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Microbiome Assessment for Breast Milk of Lactating and Non- Lactating Women from Thi-Qar Province

Aya Talib Jawad[1], Nidhal Y.Mohammed[1] and Wissal A. Alhilfi[2]
[1] Medical Laboratory Technology Dept.,College of Health & Medical Technology , Southern Technical University, Basrah, Iraq
[2]Development and Continuing Teaching Center/ University of Basrah

Abstract
The study aimed to indicate the presence of milk microbiome in both lactating and nonlactating women, in addition to investigate some of relations between related parameters. A total of 90 women (20-35 years) were selected for providing samples of breast milk after birth for different periods. Culturing, isolation, staining, and genetic identification was applied. After PCR and Sequencing for 60 isolates, the possibility of identifying 54 of them illustrated that; the most frequent isolated bacteria were *Enterococcus* spp with 26.7%, followed by *Staphylococcus* spp 11.1%, *Leuconostoc* spp 10.0%, *Bacillus* spp 3.3%, and 2.2% for both, *Proteous* spp and *Providencia* spp. While the lowest isolated bacteria were *Exiguobacterium aquaticum* with 1.1%. Other non-identified organisms were *Candida* in 10%, mixed growth for bacteria and *Candida* 15.6%, and non-identified bacteria as 15.6%. A significant differences was found between type of lactation at P. value < 0.05, but there was non-significant differences between age groups, mode of delivery (birth way), use of medication and lactation stages.
Keywords: Breastfeeding, Breast milk, Human milk microbiome, PCR,16S rRNA gene sequencing

Introduction

The human milk is fundamental for a correct newborn s' development, as it is a source not only of the vitamins and nutrients, but also of the commensal bacteria. The microbiota associated to the milk contributes to create the initial intestinal microbiota of infants, having also a pivotal role in the modulating and influencing bnof the newborns' immune system (Toscano *et al*., 2017). Its composition is the biologic norm for infant nutrition. It also contains many of distinct bioactive molecules that protect against infection and inflammation, and contribute to immune maturation, organ development, and colonization of healthy microbes. A dynamic, bioactive fluid of human milk changes in composition from colostrum to late lactation, varies within feeds, diurnally, and between mothers (Ballard and Morrow, 2012).

Institute of Medicine (1991) Set the chemical constituents in human milk as several classes, such as proteins and nonproteins nitrogen compounds, carbohydrates, lipids, fat and water-soluble vitamins, trace elements, ..etc.

Additionally; breast milk is a source of commensal bacteria, enhance infant health by preventing adhesion of pathogen and promoting gut colonisation of beneficial microbes, and was

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initially considered a sterile fluid and the microbes isolated were considered contaminants, it is now widely accepted that it is a home to its own unique microbiome (Lyons *et al*., 2020).

The human microbiome; is the full complement of microbial species and their genes and genomes that inhabit the human body (Proctor *et al*., 2013)

There was acknowledgement about the presence of bacteria in human milk since the seventies, and the microbiological analysis of human milk was only performed in case of infections for a long time, therefore the presence of non-pathogenic bacteria was yet unknown (Jeurink, *et al*., 2013).

Collado *et al*., (2009) demonstrated that, breast milk was described as a source of bacteria influencing the development of the microbiota of infant's gut. Fifty breast milk samples were analysed by the qPCR to assess the presence of different genera or clusters of bacteria, including the *Bifidobacterium*, *Lactobacillus*, *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Bacteroides, Clostridium* cluster IV and cluster XIVa–XIVb groups. *Staphylococcus, Streptococcus, Lactobacillus* and *Bifidobacterium* were the predominant and detected in all samples. *Clostridium* XIVa–XIVb and *Enterococcus* were detected in most of samples in contrast to *Bacteroides* and *Clostridium* cluster IV groups.

Milk was collected from 39 women, in the study of (Urbaniak *et al*., 2016), with microbial profiles analyzing by 16S ribosomal RNA (rRNA) sequencing. A diverse community of bacteria was found with the most dominant genera; *Staphylococcus*, *Pseudomonas*, *Streptococcus* and *Lactobacillus*.

Human milk–associated microbes as mentioned by (Fitzstevens *et al*., 2016) are among the first to colonize the infant gut and may help to shape short- and long-term infant health outcomes, suggested that and the genera, *Streptococcus* and *Staphylococcus*, probably be universally predominant in human milk, regardless of differences in geographic location or analytic methods.

Methods

Study Design

A total of 90 women were participated in the study, provided samples of breast milk after birth for different periods. Mothers were selected based on the availability of samples of human milk from health care units in health centers and Bint-Alhuda hospital of Thi-Qar province, with ages between (20-35) years. The mothers were divided into two groups according to the method of child feeding; breast feeding and artificial feeding group**.** The complete information was taken using a questionnaire list that includes; age, type of birth, type of lactation, date of birth and medication use .

This study was carried out in public health laboratory in Thi-Qar province and Al-Fayhaa Hospital in Basra province.

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Sampling

The breast (nipples and mammary areola) were sterilized with iodine cotton swab, milk then collected (2-3ml) in sterile tubes manually, after first drops were discarded according to (Cabrera-Rubio *et al*., 2012). Samples were either delivered to the laboratory within 24 hours for directly work or kept frozen until used.

