Infant gut microbiota changes during lactation and how it is shaped by human breast milk microbiota

By

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1. Abstract

Background: Human breast milk is a primary source of bacteria for the infant gut. This study aims to develop bacterial DNA extraction methods to determine whether a relationship exists between breast milk and infant gut microbiota with respect to obesogenic bacteria. **Study design:** Total of 16 breast milk and respective infants fecal samples were collected to analyze. **Methods:** Fecal and breast milk bacterial DNA were analyzed to identify the strains up to genus level. **Results:** Fermicutes was high in breast milk from overweight women and their infant's gut microbiota but Bacteroidetes increased only in infants' gut microbiota of overweight women. However, there were no significant relationships between normal-weight and overweight women' breast milk and between their respective infants' gut microbiota. **Conclusion:** This pilot study has shown means that obesogenic bacteria may be introduced into infant gut through the breast milk. However, we were impossible to answer whole concept statistically.

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3. Dedication

To the pleasant memory of my late parents Abraham de Silva and Karunawathi Hewadunuwila.

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7. List of Abbreviations

16S rRNA 16 Svedberg Unit Ribosomal Ribonucleic acid

BMI Body Mass Index

cfu Colony Forming Unit

CpG Cytosine – Phosphate – Guanine site of DNA

DNA Deoxyribonucleic acid

dNTPs The Nucleotide Triphosphates containing deoxyribose

FISH Fluorescent *in situ* Hybridization

IgA Immunoglobulin A

GABA Gamma-Aminobutyric Acid

HIV Human Immunodeficiency Virus

IGF1 Insulin-like growth factor 1

IGF2 Insulin-like growth factor 2

MiSeq In 2011 Illumina released a benchtop sequencer called the MiSeq

OTUs Operational Taxonomic Units

PCR Polymerase Chain Reaction

PhiX PhiX bacteriophage

RANK- L Receptor Activator of Nuclear Factor-kappa B -Ligand

TM7	Clones for 'Torf, Mittlere Schicht 7 phylum
V3- V4	V3/V4 region of 16s rRNA gene

8. General Introduction

It is a microbial world and humans host provides an environment and surface in which billions of microorganisms thrive. The human microbiota is highly complex and diverse, consisting of bacteria, viruses, and eukaryotes that compete for hosts' epithelium, nutrients and survival space.

Human breast milk microbiota plays a fundamental role in the well-being of infants. This microbiota can influences and shape an infant gut microbiota and/or vice versa. Contrary to traditional belief that human breast milk is a pure and sterile biological fluid, it is in fact a significant source of microbes that can be introduced to the infant's gut. Studies have shown that 800ml of breast milk can contribute between 1×10^5 and 1×10^7 bacteria to the infant's gut (1). While extensive development of non-culture based technology will continue to enable the identification of breast milk microbiota species, this technology is very dependent on high quality bacterial DNA extraction. Some obstacles faced in DNA extraction are the practical dilemma of removing lipids and precipitating proteins from breast milk samples. Also, commercially available kits remain limited in targeting and identifying only specific bacterial groups rather than extensive breast milk microbiota. Therefore, due to these limitations related to the extraction of good quality bacterial DNA from breast milk, the development of new techniques is essential. One of objective of this study was the development of a successful method for extracting bacterial DNA from breast milk.

Many details regarding the origin of bacteria in human breast milk are not yet fully

understood. It has been suggested that bacteria in breast milk originates from the bacterial contamination that occurs when the maternal skin and infant's oral cavity meet (2). Bacteria such as *Staphylococcus, Propionibacteria* and *Corynebacteria* have been shown to be present in maternal skin (3,4) as well as abundant in breast milk (5). This lends evidence to the fact that maternal skin microbiota may influence the development of breast milk microbiota. Also the oral cavity bacteria of infants may transfer into the mammary gland via retrograde milk flow of the mammary duct while the infant is suckling (6). Interestingly, another suggestion is that the maternal gut bacteria contribute to the creation of breast milk microbiota, which may arrive into the breast milk through a hypothesized route termed the "bacterial entero-mammary pathway" (5).

Diverse bacterial communities, at levels as high as 1×10^{14} and representing up to 15,000 different species, colonize the entire region of the gastro - intestinal tract (7) with 70% situated in the colon (8). Human gut microbiota influence many aspects of nutrition and health. The gut bacteria are associated with the energy storage of the host, as they ferment non-digestible dietary ingredients in the colon. The balance of the gut bacteria composition is important in maintaining many physiological requirements of the body. An imbalance of bacterial composition may cause inflammation and infection, which may enhance the risk for the development of gastrointestinal tract diseases, and diabetes and obesity (9).

Today, childhood overweight and obesity are major health concerns as they may lead to development of diabetes and cardiovascular diseases in later life. Recently, some experimental studies have suggested that obesogenic bacteria, such as *Bacteroides* and

Staphylococcus, that are dominant in overweight and obese maternal guts, may influence the development of obesity in their respective children (10). This has been suggested based on the claim that obesogenic bacteria of the maternal gut may be transferred into the mammary gland through the bacterial entero-mammary pathway and then into the infant's gut through human breast milk. Further to this, research has shown that *Bifidobacterium* in the infant gut may in fact normalize the impact of obesogenic bacteria and contribute to maintaining the normal weight of the children (10).

Knowledge of the structural development and cellular functions of the mammary gland and intestine is important in understanding the development of childhood obesity. However, characterizing the composition and diversity of breast milk and infant gut microbiota, as well as the defining the factors that influence the composition and diversity of each, may play an even greater role. Furthermore, understanding the impact of an imbalance in the composition and diversity of these microbiota may be critical in associating the obesogenic bacterial effects on childhood obesity and the early origins of some human diseases.

9. Literature Review

9.1 Mammary Gland Development & Lactogenesis

The mammary gland evolved, along with other skin appendages such as hair, sweat and oil glands, during the sequence of transitional lineages between early tetrapod and true mammals (11,12). The mammary gland develops in four phases that can be roughly mapped into distinct life-cycle stages; embryo, puberty, pregnancy, and lactation (13). Embryonic development of the mammary gland is first evidenced by lines of thickened

epithelium on the ventral surface, lateral to the midline, known as “milk lines” or “mammary ridges” (13). During the early stage of embryonic development, the mammary anlagen are organized into a small, branched epithelial network that is linked to the surface at the embryonic nipples. These juvenile glands may biosynthesize milk by stimulation of high levels of maternal reproductive hormones in utero. This condition is called neonatal galactorrhea (13).

There is no significant development that can be seen in the mammary gland until puberty is reached. At puberty, under the influence of estrogen, growth hormone, and insulin-like growth factor 1 (IGF1), the mammary glands undergo an allometric growth, which is characterized by ductal elongation (14-17). During pregnancy, the mammary gland developmental is significant. Extensive alveolar luminal cell proliferations can be observed in the pregnancy stage. Estrogen, progesterone and prolactin are responsible for the mammary gland development during pregnancy. The alveolar epithelial cell proliferations are mediated by Receptor activator of Nuclear Factor Kappa B- ligand (RANK-L) and insulin-like growth factor 2 (IGF2) (18,19).

