	20(34)
NDAMC	22 (69)	10 (31)	32(33)	19(86)	3(14)	22(38)
BPHCC	16 (73)	6 (27)	22(23)	16(100)	0 (0)	16(28)
Total	58 (60)	38 (40)	96	52(90)	6(10)	58

Keys: YDMH: Yusuf Dantsoho Memorial Hospital, Kaduna NDAMC: Nigerian Defence Academy, Medical Centre BPHCC: Badarawa Public Health Care Centre, Kaduna

Table 2. Distribution of Bacterial Isolates obtained from Saliva of Infants Aged 0-2yrs in relation to Age and Sex in selected Hospitals in Kaduna Metropolis, Kaduna

Age (Months)	No of Infants Examined (%)		Sex
	Male		Female
≤ 1 Month	7 (12)	5 (71)	2 (29)
≤ 6 months	36 (62)	19 (53)	17 (47)
≤ 12 months	13 (23)	4 (31)	9 (69)
≤ 18 months	2 (3)	1 (50)	1 (50)
Total	58	29 (50)	29 (50)

Table3. Biochemical Screening of Bacteria Isolated from Saliva of infants 0-2yrs for Bifidobacterium sp

Number of enzymes producers (%)						Samples Suspected to contain *Bifidobacterium (SB)*
	Catalase		Indole			
Hospital	+ve	-ve	+ve	-ve	F6PP	
YDMH n= 20	16 (80)	4 (20)	7 (35)	13(65)	2 (16)	SBI9andSBI11
NDAMC n= 22	12 (55)	10(45)	11(50)	11(50)	5 (42)	SBI4,SBI5,SBI6,SBI7and SBI8
BPHCC n= 16	9 (56)	7 (44)	8 (50)	8 (50)	5 (42)	SBI1,SBI2,SBI3, SBI10andSBI12
Total(58)	37 (64)	21(36)	26(45)	32(55)	12	

Keys: YDMH: Yusuf Dantsoho Memorial Hospital, Kaduna NDAMC: Nigerian Defence Academy, Medical Centre BPHCC: Badarawa Public Health Care Centre, Kaduna

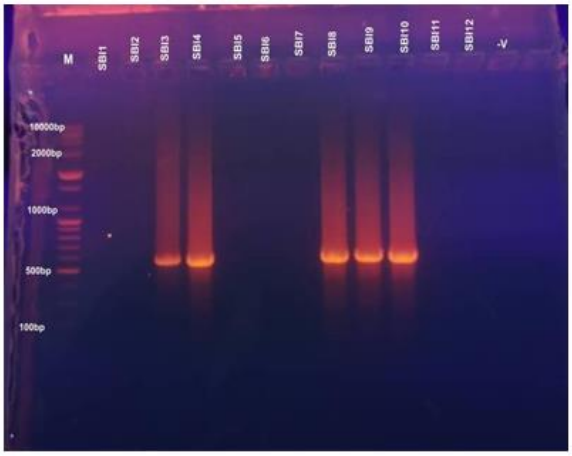

Plate 1: Agarose gel electrophoresis of PCR amplified G-Bifid in 12 bacteria isolates.

KEY: M: Molecular Ladder: 100bp (Bioneer USA)
SBI1-SBI12 – DNA Samples of Bacteria Isolates

Table 4. Distribution of bifidobacterial sp based on Age and Sex

ISOLATE CODE	Age(months)of infants examined	Sex	Bacteria species
SB13	0-6	Male	*Bifidobacteria* sp
SBI4	0-6	Male	*Bifidobacteria* sp
SBI8	0-6	Male	*Bifidobacteria* sp
SBI9	0-6	Male	*Bifidobacteria* sp
SBI10	0-6	Male	*Bifidobacteria* sp

Table 5. Sequencing Results of Some PCR Positive Samples from Saliva of Infants in selected hospitals in Kaduna metropolis, Kaduna

Isolate Code	Closest type strain in NCBI database	Accession Number	E-value	16S rRNA identity (%)	Isolates Identity
SBI9	*Bifidobacteria longum* subsp. Infantis strainFC13644	MK962475.1	0.0	99	*Bifidobacteria longum*
SBI10	*Bifidobacteria longum* subsp. Infantis strainFC13644	MK962475.1	0.0	99	*Bifidobacteria longum*

DISCUSSION

The observed growth of other bacteria other than Bifidobacteria despite the use of a selective media for Bifidobacteria could be due to the lack of propionic acid contained in the modified Bifidobacteria Agar. The propionic acid is an inhibitor of many other bacteria other than Bifidobacteria (Bjorksten *et al.,* 2001; Guarner and Malagelada *et al*., 2013).

The occurrence of more bacteria in saliva of infants of Age < 6months; 35(60%) and the least occurrence in infants <18 months; 2(39%).In agreement with the work of (Hill *et al.,* 2017) which reported that; during the first week after birth, a dominance of Actinobacteria (Mainly comprising the genus *Bifidobacterium* has been observed as the most prevalent microbial population for caesarian section infants.

Moreover, the prevalence of bifidobacteria continuously increased in both Vaginal Delivery (VD) and CS infants' overtime. This observation could be explained by the findings of (Yoshioko *et al.,* 1991) which stated that; bifidobacteria were found in low numbers on day 1, with gradual increase in number of anaerobic bacteria after few days to some weeks. This was due to decomposition of oxygen in the intestine by enteric microorganism which reduces the oxidation-reduction potential and this provides the conditions for the settlement of a more diversified microflora that include anaerobic bacteria.

Although there was an equal distribution (50%) of bacteria Isolates in both Male and Female infants, Bifidobacteria were isolated more from the male infants which corresponds with the findings of (Mueller *et al.,* 2006), that boys had higher total bacteria count than girls at birth and there are larger colonization by bacteriodes in males than in females.

The inability of the 12 isolates suspected to be bifidobacterial to convert hydrogen peroxide to water and oxygen and at the same time not being able to convert typtophan to indole and the possession of fructose 6-phosphate phosposketolase (F6PPK) is considered a reliable indication of a gram positive Rod bacteria belonging to the genus *Bifidobacterium*. Recent phylogenetic studies using 16SrRNA sequencing revealed a close taxonomic relationship between the species *Gardnerella vaginalis* and species of *Bifidobacterium.* Therefore, F6PPK production can be considered a pointer for both Bifidobacteria and *Gardnerella vaginalis* strains.

The identification of *Bifidobacterium longum* using the molecular identification with PCR and the blast of the nucleotide sequence is in line with the work of (Wasilewska and Bieleck *et al*., 2003), which reported *B.breve* and *B.longum* as most common species found in the gut of breast-fed infants. Although (Matsuki *et al.,* 1998, 1999), consider them as predominant in the infant gut using a new identification method*, B.infantis* strains were not detected. A 16S rRNA gene target species specific PCR technique, affirmed that *B. breve* is the most commonly found taxon followed by *B.longum* and *B. infantis* in the intestinal tract of 27 breastfed infants. (Zavaglia *et al.,* 1998), identified 25 bifidobacterium strains isolated from infant faeces mainly as *B. bifidum, B. longum* and *B. breve*, whereas only single strains *of B.adolescentis*, *B. infantis* and *B.pseudolongum* were detected based on numerical analysis of whole-cell protein electrophoresis.

Conclusion

It can be concluded that *B.longum* is probably the most central bifidobacterial clade in Kaduna Metropolis infant gut. The presence of microbes during the neonatal period, gestational age and the delivery mode, influence the microbial colonization in new borns. Most of the changes in the microbiota of infants could be corrected or reduced by administration of probiotic supplementation to both mother and infant.

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