Microbial culture Examination

a. Culturing and Isolation of Bacteria

The de-Man, Rogosa, Sharpe (M.R.S) and nutrient agar were prepared and sterilized according to the manual of manufacturers instruction, and a volume of 100µl of milk samples were cultured on media by spread-plate technique using L–shape spreader (Harley and Prescott, 2002), the plates incubated at 37ºC for 24-48 hours under anaerobic condition using anaerobic jar and an aerogen.

Different colonies were picked up and sub cultured by streak-plate technique (Harley and Prescott, 2002) several times for purification.

b. Identification of Bacteria

Gram staining and catalase test were done according to the Gram staining kit (Jourilabs, Jordan) company information, and (Forbes *et al*., 1998).

Whereas genetic identification included; the preparing of agarose and electrophoresis process for extraction according to (Sambrook and Russell, 2001), the extraction of genomic DNA samples, due to the manufacturing procedures information steps of ZR fungal /yeast /bacterial DNA MiniPrep kit (ZYMO, USA) as possible, Primers preparations following the (Integrated DNA Technologies company, Canada). The primers were chosen according to (Srivastava *et al*., 2008).

Table (1): The specific primer 16s RNA of gene

Primer	Sequence	Tm(°C)	GC(%)	Product size
Forward	5'- AGAGTTTGATCCTGGCTCAG-3'	54.3	50.0	1250 base pair
Reverse	5'- GGTTACCTTGTTACGACTT- 3’	49.4	42.1	

The PCR steps technique was performed according to (Intron's Maxime PCR PreMix Kit (Korea) .

Table (2): Mixture of the specific interaction for diagnosis gene

Components	**Concentration**
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl

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Distill water	16.5 µl
Final volume	25µl

Changes in temperatures and the concentration of DNA template between (1.5-2µl) and, are considered during the primer annealing with complement**.**

Table (3): The optimum condition of detection

No.	Phase	Phase	Time	No. of cycle
1-	Initial Denaturation	94°C	3 min.	1 cycle
2-	Denaturation-2	94°C	45sec	35 cycle
3-	Annealing	56°C	45sec	
4-	Extension-1	72°C	1min	
5-	Extension -2	72°C	7 min.	1 cycle

Statistical analysis:

All data of the current study were statistically analyzed using Microsoft Windows Excel 2010 with Chi-square for independent sample.

Results and Discussion

Incidence of total isolated microbiome in both breast and artificial feeding

A total of 90 breast and artificial feeding women were subjected for cutlturing and isolation. However, after PCR and Sequencing for 60 isolates, the possibility of identifying 54 of them illustrated that; the most frequent isolated bacteria were *Enterococcus* spp with 26.7%, followed by *Staphylococcus* spp 11.1%, *Leuconostoc* spp 10.0%, *Bacillus* spp 3.3%, and 2.2% for both, *Proteous* spp and *Providencia* spp. While the lowest isolated bacteria were *Exiguobacterium aquaticum* with 1.1%. The figures (1 and 2) revealed the gel electrophoresis of DNA genome extraction and PCR product for selected isolates. Other non-identified organisms were *Candida* in 10%, mixed growth for bacteria and *Candida* 15.6%, and non-identified bacteria as 15.6%.

Figure (1): Gel electrophoresis of genomic DNA extraction from bacteria , 1.5% agarose gel at 5 vol /cm for 1hour

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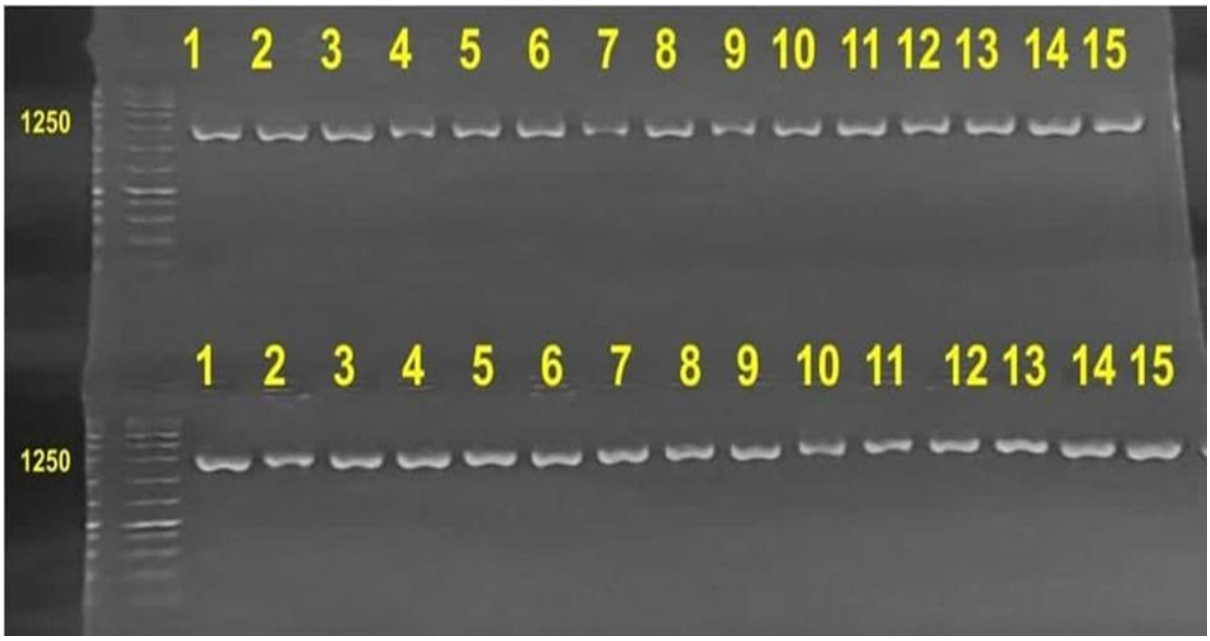

Figure (2): PCR product the band size 1250 bp. The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. N: DNA ladder (1000).