Lactogenesis

The process of milk biosynthesis and secretion are known as lactogenesis (20). Lactation is a stable and cyclical process, which occurs in the luminal spaces of the gland such as alveoli, ductules and ducts. The luminal space is rhythmically emptied and filled by stimulation of several hormones; oxytocin, prolactin and serotonin (13). In addition, these hormones are involved in lactation homeostasis (13). Recently, some studies have revealed that dilatation of alveolar spaces within the mammary gland is responsible for the production of serotonin (13). Two homeostatic functions are associated with

mammary serotonin: regulation of lactation and communication with the parathyroid hormones to mobilize calcium from bones (13). The mammary epithelium is stimulated by prolactin or placental lactogens to synthesize milk (13). The characteristic functions of mammary epithelium are milk biosynthesize, transport, and exocytosis (13). These unique features of mammary epithelium render mammals the ability to produce large amounts of milk, comprised nutrient-rich, complex macromolecules and a high level of bacterial diversity (13).

Two important stages of lactogenesis can be seen in ruminants (21) and can be also seen in human. The first stage is developed during pregnancy where the mammary gland produces small amount of milk components such as casein and lactose. This stage of lactogenesis is important for formation of colostrum. The alveolar epithelial cells are responsible for secreting milk proteins (13). The second stage of lactogenesis occurs during the peripartum period where copious amount of milk is synthesized. This second stage is initiated when progesterone levels in the blood are suppressed by high prolactin concentrations (13) and when tight junctions of mammary epithelium cells are closed (22).

Cellular physiology of milk biosynthesis is poorly understood (13). The breast milk is biosynthesized by lactocytes, also known as secretory mammary epithelial cells, which line in the inner surface of alveoli of the lactating mammary gland (23). The mechanisms of milk biosynthesis have some similarities with other secretory epithelial mechanisms (24), but two important exceptions. One exception is secreted milk fluid that is not modified after secretion by mammary epithelial cells and second is secreted lipids, which

are secreted only after it is covered by multilayered cell membranes with small amounts of cytoplasm (25). The main compositions of mature milk are: proteins, simple sugars (especially lactose), oligosaccharides, ionic solutes, and lipids (13). Secreted milk proteins are comprised mainly of caseins and other milk-specific proteins, bioactive peptides, glycoproteins, and proteoglycans (26). Secretory mammary epithelial cells biosynthesize milk through five different pathways: paracellular flow, transcytosis, conventional exocytosis, transporter-mediated secretion, and milk fat globule extrusion (25,27,28), and many nutrients are secreted via the use of more than one pathway. The pathways are differentially used for secreting colostrum and mature milk (22).

Biosynthesized or secreted milk needs to be removed from the mammary gland. Some species, like cows, ewes, and goats have milk cisterns in their own mammary glands that facilitate the expulsion of milk prior to milk ejection. In other species such as sows and humans who do not have milk cisterns, milk is removed only by milk ejection reflex (29). Oxytocin initiates the milk ejection reflex, constricting the myoepithelial cells located around the alveoli. When myoepithelial cells contract, the milk is injected into the milk duct. During suckling, an impulse is generated that travels from the nipple to the posterior pituitary gland, releasing oxytocin to commence the milk ejection. Milk ejection is an important biological process for milk biosynthesis (30). Milk ejection has rhythmic patterns due to the pulsatile release of oxytocin ((31-33), where each cycle is comprised of multiple ejections and one ejection is completed in a time frame of between 45 seconds to 3.5 minutes (31-33).

In lactating mothers, milk is stored in the alveolar area (23). Widening and shortening of

the duct diameter due to release of oxytocin on pulsatile pattern at milk ejection, lead to removal of the milk from the alveoli (34). It has been suggested that the decrease in diameter of larger ducts following milk ejection is due to breast milk not accumulating in the larger ducts adjacent to the nipple but rather there is a reversal milk flow that is observed in small ducts because there is a little amount of milk in the smaller ducts (6). Therefore, a small amount of milk, needs to be present in the ducts and ductules to activate the retrograde milk flow prior to milk ejection. In animals such as rat, mouse, guinea pig, and rabbits, studies have showed that such species may also have retrograde milk flow (34). Interestingly, reverse milk flow may be a real transporter of microorganisms from an infant's mouth to the mother's mammary gland (5). Rhythm of milk ejection and strength of suckling are directly associated with milk duct diameter (6). The milk duct diameter of first- offered breast (1.63 ± 0.11 to 2.75 ± 0.17 mm) is higher than the duct diameter of second- offered breast (1.56 ± 0.11 to 2.09 ± 0.15mm), and the first- offered breast provides more milk compared with second-offered breast (first-offered breast: 65 ± 8 g, second-offered breast: 39 ±5 g) (6). More studies need to be conducted to determine whether an increase milk duct diameter is enough to facilitate more microbes being introduced from the infant oral cavity into the first- offered breast.

9.2 Human Breast Milk Microbiota

The mammary gland has undergone several phases of development to reach the state of lactation. After such development, the mammary gland can produce a small amount of colostrum and can be ready for lactation just a few weeks prior to delivery (5). During the pregnancy period, the nipple and areola of the mammary gland are enlarged, and

sebaceous glands surrounding the nipple area become significantly prominent, and lymph and blood circulation into the mammary gland is increased (35). Some of these changes create a favorable atmosphere for the formation of a biofilm in the areola area and milk duct system, which encourage the production of specific and transitory mammary microbiota. Transitory mammary microbiota begin to grow during the third trimester of pregnancy (5). Maximum complexity of the microbiota is achieved by the end of the third trimester and this complexity level is maintained during the entire lactation period (5). The mammary microbiota starts decreasing gradually by the weaning period and vanishes when milk is not available in the gland and it is followed by the mammary involution, which is initiated by the apoptosis process (5).

An average infant feeds approximately 800ml of human milk per day. This volume of milk can contain between 1×10^5 to 1×10^7 number of bacteria that can be introduced into the infant's gut daily (1). It is a reason that infant gut and breast milk microbiota have similar bacterial diversity (5). Culture media studies have revealed that the dominant microbial species found in the breast milk of healthy lactating mothers are: *Staphylococci, Streptococci*, Lactic acid bacteria, Propionibacteria, and other Gram positive- like bacteria (1,2,36,37) such as *Streptococcus lactarius* (38). Human breast milk is also a unique source to supply live Bifidobacteria into the infant intestine (39). Several studies have proven that some bacterial strains such as Lactobacillus, Staphylococcus, Enterococcus, and Bifidobacterium genera are exchanged from mother to infant via breast milk (36,39-44). Due to the hundreds of microbial strains that are introduced through breast milk, breast-fed infants have been found to be resistant to developing diarrhea, respiratory tract diseases and other non-communicable diseases such

as diabetes and obesity (45,46). Hundreds of operational taxonomic units (OTUs) can be identified in breast milk but the most common strains of bacteria in breast milk are *streptococcus, staphylococcus, pseudomonas, serratia, corynebacteria, propionibacterium, bradyrhizobiaceae, ralstonia, and sphingomonas* (5). However, the mammary microbiota has shown that its bacterial populations are often stabilized and highly personalized (47). Therefore, it has been widely accepted that the composition and diversity of breast milk microbiota is dependent on individual variances of microbiota within the maternal gut, skin, vagina and microbiota of the infant oral cavity.

9.3 Origin of Human Breast Milk Microbiota

The origin of the microbiota in human breast milk can be explained by several hypotheses. Interestingly, some studies have suggested that maternal gut bacteria may be influenced by the travel of maternal dendritic cells and macrophages into the mammary gland through the endogenous route known as bacterial entero-mammary pathway (5). In addition, a certain degree of backward flow of breast milk into the mammary ducts also involves the exchange of bacteria from infant oral cavity to the mammary gland during infant suckling (6). Some common bacterial strains associated with adult skin, such as Staphylococcus, Corynebacteria, and Propionibacteria, have also been identified in human breast milk. Therefore, this may also suggest that bacterial contamination of adult skin may dictate and play a role in the composition of breast milk microbiota (5). Some other studies have emphasized that the antigen-stimulated cells in maternal gut mucosa may travel into distant areas of the body and colonize in the mucosa of the genitourinary and respiratory tracts as well as the salivary and lactating mammary glands (5). Overall,

gut microbiota associate and communicate with all other microbial populations within the human body.

9.3.1 Bacterial Entero-Mammary Pathway

The bacterial entero-mammary pathway is not yet fully understood. Some studies have proven that viable non-pathogenic microbes in the dendritic cells survive for short periods in the mesenteric lymph nodes (48). Dendritic cells and/or macrophages are responsible for transporting gut microbes into other sites of the body through the lymphatic circulation of the mucosa. Other research has identified different strains of lactobacilli or bifidobacteria found in both breast milk and breast skin of lactating mothers (39,49). It has been shown that the bacteria found in the gut of lactating mice may travel into the mammary gland through the mesenteric lymph nodes during late pregnancy (50). Similarly, another study has isolated live bacteria and bacterial DNA in human breast milk and also in the maternal peripheral blood mononuclear cells (5). More recently, two studies have further given credence to the existence of the bacterial entero-mammary pathway after isolating three orally administrated lactobacilli strains (*L. salivarius* CECT 5713, *L. gasseri* CECT 5714, and *L. fermentum* CECT 5716) from associated human breast milk samples (51,52). The above research emphasizes that living microorganisms within human breast milk may originate in the maternal intestine and travel to the lactating mammary gland via the hypothesized endogenous route under the influenced of maternal dendritic cells and macrophages.

9.3.2 Retrograde Milk Flow of Breast Milk in Milk Duct

A reversal of milk flow into the milk duct can be seen during infant suckling (6).

Therefore, human breast milk could be a bacterial exchange medium between the infant oral cavity or saliva and the mammary gland. The infant salivary microbiota is poorly understood however studies on adults have shown that the predominant bacterial phylotype of salivary microbiota is streptococcus (53-55), which is also an abundant strain in the saliva of edentulous infants (56). Human breast milk bacteria are directly associated with infant oral cavity microbiota because streptococcus is the predominant species that can be seen in breast milk, colostrum as well as in infants salivary microbiota (40,57,58). This evidences supports the hypothesis that the maternal milk bacteria may play an important role in establishing salivary bacterial communities and/or vice versa.

Mastitis is a common illness that is suffered by 33% of lactating women during the lactating period (59,60). Mastitis is an inflammation that occurs locally within one or several lobules of the mammary gland and caused by staphylococci, streptococci and/or corynebacteria (60,61). More investigations must be done to determine whether causative bacteria of mastitis are introduced by the infant oral microbiota. The dominant bacterial phyla of all edentulous infants are: Proteobacteria, Firmicutes, Fusobacteria and Actinobacteria as well as genera Streptococcus (62). The development of new strategies for treatment of mastitis based on the replacement of antibiotics by probiotics is another area of interesting study (5).

9.4 Salivary/ Oral Microbiota

The oral microbiota is responsible for causing several diseases systematically: bacterial endocarditis (63), aspiration pneumonia (64), childhood osteomyelitis (65), low birth weight of premature deliveries (66,67), cardiovascular disorders (68,69) as well as locally

such as periodontal diseases (70). *Actinomyces naselundii* has been associated with premature deliveries and low birth weights while *Lactobacillus casei* caused slightly higher birth weights and normal vaginal deliveries (71). Recently, some studies have stated that microbes in the mouth interestingly grow in different areas of the oral cavity due to the affinity of oral bacteria to bind to specific receptors within the mucosal surface (72,73). The 141 various bacterial taxa included six different bacterial phyla such as Actinobacteria (species *Atopobium Actinomyces* and *Rothia*), Firmicute*s* (species *Streptococcus*, *Selenomonas*, *Gemella*, *Eubacterium* and *Veillonella*), Proteobacteria (species *Neisseria*, *Campylobacter* and *Eikenella*), Bacteroidetes (species *Porphyromonas*, *Capnocytophaga and Prevotella*), Fusobacteria (species *Leptotrichia and Fusobacterium*) and the TM7 phylum (they were not identified on culture media), were isolated from nine different areas of the adult' oral cavity (74).

9.5 Gut Developments

The gastrointestinal tract is a complex system that is comprised of several vital organs. It is derived from a simple tubular structure (75) that can be differentiated in the human embryo at 4 weeks of development (76). The gut is formed by three germ layers known as the endoderm, mesoderm and ectoderm (76). The primitive gut is differentiated into the foregut (which forms the pharynx, esophagus and stomach), the midgut (small intestines) and hindgut (colon) (76). The epithelium, mesenchyme and muscle layer can be demonstrated between 10 and 17 weeks of gestation (77). The gut epithelium is continuously regenerating throughout life (76).

9.6 Infants Gut Microbiota

The fetal gut lumen is devoid of significant number of bacteria but it colonizes microorganisms within a few hours of delivery (78). The number of bacteria can be identified in infant feces within hours of birth and it contains greater than 10 colony forming units (cfu) of bacteria for every gram of stool within the first week (79,80**)**. Culture based studies showed that infant gut was first cultivated by facultative anaerobes such as Enterococci and *Escherichia coli* at concentrations exceeding 10 cfu/g feces (79). As well, staphylococci and streptococci have also been isolated in significant concentrations (81). Initially, the presence of oxygen in the neonatal intestine suppresses the establishment of strict anaerobes, but facultative aerobic populations of bacteria expand and they consume oxygen that creates an anaerobic environment later which supports the growth of anaerobes (78**)**. In the first week of life, *Bifidobacterium*, *Clostridium* spp. and *Bacteroides* can be isolated from healthy neonatal fecal samples and anaerobic species such as bifidobacteria have been recognized as the predominant component of the gastrointestinal bacterial mass (78). The human gastrointestinal tract is dominated by 4 recognized phyla of bacterial kingdom such as firmicutes, bacteroidetes, actinobacteria, and proteobacteria although approximately 10% of gut bacterial population cannot be identified under culture methods in the first 2 months of life (82). This may be a reason why gut microbiota studies are depending on non-culture base methods.