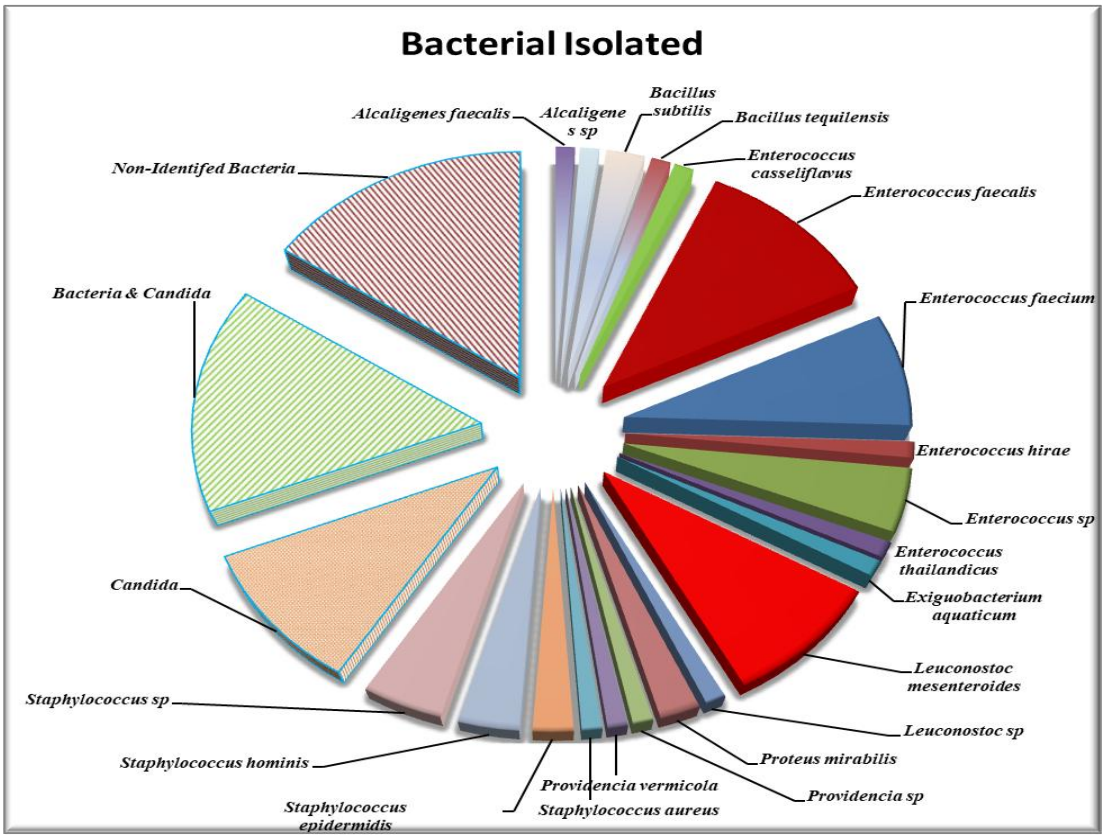

Figure (3): Incidence of total isolated microbiome in both breast and artificial feeding.

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Regarding the total microbiome isolated from milk samples in the present study, a number of different microorganisms obtained, mostly of beneficial effect to the infant health throughout his life with non- suspected contaminated origin, and that probably conducted in somewhat with different previous researches such as (Cabrera-Rubio *et al*., 2012) whom proved that the milk microbiome is compositionally distinct from any other human niche and that it is not a simple contaminant from the skin, although part of the differences could be a result of the different sampling procedures.

Talhi-Mekhici *et al*. (2017) noticed that according to the mother's lifestyle the genus *Enterococcus* was the most frequently isolated from rural mother's milk as well as urban mother's milk. *Enterococcus faecium* was the most frequently isolated species, which may had a similarity with our findings, in which the genus *Enterococcus* (26.7%) was the most frequently isolated bacteria, although; *Enterococcus faecalis* was the predominant than *Enterococcus faecium.*

In concern with *Staphylococcus* which appeared in recent investigation in relatively higher percent, different studies also, considered it as one of the most predominant genera in human breast milk (Murphy *et al*., 2017; Li *et al*., 2017; Urbaniak *et al*., 2016), which may agree with our investigation.

Leuconostoc, on the other side, had a relative abundance in recent study, appeared also in the study of (Cabrera-Rubio *et al*., 2012) and found predominant in colostrum samples with other genera.