Major bacterial source of infant gut microbita is human milk; it supplies hundreds of bacterial phylotypes (5). However, establishment and composition of infant gut

microbiota is related by several factors; prenatal-uterine environment, mode of delivery, type of feeding, antibiotic use, and the immediate environment. There are several functions associate with human milk microbiota to enhance the infant health such as decreasing incidence and severity of diseases (83), synthesis of antimicrobial compounds (83-86), or promotion of the gut barrier function by increasing mucine production and lowering intestinal permeability (83). Interestingly, it has been witnessed that introducing human breast milk Lactobacillus strains into the infant's gut before 6 months of age results in reductions of gastrointestinal diseases, upper respiratory tract diseases, and total number of diseases, by 46%, 27%, and 30% respectively (87). Administrating viridans Streptococci and commensal coagulase-negative Staphylococci through the human breast milk have been shown to reduce the risk of pathogenicity of microbes in hospitalized infants (5). Similarly, another study has suggested that methicillin-resistant *Staphylococcus aureus* which can be isolated from the in oral cavity of hospitalized newborns, may be prevented by introducing viridans streptococci (88). In addition, viridans streptococci were one of the dominant microbes groups in human breast milk that facilitate in maintaining a healthy infant gut (89).

9.7 Determinant Factors of Infant Gut Microbiota

9.7.1 Prenatal Microbial Exposure

The prenatal environment is related to later development of chronic diseases, such as obesity, type 2 diabetes, hypertension, and coronary artery disease (78**)**. Direct bacterial exposure to the fetus through the amniotic fluid, which is typically sterile, has given rise to some adverse effects. Bacterial ribosomal DNA and bacteria from *Leptotrichia* spp.

and other relevant species were identified in amniotic fluid of pregnant women at premature deliveries (90,91). Studies revealed that infant prematurity is positively associated with bacterial load, even in the absence of ruptured amniotic membranes (90). Although the clinical significance of microbial identification in amniotic fluid has not been fully understood. It has been suggested that microbes of amniotic fluid may cause gut inflammation of fetus because fetus is often swallowing amniotic fluid and the gut inflammation lead to stimulate uterine contraction and final outcome is premature delivery (92).

9.7.2 Mode of Delivery

During vaginal delivery, microbes from the birth canal, the perineum and the mother's skin contribute the first bacterial inocula for colonization of the infant's gastrointestinal tract (78). Therefore, the infant's early gut microbiota profile is similar to the pregnant mother's fecal and vaginal microbes (93,94). In contrast, infants born by cesarean section showed delayed colonization and lack of microbial diversity (93,95). The early gut microbiota of cesarean sectioned- infant is similar to some bacterial species in the hospital environment, including reduced levels of strict anaerobes such as Bifidobacteria (96,97). Comparison of infants born by vaginal delivery to those by cesarean sectioned, showed that those born by cesarean section tend to harbor higher stool quantities and/or increased prevalence of *Staphylococcus* spp, *Streptococcus* spp, (98) *C difficile,* klebsiella, and enterobacteria, and a reduced prevalence of bacteroides, (95,96,99) and reduced or delayed colonization of bifidobacteria and lactobacilli (100-102). These altered patterns have been documented to be present up to 7 years of age (103). Some studies emphasize that changes in composition of intestinal microbiota may be related to

obesity and metabolic syndrome (104,105). In addition low-grade systemic inflammation may be associated with obesity or overweight and some disorders, which are related with adiposity such as type II diabetes and insulin resistance (78). Obesity has been shown to increase two fold among 3 years children who were delivered by cesarean section even after correction for modified relevant factors such as birth weight, mothers' body mass index, and other related variables (106). Several studies have shown that obese individuals' major gut bacterial phylum is Firmicutes (107,108) and when compared obese individuals with lean, Firmicutes to Bacteroidetes ratio was increased from 3:1 to 35:1 (109). This changed bacterial quantity has been implicated in obesity development through its effects on increasing energy harvest from food and its interactions with endocrine and epithelial cells that increase insulin resistance and inflammation and increase adipocyte generation (110-112). Recently, one prospective trial studied 138 infants between 3 weeks and 1 year and the infants were identified as low Staphylococcus and high *Bacteroides fragilis* concentrations that related a greater incidence of obesity during preschool age (113). A separate longitudinal study was done to understand microbiota composition during infancy (at 6 and 12 months) and 7 years later. The study had revealed that 25 children who became obese or over weight at age 7 and 24 children who remained in normal weight. Results from Fluorescent *in situ* Hybridization (FISH) and quantitative Polymerase Chain Reaction (qPCR) analysis showed that high fecal bifidobacteria and low concentration of *Staphylococcus aureus* reducing in childhood overweight (114). The authors, Saavedra and Dattilo (2012) proposed that "protection from obesity noted with higher bifidobacteria may be partly mediated by its potential anti-inflammatory effects, whereas *Staphylococcus aureus* may act as a trigger of low-

grade inflammation, contributing to difficulty with weight management" (p, 724) (78).

9.7.3 Dietary Intake

Microbial diversity of the intestine depends on several factors; diet is one of main factor among others (115). In the full-term breast-fed infant, anaerobic bacteria such as *Bifidobacterium* begin to colonize in the gut within the first week of life (78). Bifidobacteria can constitute more than 60% of the fecal bacteria in breast-fed infants within 6 days of nursing (116), and up to 72% by the third week of exclusive breast-feeding (117). Presence of higher concentration of Bifidobacteria in the infant's stool depends on concentration of multiple *Bifidobacterium* spp in maternal milk that provide a constant inocula to the infant gut (118-121) as well as several other breast milk components, including galacto-oligosaccharides, which selectively foster the growth of Bifidobacteria in the infant gut (78). Formula-fed infants are less frequently colonized with Bifidobacteria, and they are more often colonized with potentially pathogenic species of enterococci, coliforms, and clostridia when compared with breast-fed infants (78). Specifically, populations of *Escherichia coli*, *Clostridium difficile*, and bacteroides have been consistently reported as more prevalent species in the stool of formula-fed infants (113,116,122). By approximately 2 years of age, when children are consuming an adult-type diet, the gut flora becomes similar to the adult microbiota (78). However, diversity of microbiota depends on clinically induced variations of macronutrient contribution in the diet (78). Increased density of firmicutes and decreased densities of bacteroidetes (123-125) and bifidobacteria (124) have been documented with high-fat feeding in both animal and human studies, although there are no studies follow-up for long period to understand whether these changes are persist (78).

9.7.4 Antibiotic Exposure

If antibiotic is administrated within the first year of life, the microbial diversity is dramatically decreased in term infants (126). When antibiotics (primarily Cephalosporins or oral Amoxicillin) were given during the first month of life, the fecal Bacteroides and Bifidobacteria were reduced and *Clostridium difficile* was increased (113). The duration of antibiotic treatment and bacterial diversity in a month old premature infants are negatively associated (127). Enterococci populations were increased by 4-days treatment of broad-spectrum antibiotics in month old neonates (128).

9.7.5 Other Factors

The immediate environment (78) and geographical variances in early life can also affect composition and diversity of microbiota. In addition, individual differences in the luminal environment of the gut influence the change in the diversity of intestinal microbiota (78). Unique individual factors such as luminal oxygen tension and lumen redox potential, gut pH, the composition and concentration of digestive enzymes, biliary secretions, mucus and mucin, and IgA production are all specific and phenotypically unique to each individual host, and these variations can define the composition and diversity of gut microbiota in different ways (129).

9.8 Skin microbiota

Skin is the largest organ in human and the skin microbiota is one of the major bacterial populations in the human body. The total human skin area is approximately 1.8m^2 and it is characterized with multiple folders, invaginations, specialized niches and complex

habitats, and provides surface for a wide range of microbes (130). Cultivation of microbes is induced by the ecology of the skin surface, which is determined by endogenous host factors, topographical location, and exogenous environmental factors (30). Knowledge of skin microbiota is important in order to understand the origin of human breast milk microbiota. Furthermore, this knowledge can be used to understand dermatological diseases and to make treatment choice for choosing whether to use antibiotics or probiotics.

Tagami (2008) stated that "generally, the skin is cool, acidic and desiccated but distinct microbial habitats are determined by skin thickness, folds and the density of hair follicles and glands" (as cited in Grice & Segre, 2011, p.244) (131). Each individual have a unique and characteristic skin microbiota that related with it's own cutaneous invaginations and appendages, including sweat glands (eccrine and apocrine), sebaceous glands and hair follicles (132). The eccrine glands also have other functions including excretion of electrolytes and water and skin acidification, which inhibits the cultivations and multiplications of microbes (130). The axillary vault (armpit), nipple and genito-anal regions are common sites for the apocrine glands. Skin bacteria use the secretions of the apocrine glands and sweat derives which may produce characteristics odor (133-136). One of the non-pathogenic bacteria on the skin is *Propionibacterium acnes,* which lives living in the sebaceous glands because it is usually anoxic (132,137). *Propionibacterium acnes* releases free fatty acids onto the skin after hydrolysis of the triglycerides available in sebum (138,139). Bacterial growth is enhanced by free fatty acids, and it may be the reason bacteria are growing within the sebaceous gland (140). These free fatty acids maintain acidic pH (~5) in the skin surface (141,142). An acidic pH in the skin

suppresses harmful microbes such as *Streptococcus pyogenes and Staphylococcus aureus* and induces growth of coagulase-negative staphylococci and corynebacteria (142-145). Although, increased skin pH promotes the growth of *Staphylococcus aureus* and *Staphylococcus pyogenes* (143). Some bacteria grows in axillary vaults, groin and toe webs because of the higher temperature and humidity. Another promoting factor of the skin microbiota is the number of sebaceous glands. Generally, the sebaceous glands and its surrounding area show low microbial density and diversity since only few bacteria can adapt those environmental conditions (130). Actinobacteria, proteobacteria, firmicutes and bacteriodetes bacterial phyla are common in dry areas of the skin (3,4,146). Various parts of the hand, forearm and buttock are examples of dry skin areas. There are several factors such as degree of exposure to environment elements, frequency of hand washing, and handedness preference change in the skin microbiota of the hand (130). Women's hands are more susceptible for colonization and diversity of bacteria compared with mens's hands but the reason is not known (130). The skin microbiota is also associated with occupation, type of clothes, use of antibiotics of the person (130). Age directly affects in the microenvironment of the skin and cultivation of microbes (147,148). High-temperature and high-humidity enhance the growth of microbes on the axillary vaults, back and feet when compare with low-humidity and high-temperature conditions (149). In the same study, high humidity and low temperature environments favor the growth of Gram-negative bacteria, found on the feet and back (149).

In the uterine cavity, the skin of the fetus is sterile however just after delivery the skin is harbored by several bacteria, although it does not depend on the mode delivery (150,151). According to the 16S ribosomal RNA metagenomic sequencing method, the majority of

skin microbes can be classified into four different phyla; which are firmicutes, actinobacteria, proteobacteria and bacteroidetes. In addition, the same major bacterial phyla are also present in mucosa of human gut and mouth (152-157). Composition of major bacterial phyla changed on different surface areas of the body; Actinobacteria species are common in the skin, while species of Bacteroidetes and Firmicutes are predominant in the gut (152-157).

Most of the dermatological diseases have a specific topographical location and/or specific microbes, and a specific stage of life (130). Whether these specific factors related to the skin diseases are directly associated with endogenous microbial population is not yet answered (130).

9.9 Vaginal Microbiota

Vaginal microbiota change periodically on physiological milestones of woman life; alteration of microbiota takes place through puberty, reproductive period and menopause (158). These changes may interconnect with vaginal microbiota and host or vagina and with health or disease (158). The indigenous bacterial populations, which are harbored in the early physiological milestone of a woman's life, are acting as defense organisms against cultivation of pathogenic bacteria (158). Lactobacilli becomes the dominant bacteria in vaginal microbiota during the reproductive age of a woman (160). There are four common lactobacillus species such as *Lactobacilli crispatus*, *Lactobacilli iners*, *Lactobacilli gasseri*, and *Lactobacilli jensenii* that can be seen in vaginal microbiota (159). Some studies have suggested that Lactobacillus have an important role to protect the vagina from non-indigenous and pathogenic microbes such as Lactobacillus, which

can reduce vaginal pH up to 3.5- 4.5 due to production of lactic acid (160). Interestingly, lactic acid plays an important role as a microbicide, which is effective against *Neisseria gonorrhoeae* or HIV (161,162). The lactic acid is also harmful for the gram –negative microorganisms (163). *Lactobacilli crispatus*, *Atopobium vaginae* and *Prevotella bivia* are associated with enhancing the innate immunity, which was proven by a recent study in vitro (164). Previously studies observed that *Lactobacilli crispatus* was a dominant species but later, non-culture based technique revealed that *Lactobacilli iners* is a common lactobacillus in vaginal microbiota (165,166). Knowledge of genetic variation of *Lactobacillus* species (or strains) and strength of their functions may be meaningful impact on human health and illnesses (158).

The vagina an has anaerobic condition because of reduced oxygen concentration. In addition to the lactic acid, target-specific bacteriocins (167,168) and broad-spectrum hydrogen peroxide (169,170) are also released by vaginal lactobacillus. However, because of anaerobic condition, there is no possibility to release hydrogen peroxide in higher concentration to cause toxic effects into the vagina (158). Hydrogen peroxide have antibacterial effects to prevent the growth of pathogenic microbes in vivo when they grow in aerobic atmosphere (171-174).