However; variable records for above isolates in other studies, (Heikkila and Saris, 2003) collected random isolates from breast milk samples of healthy lactating women and tested for antimicrobial activity against *Staph. aureus*. Commensal staphylococci 64%, oral streptococci 30%, *Staph. epidermidis*, *Strep. salivarius*, and *Strep. mitis* as the most frequent isolates, were the predominant bacterial species in breast milk. *Enterococci* (*Ent. faecalis*), isolated from 7.5% of samples, and lactic acid bacteria (LAB) (*Lactobacillus rhamnosus, Lact. crispatus, Lactococcus lactis, Leuconoctoc mesenteroides*), isolated from 12.5% of samples.

In addition; the presence of *Candida* sp in recent work, met relatively the findings of (Boix-Amorós *et al*., 2017) in which samples of healthy lactating mothers` milk within 1 month after birth were analyzed, 33 strains were isolated and identifed, confrmed the presence of viable fungal species, showed that the most common genera were *Malassezia* (44%), followed by *Candida* (19%) and *Saccharomyces* (12%). Yeast cells were observed by fuorescence microscopy.

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It had been mentioned by (Boix-Amorós *et al*., 2019) that recent studies report the presence of fungal species in the breast milk of healthy mothers, suggesting a potential role in the infant mycobiome development. The study investigated the influencing of geographical location and mode of delivery on the healthy human breast milk mycobiota. Basidiomycota and Ascomycota were found to be the dominant phyla, with *Malassezia* and *Davidiella* were the most prevalent genera across countries.

Incidence of diagnosed bacteria according to method of delivery

The study indicated that the higher bacterial level was in the women of natural birth with percentage 64.2%, whereas in caesarean was 35.8%. in addition; there was a non-significant difference between birth ways at P. value < 0.05. As found in table (4) and figure (4).

Table (4): Incidence of diagnosed bacteria according to method of delivery

Birth way / **Bacteria**	**Caesarean birth**		**Natural Birth**		**Total**	
	No.	%	No.	%	No.	%
Alcaligenes spp	1.0	1.9	1.0	1.9	2.0	3.77
Bacillus spp	1.0	1.9	2.0	3.8	3.0	5.66
Enterococcus spp	8.0	15.1	16.0	30.2	24.0	45.28
Exiguobacterium aquaticum	0.0	0.0	1.0	1.9	1.0	1.89
Leuconostoc spp	2.0	3.8	7.0	13.2	9.0	16.98
Proteus mirabilis	0.0	0.0	2.0	3.8	2.0	3.77
Providencia spp	1.0	1.9	1.0	1.9	2.0	3.77
Staphylococcus spp	6.0	11.3	4.0	7.5	10.0	18.88
Total	19.0	35.8	34.0	64.2	53.0	100
$CalX^2 = 5.632$	**$TalX^2 = 14.07$**		**DF = 7**		**P. Value = 0.616**	

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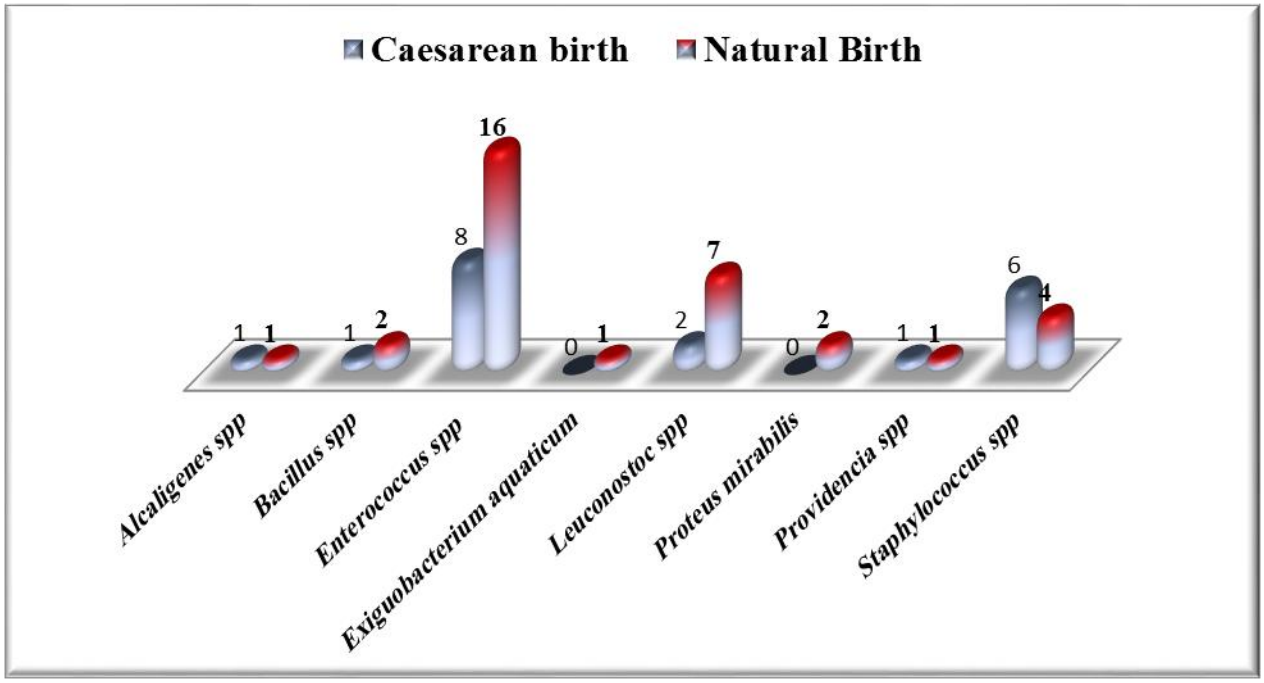

Figure (4): Incidence of diagnosed bacteria according to method of delivery.