Interestingly, very few mammals, including human have *Lactobacillus* spp. as a common bacterial population links to maintains a low pH in vagina (158). In addition, some other factors may also affect the diversity of the vaginal microbiota such as immune stimulation or microbial protection during the first few days of newborn (158). Understanding of these determinant factors of vaginal microbiota is important to implement new prevention strategies to minimize the vaginal disorders. These strategies

support protecting vaginal microbiota or may use probiotics or prebiotics individually to prevent vaginal diseases (158). Although, comprehensive molecular community surveys facilitate to understand significant features of composition of vaginal microbiota, which lead to communicate with its host are not yet understood. Co-evolutionary processes between the human host and specific bacterial partner are regulated by vaginal ecosystem, however the selective forces (traits) related this mutualistic associations are also not yet verified (158).

9.10 Maternal (Adult) Gut Microbiota

Human diet is associated with biological diversity of gut microbiota. The function of the gut is altered by gene expression changes in mammalian intestinal mucosa by gut microorganisms (175). A study that compared, mice grown in germ free environment to those conventionally raised showed that intestine microbiota facilitate gene expression of nutrient absorption, immunity, energy metabolism and intestinal barrier function in mouse gut. (176). These changes can be seen significantly in the small intestinal mucosa (175). Recently it has been proved that probiotics can change genes expression pattern in human gut (177).

Human meals may lead to changes in the composition of gut microbiota, which finally alter the patterns of biochemical reactions in the gut (175). A study demonstrated that after transplant human fecal microbiota into germ free mice and then they were introduced a high-fat, high- sugar Western diet, Firmicutes became the dominant phylum instead of Bacteroidetes (175). In addition, gut microbiota returned to the original composition within one week after replacing the current diet with the usual one (178) and

this mechanism is named ‘luminal conversion’ (175). The luminal conversion produce some compounds such as short-chain fatty acids, biogenic amines (such as histamine) or other amino-acid-derived metabolites such as serotonin or Gamma-AminoButyric Acid (GABA) that may influence health and diseases as well as these products may influenced the composition of gut microbiota (175). Dietary non-digestible polysaccharides in the gut involve the synthesis of short chain fatty acids such as butyrate, acetate and propionate (175) and human gut epithelium utilize short chain fatty acids (175). Some microbes’ proliferations, especially *Bifidobacterium* spp. and *Lactobacillus* spp. are stimulated by fermentation of prebiotic carbohydrates such as fructo-oligosaccharides and inulin in the gut (175). Overall, diet may link to define function, composition and diversity of the gut microbiota.

Today, the multi-dimensional cluster analysis and principal components analysis are useful methods to study the fecal metagenomes of individuals (179). The fecal metagenomes can be clustered into three different enterotypes (175). Bacteroides (enterotype 1), Prevotella (enterotype 2) and Ruminococcus (enterotype 3) are examples for the three common genera (175). These major enterotypes are not dependent on nationality, body mass index, sex or age (175). Although, another study has claimed that enterotypes may be directly related with long-term dietary composition (180). Bacteroide*s*-related enterotype 1 has been linked with digestion of saturated fats and animal proteins, and Prevotella-related enterotype 2 may be related to carbohydrate diet such as simple sugars and fiber (175). However, little is known about whether enterotypes cause diseases, but it is suggests that changing long-term dietary patterns may affect enterotype status, nutrition and microbiome relationship, and pathophysiology

of an individual (175).

9.11 The Gut-Brain Axis

The human gut consists of a large and complex interconnected nervous net, named the enteric nervous system. The nervous system maintains physiological functions of the intestine, exchanging impulses between the intestine and the central nervous system by two pathways: upward (gut-to-brain) and downward (brain-to-gut) (181). This impulse exchange system is known as a gut–brain axis, which include a highly complicated loop of neurological reflexes (182). To regulate the intestine functions, the gut-brain axis communicates with the brain, gut, immune and endocrine systems (183). Disturbances of the gut-brain axis are connected with psychiatric symptoms such as anxiety and functional gastrointestinal disorders such as inflammatory bowel syndrome (184). Some neuroactive and neuroendocrine molecules such as GABA, serotonin, histamine, adrenaline and noradrenaline are produced by gut microbiota to connect the gut–brain axis (183,185). A metabolomics study suggest that conventional mice have 2.8 fold higher level of serum serotonin than germ free mice however synthesis of serotonin by gut microbes is not yet understood (185). GABA can be converted from glutamate by action of some gut microbes such as Lactobacilli and it suppresses the neurotransmitters in the central nervous system and works as a painkiller (186-191). When *Lactobacill rhamnosus* JB-1 was introduced into the mice, they changed the receptors of GABA in the mice brain and facilitated suppress stress-induced corticosterone and decreased depression related behavior and anxiety, although neurochemical and behavior features were disappeared in vagus nerve-dissected *Lactobacillus*-treated animals because vagus

nerve communicate between gut and brain (189). Visceral pain in the gut can be suppressed by intestinal microbes, which were identified in a colorectal distension model in Sprague–Dawley rats (190). The pain was suppressed in colonic distension rats when they were treated with *Lactobacill rhamnosus* ATCC 23272 for 9 consecutive days (192). Therefore, gut microbes link with nervous system and mental health and disorders.

9.12 Maternal and Childhood Obesity

Currently, overweight and obesity are major health concerns all over the world, but it is a preventable nutritional disorder. Obesity is believed to affect both mental and physical health and it is especially a risk factor for developing hypertension, cardiovascular diseases, diabetes, depression, orthopedic disorders and sleep apnea (193).

Development of obesity during childhood may cause more severe health problems when compared with adulthood obesity (194). Obese children between the ages of 10 to 13 years old have a 65% chance to develop obesity in their adulthood (194). In addition, each 1 kg increase in birth weight of newborn is associated with about a 5% chance to increase overweight in adolescence (195). Among other factors that can contribute to the development of childhood obesity, prenatal maternal obesity, maternal over nutrition, genetic influence and maternal gut microbiota are significant to define childhood obesity. In fact, overweight mothers may deliver overweight infants (196-199).

Pre-pregnancy obesity or increased weight gain during pregnancy may be linked with several maternal and child complications such as premature delivery, miscarriage and preterm rupture of the membrane (200,201). There is also a positive correlation between

pre-pregnancy BMI and cesarean delivery (202) because pre-pregnancy obesity also leads to development of gestational diabetes, which may concurrently large for the gestational age of infant (203,204). Healthy dietary pattern during pregnancy may lead to healthier outcome while preventing birth weight related abnormalities (205,206). Interestingly, maternal over nutrition related childhood overweight and obesity may be regulated by epigenetically (207,208). Since the availability of amino acids may decide DNA methylation in fetus development, maternal diet is an important factor in epigenetic regulation of fetal genome (209). Epigenetic DNA methylation, especially CpG methylation, which is a specific gene promoter in neonates, is associated with childhood obesity risk (210). Development of fetus adipose tissues, which also causes fetal over nutrition, is directly influenced by maternal over-nutrition as well as increase number of adipocytes in the fetus may persist and it is carrying rest of the their life (211).

Genetic influence is another important factor for developing childhood obesity (212). One systematic review suggested that they had clear evidence that childhood obesity is depends on genetic and common environmental factors; however the effect of common environment may gradually disappears during adolescence (212). In other words, genetic factors from childhood to adulthood have significant effects (212).

Another avenue for childhood obesity is the effect of gut microbiota. The human gastrointestinal tract provides surface to colonize 10^{13} to 10^{14} number of microbes, which are important as they are associated with health and disorders (213). The gut microbiota is associated with the development of obesity because it may lead to increase energy harvest from diet, changing gut transit time for facilitating short chain fatty acids absorptions, regulating host cellular signaling pathways related to lipogenesis, energy

storage and inflammation (193). Initially, relatively, lower abundance population of phylum Bacteroidetes and higher level of phylum Firmicutes may enhance to develop obesity (109,124,214). Although, recently, other studies revealed that higher level of phylum Bacteroidetes were isolated from obese cohorts (215,216).

Previous studies have stated that childhood obesity is strongly related with maternal weight rather than paternal weight (217). Another study has reported that maternal pre-pregnancy Body Mass Index (BMI) is positively associated with childhood obesity because composition and diversity of infants gut microbiota is altered based on the BMI of respective mothers (10). Bacteroides, Staphylococcus and Clostridium concentration of infants fecal samples are high and Bifidobacterium is low in mothers whose BMI are higher (10). The same study suggested that high Bifidobacterium concentrations of infant gut microbiota may control weight gain of infants (10). Overall, diversity and composition of gut microbiota define developing childhood obesity. However, available bacterial profile of gut microbiota is not always evidence to prove the difference between obese and normal weight human population (193).

10. Hypothesis and Objectives

10.1 Hypotheses

- There is a significant relationship between human breast milk microbiota and infant gut microbiota.
- Breast milk is a rich source and vehicle to introduce obesogenic maternal gut bacteria into an infant gut to initiate childhood obesity.

10. 2 Objectives

- This study aims to understand whether any significant relationships exist between human breast milk microbiota and the infant gut microbiota, and whether maternal obesity is an intervening variable.

- This study will focus on developing a method that allows for sufficient extract of bacterial DNA from human breast milk microbiota.

11. Materials and Methods

11. 1 Study Design and Collection of the Samples

This cross sectional study included 16 healthy (i.e. free of diseases) lactating women (Table 1.) from Winnipeg, Manitoba, Canada and their respective exclusively -breastfed infants aged 6 months or earlier (Table 2.). Selected mothers and infants did not use antibiotics, immunosuppressive medications, prebiotics and probiotics and infants were born via normal vaginal delivery. Breast milk samples (10ml) were collected, by the mothers utilizing proper sterile techniques, into a sterile tube by manual expression using sterile gloves. Prior to the collection, the nipples and mammary areola region were cleaned by soap and sterile water and first milk drops (500 µl) were discarded. Fecal samples (5 g) were collected into individual sterile feces containers from their respective infants. All the milk and fecal samples were kept under 4°C refrigeration until delivery to the laboratory. Samples were then stored at - 80°C and aliquots were stored at - 20 °C prior to DNA extraction.

11. 2 DNA Isolation from Milk and Fecal Samples

Prior to DNA extraction, 1ml of each human breast milk sample was centrifuged three times at room temperature at 10,000 x g for 10 minutes. The top layer of the supernatant, which was comprised of milk lipids, was removed each time. This procedure is subjective, thus how many cycle is needed for removing the lipids is depended on the amount of lipid in the milk samples. The total DNA was then purified and precipitated from isolated bacteria within the samples by using MasterPureTM Gram Positive DNA purification kit (Epicentre® Biotechnologies, Madison, WI, USA) according to the instruction by the supplier. However, the protocol was modified for obtaining maximum DNA concentration. Purified breast milk DNA extracts were stored at -20°C.

A samples size of 150mg of each individual infant feces was used for the DNA extraction process. To extract total DNA, Zymo Fecal DNA MiniPrep kit was used and followed method, which was recommended by the supplier, the Epicentre. Purified infant fecal DNA was stored at -20°C for later analysis of PCR and further downstream examinations.

11. 3 Gel Electrophoresis, Polymerase Chain Reaction (PCR) Assays and Sequencing

DNA samples of infant fecal and breast milk were normalized to 20 ng/µl, and quality checked by PCR amplification of the 16S rRNA gene using universal primers 27F (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as described by Khafipour et al. (218). Amplicons were verified by agarose gel electrophoresis.

The PCR was used to assess the presence of DNA from infant gut and breast milk microbiota targeting especially *Bacteroides, Staphylococcus and Bifidobacterium* in milk and fecal samples. For this purpose, the V3-V4 region of 16S rRNA gene was targeted for PCR amplification using a modified F338 primer (5´-AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTACTCCTACGGGAGGCAG-3´) for forward primer and modified barcoded R806 primers as described by Caporaso et al. (219). PCR reaction for each sample was performed in duplicate and contained 1.0 µl of pre-normalized DNA, 1.0 µl of each forward and reverse primers (10 µM), 12 µl HPLC grade water (Fisher Scientific, ON, Canada) and 10 µl 5 Prime Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial denaturing step at 94°C for 3 min followed by 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min in an Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) to remove primers, dNTPs and reaction components. PCR reaction conditions were optimized as follows: an initial denaturing step at 94°C for 3 min followed by 30 amplification cycles at 94°C for 45 sec, 62°C for 60 sec, and 72°C for 90 sec; finalized by an extension step at 72°C for 10 min. The V3-V4 libraries were then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (Invitrogen, NY, USA) and diluted to a final concentration of 5 pM, measured by Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). In order to improve the unbalanced and biased base composition of the generated 16S rRNA libraries, 15% of PhiX control library was spiked into each amplicon pool. Customized sequencing primers (Table 3.) were designed and added to

the MiSeq Reagent Kit V3 (600-cycle) (Illumina, CA, USA). All the primers used in this study were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA). The 150 and 300 paired-end sequencing reactions were performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

11. 4 Bioinformatics and statistical analysis

The FLASH assembler (220) was used to merge overlapping paired-end Illumina fastq files. All sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source software package QIIME (221). Assembled reads were demultiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with more than 3 consecutive bases with quality scores below 1e-5 were truncated, and those with a read length shorter than 75 bases were removed from the downstream analysis. Chimeric reads were filtered using UCHIME (222) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST (223) at 97% pairwise identity threshold. Taxonomies assignment of representative OTUs and alignment to Greengenes Core reference database (224) was performed using PyNAST algorithms (225). Phylogenetic trees were built with FastTree 2.1.3. (226) for further comparisons between microbial communities. Within community diversity (α-diversity) was calculated using QIIME scripts. Alpha rarefaction curve was generated using Chao 1 estimator of species richness (227) with ten sampling repetitions

at each sampling depth. An even depth of approximately 14,500 sequences per sample was used for calculation of richness and diversity indices. β-diversity between microbial communities were compared using weighted and un-weighted UniFrac distances (228) based on phylogenetic differences. Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using PRIMER V6 software (229) and permutational multivariate analysis of variance (PERMANOVA) (230) was used to calculate *P*-values and test for differences between microbial communities.