The results of the present study indicated that the method of delivery (birth way) has an effect on the microbial content of breast milk, as it was observed with a decrease in the diversity and richness of the beneficial microbial content of the mother`s milk .These results agreed with results of previous study (Hermansson *et al*., 2019) on milk samples of 1 month after delivery, as their results suggested that mode of delivery had an independent impact on the microbial composition of the breast milk and the reduced breast milk microbiota diversity and richness was associated with cesarean section delivery. Another study also, (Cabrera-Rubio *et al*., 2015), found that the high bacterial richness and diversity found in milk samples of vaginal deliveries compared with those of C-section birth.

Whereas; Cabrera-Rubio *et al*., (2012) reported that milk samples of elective but not nonelective mothers who underwent the cesarean delivery contained a different community of bacteria than did samples from individuals of vaginal delivery, suggested that it is not the operation per se, but rather the absence of the physiological stress or hormonal signals, that could influence the microbial transmission process to milk.

Incidence of diagnosed bacteria according to type of feeding

The study showed that the higher bacterial number was in the women with breast feeding with percentage 71.7%, while in artificial feeding was 28.3%, also; there was a significant difference between type of feeding at P. value < 0.05. As in table (5) and figure (5).

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Table (5): Incidence of diagnosed bacteria according to type of feeding

Type of Feeding / Bacteria	Breast Feeding		Artificial Feeding		Total	
	No.	%	No.	%	No.	%
Alcaligenes spp	2.0	3.8	0.0	0.0	2.0	3.77
Bacillus spp	3.0	5.7	0.0	0.0	3.0	5.66
Enterococcus spp	17.0	32.1	7.0	13.2	24.0	45.28
Exiguobacterium aquaticum	1.0	1.9	0.0	0.0	1.0	1.89
Leuconostoc spp	9.0	17.0	0.0	0.0	9.0	16.98
Proteus mirabilis	2.0	3.8	0.0	0.0	2.0	3.77
Providencia spp	2.0	3.8	0.0	0.0	2.0	3.77
Staphylococcus spp	2.0	3.8	8.0	15.1	10.0	18.88
Total	38.0	71.7	15.0	28.3	53.0	100
$CalX^2 = 20.68$	**$TalX^2 = 14.07$**		**DF = 7**		**P. Value = 0.004**	

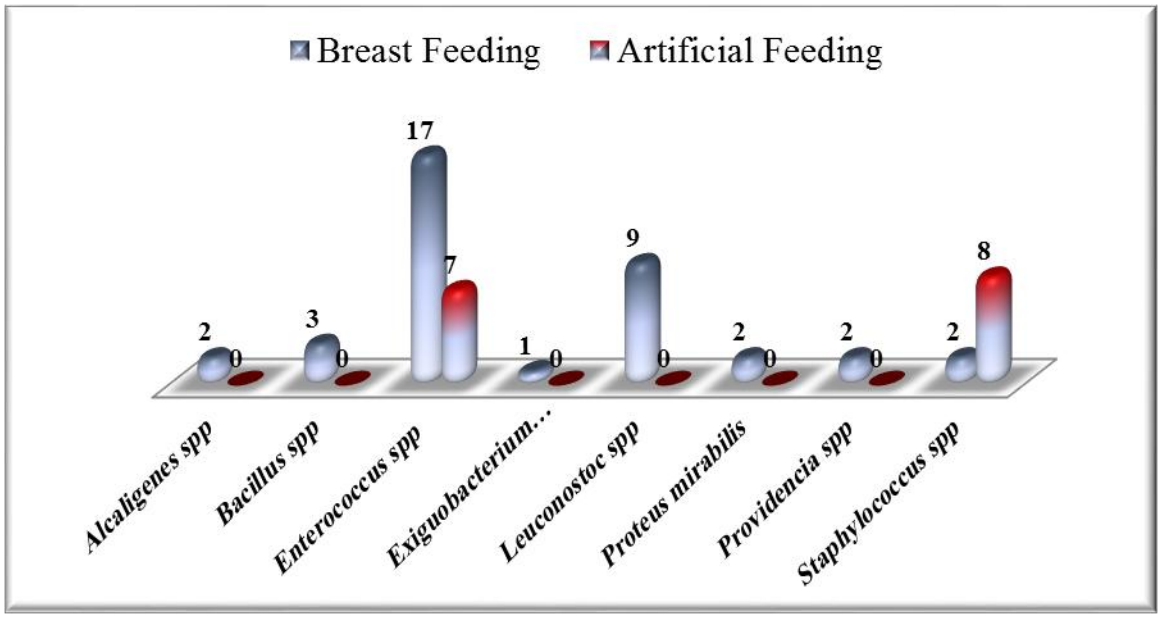

Figure (5): Incidence of diagnosed bacteria according to type of feeding

The results of the current study showed a statistically significant difference in the percentage of bacteria present in the samples that were taken from lactating mothers, it was more than the percentage of bacteria in the samples taken from non-lactating mothers .This shows the effect of

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type of feeding on the percentage and diversity of the beneficial microbiota in human breast milk .

Our study come in agreement with (Moossavi *et al*., 2019) whom mentioned that; human milk contains a bacterial diverse community and the mode of breast milk feeding was significantly associated with the milk microbiota composition, an observation which could reflect an increased exposure to the pumps and/or a decreased exposure to the mouth of infant, either way, it provides evidence for the retrograde mechanism of the milk inoculation.

Furthermore, Kordy *et al*., (2020) investigated the role of mother-infant interaction on breast milk microbes, and the data of his study suggested that the process of breastfeeding and interaction between the areolar skin and infant oral cavity are potentially critical for seeding the microbiome of milk. Previous report provided intriguing evidence suggestive of the enteromammary pathway in humans with the transfer of a single strain of *Bifidobacterium* breve in the maternal intestine, breastmilk and the infant stool in an infant delivered via Caesarian section. Also recorded that the breast milk bacteria were largely comprised of *Staphylococcus*, *Streptococcus*, *Acinetobacter*, and *Enterobacter* were primarily derived from the maternal areolar skin and the infant oral sites in breastfeeding pairs, which suggested that the breastfeeding process is a potentially important mechanism for the propagation of breast milk microbes through the retrograde flux via the infant oral and areolar skin contact.

Incidence of diagnosed bacteria according to age groups

The study indicated that the most bacteria isolated in the women was with third age group with percentage 37.7%, followed by the women with first age group 32.1%, and the lowest was in the second age group 30.2%, with a non-significant difference between type of birth at P. value < 0.05. As in table (6) and figure (6).

Table (6): Incidence of diagnosed bacteria according to age groups

Age groups / Bacteria	1st Age Group		2nd Age Group		3rd Age Group		Total	
	No.	%	No.	%	No.	%	No.	%
Alcaligenes spp	2.0	3.8	0.0	0.0	0.0	0.0	2.0	3.77
Bacillus spp	1.0	1.9	2.0	3.8	0.0	0.0	3.0	5.66
Enterococcus spp	11.0	20.8	6.0	11.3	7.0	13.2	24.0	45.28
Exiguobacterium aquaticum	0.0	0.0	1.0	1.9	0.0	0.0	1.0	1.89
Leuconostoc spp	0.0	0.0	3.0	5.7	6.0	11.3	9.0	16.98

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Proteus mirabilis	1.0	1.9	1.0	1.9	0.0	0.0	2.0	3.77
Providencia spp	0.0	0.0	1.0	1.9	1.0	1.9	2.0	3.77
Staphylococcus spp	2.0	3.8	2.0	3.8	6.0	11.3	10.0	18.88
Total	17.0	32.1	16.0	30.2	20.0	37.7	53.0	100
CalX^2 = 20.322		**TalX^2 = 23.68**		**DF = 14**		**P. Value = 0.120**		

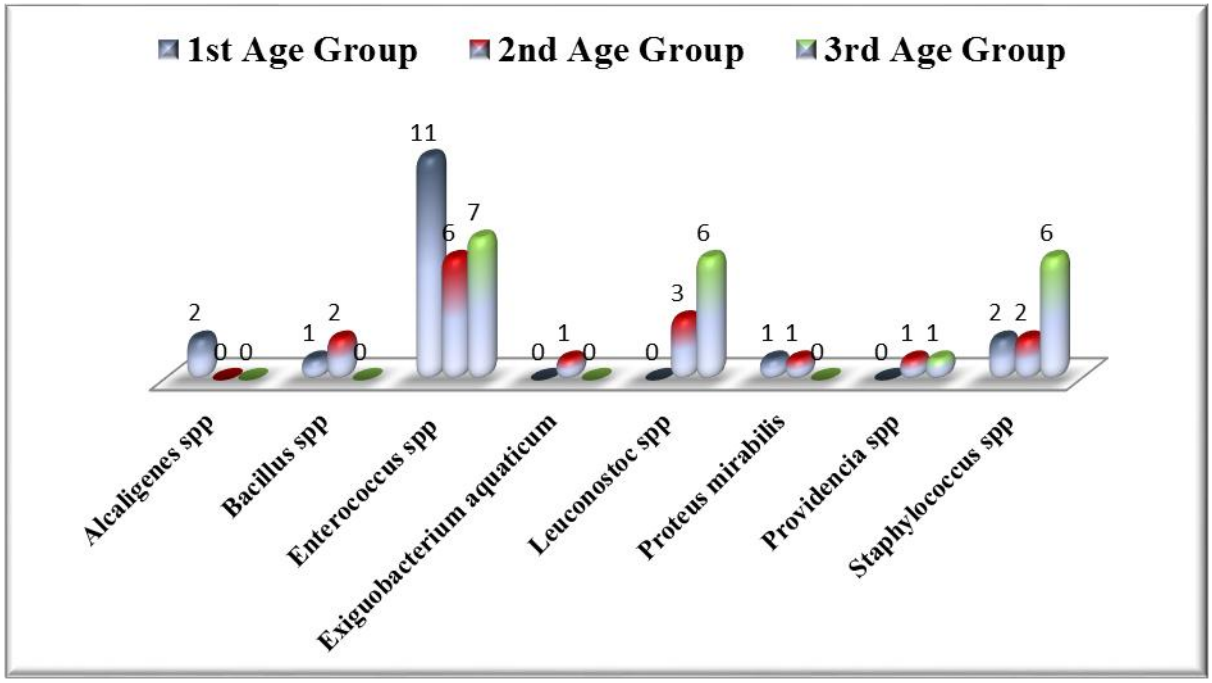

Figure (6): Incidence of diagnosed bacteria according to age group

Regarding with the findings of beneficial bacteria of the current study and the absence of a statistically significant difference between age groups, we suggested that there is no effect of the mother`s age on the microbiome present in breast milk, however; we did not get any previous study that discuss this subject.

Incidence of Bacteria according to Use of Medication

Concerning with the use of medication, there was a non- significant difference between women taking medication and not taken after delivery process, and the most bacteria isolated was *Enterococcus* in non-medication use women with percentage 24.53%, and also *Enterococcus* in medication use women with percentage 20.75%.

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Table (7): Incidence of diagnosed bacteria according to Use of Medication

Medication / Bacteria	Medication Use		Non-Medication		Total	
	No.	%	No.	%	No.	%
Alcaligenes spp	1	1.89	1	1.89	2.0	3.78
Bacillus spp	3	5.66	0	0.00	3.0	5.66
Enterococcus spp	11	20.75	13	24.53	24.0	45.28
Exiguobacterium aquaticum	1	1.89	0	0.00	1.0	1.89
Leuconostoc spp	5	9.43	4	7.55	9.0	16.98
Proteus mirabilis	0	0.00	2	3.77	2.0	3.77
Providencia spp	1	1.89	1	1.89	2.0	3.77
Staphylococcus spp	5	9.43	5	9.43	10.0	18.87
Total	27	50.94	26	49.06	53.0	100
CalX^2 = 6.261	**TalX^2 = 14.07**		**DF = 7**		**P. Value =0. 514**	

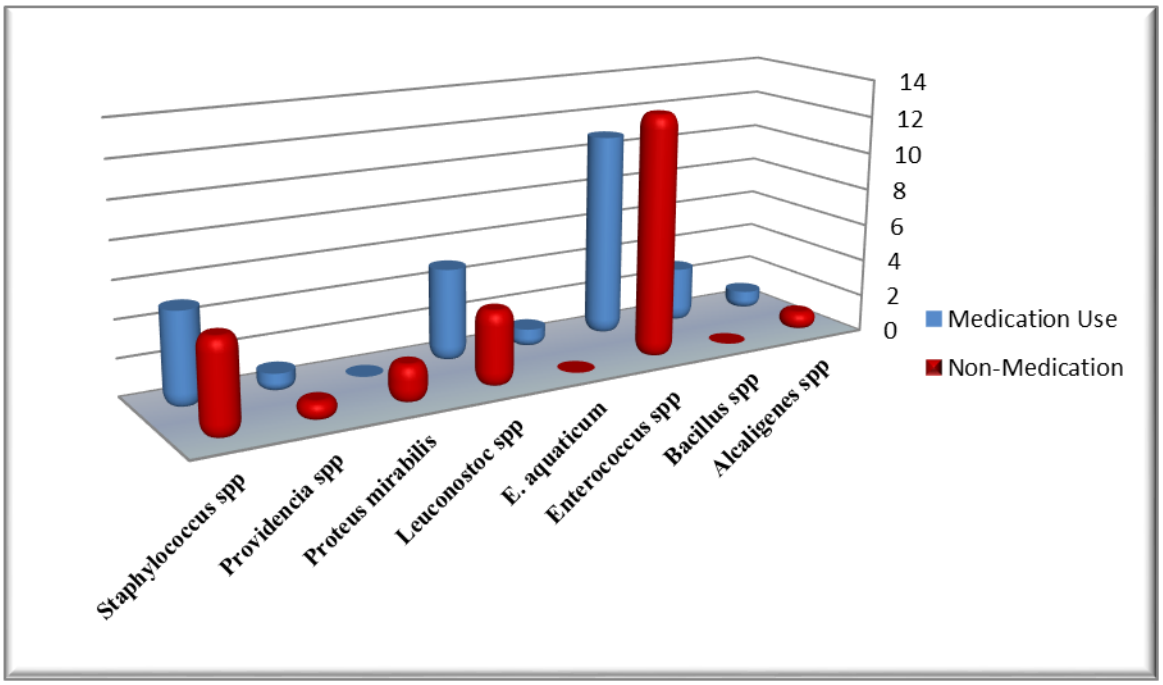

Figure (7): Incidence of diagnosed bacteria according to Use of Medication

Results indicate that there is no effect of medication use on the beneficial microbiome present in breast milk, and this disagreed with previous studies which confirmed the existence of

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the chemotherapy and antibiotics impact on the microbiome (Urbaniak *et al*., 2014; Hermansson *et al*., 2019).

Incidence of Bacteria according to Lactation Stages

Current results found that the most bacteria isolated in the women with mature milk (Third lactation stage) with percentage 56.6 %, followed by women with transitional milk (Second lactation stage) 32.08%, and lowest isolated in colostrum (First lactation stage) 11.32%. The results showed a non-significant difference between type of lactation and isolated bacteria at P. value < 0.05.

Table (8): Incidence of bacteria according to lactation stages

Lactation stage / **Bacteria**	**Mature Milk**		**Transitional Milk**		**Colostrum**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Alcaligenes spp	2	3.77	0	0.00	0	0.00	2.0	3.77
Bacillus spp	1	1.89	2	3.77	0	0.00	3.0	5.66
Enterococcus spp	12	22.64	10	18.87	2	3.77	24.0	45.28
Exiguobacterium aquaticum	1	1.89	0	0.00	0	0.00	1.0	1.89
Leuconostoc spp	6	11.33	3	5.66	0	0.00	9.0	16.99
Proteus mirabilis	2	3.77	0	0.00	0	0.00	2.0	3.77
Providencia spp	2	3.77	0	0.00	0	0.00	2.0	3.77
Staphylococcus spp	4	7.55	2	3.77	4	7.55	10.0	18.87
Total	30	56.6	17	32.08	6	11.32	53.0	100
CalX^2 = 17.566		**TalX^2 = 23.68**		**DF = 14**		**P. Value = 0.227**		

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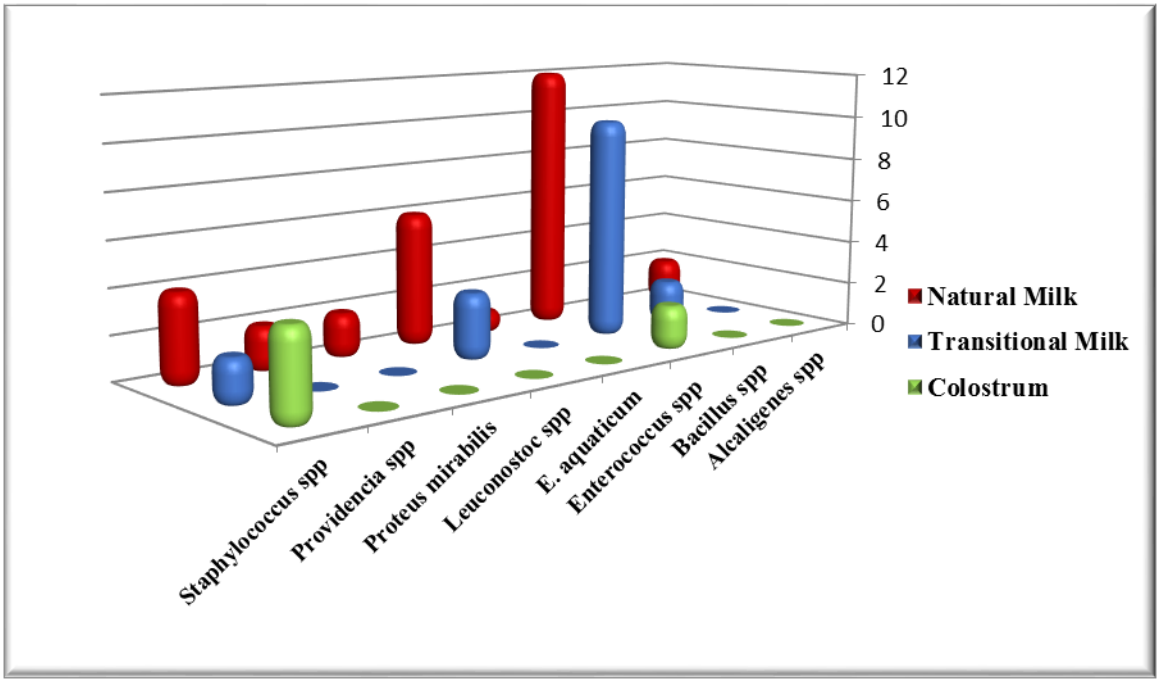

Figure(8): Incidence of bacteria according to lactation stages

Composition and diversity of the milk microbiota changed between colostrum , transitional and mature milk. Despite the differences between the types and percentage of bacteria in the different stages of lactation in current study, however; these were non-statistical differences. The *Enterococcus* spp and *Leuconostoc* were the most dominant bacteria in mature milk with another species of bacteria in different proportions. Colostrum less bacterial diversity than transitional and mature milk, *Staphylococcus* spp and *Enterococcus* spp only were in colostrum. This result met in some way with the results of a previous study (Cabrera-Rubio *et al*., 2012) in which, human milk microbiome changes over lactation, and the predominant bacteria were; *Weisella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus* in the colostrum samples, whereas in 1- and 6-month milk samples, the typical inhabitants of the oral cavity such as *Veillonella*, *Leptotrichia*, and *Prevotella* increased significantly.

In the current study, given that the colostrum samples were taken from non-lactating mothers, the reason for the difference in bacterial diversity between the different stages of lactation and the lack of bacterial diversity in the first stage (colostrum) probable due to the absence of the breastfeeding process and retrograde flow theory .

Conclusion

Breast milk is considered as an elemental nutritional source for infant health and growth. Different of beneficial microorganisms was found in milk both in lactating and non-lactating women, mostly of non-contaminated origin, supporting that milk is not sterilized, but provided naturally by a variety of healthy microbiome, especially lactic acid bacteria.

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