12. Results

The richness of microbiota were observed in all groups of normal-weight, overweight and obese lactating women' breast milk and their respective infants gut (Tables 4, 5, 6, 7). Because of less samples size (obesity I (n= 2) and obesity II (n=1)) in obesity groups, all subjects were divided into two groups; normal-weight and overweight lactating women and their normal-weight and overweight lactating women' respective infants. All groups of normal-weight and overweight lactating women' breast milk and their infants' gut microbiota richness were high (Figures 1 and 2).

Figure 1. Stack Bar chart representing the Operational Taxonomic Units (OTUs) of breast milk bacterial populations of normal-weight and overweight lactating women.

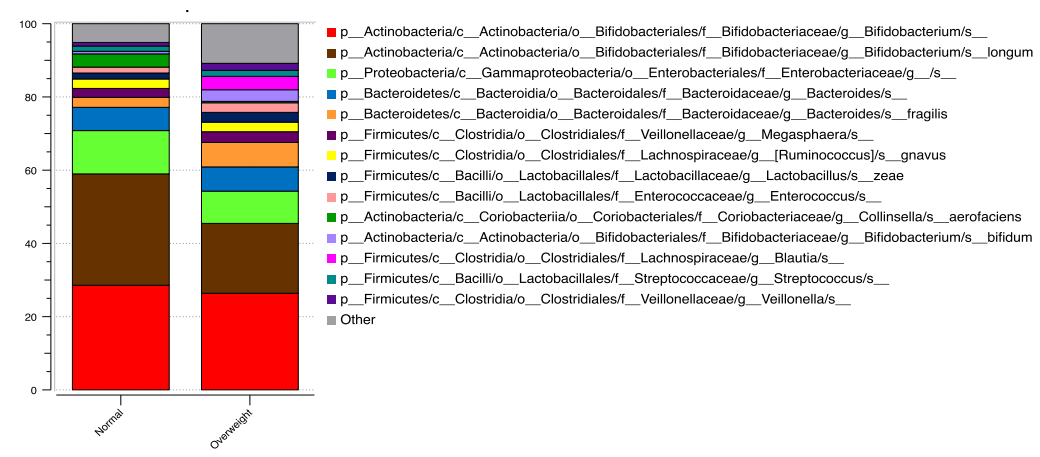

Figure 2. Stack bar chart representing the Operational Taxonomic Units (OTUs) of infant fecal bacterial populations associated with the infants of normal-weight and overweight lactating women.

Principal Coordinates Analysis (PCoA) was used for visualization of the data present in the beta diversity of breast milk and infant gut microbiota. There were no significant statistical relationship exist ($p > 0.05$) between normal-weight and overweight group of lactating women breast milk microbiota (Figure 3) and among normal-weight and overweight groups lactating women' respective infants' gut microbiota (Figure 4).

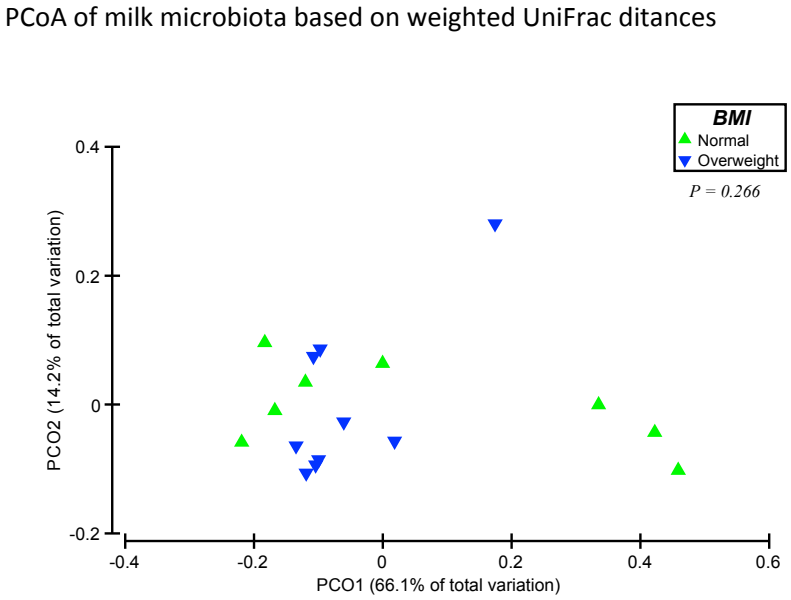

Figure 3. The PCoA analysis of breast milk microbiota has been performed based on the UniFrac distances of microbial communities and the p-values have been calculated based on PERMANOVA analysis.

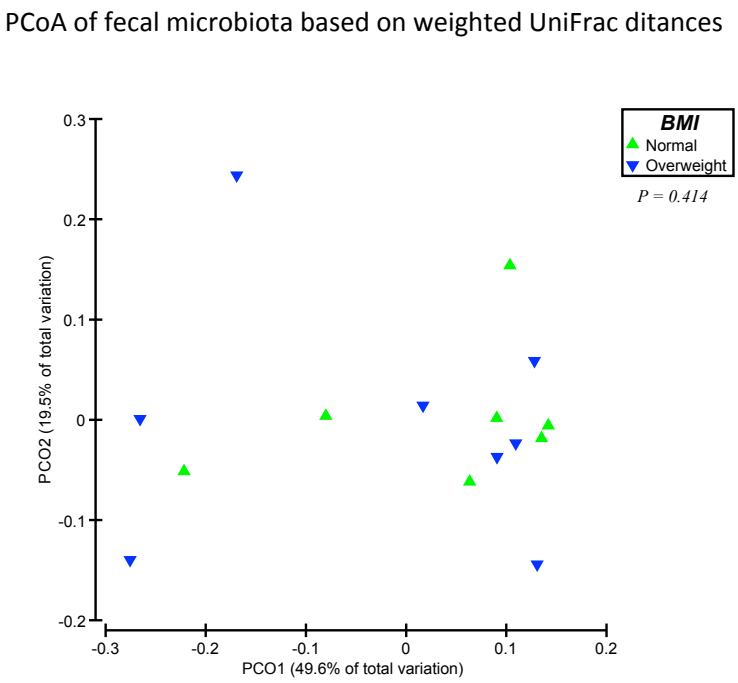

Figure 4. The PCoA analysis of infant gut microbiota has been performed based on the UniFrac distances of microbial communities and the p-values have been calculated based on PERMANOVA analysis.

Dominant bacterial phylum of breast milk microbiota was Proteobacteria (Figure 5) and its percentage was relatively high in both normal-weight (84%) and overweight (58%) lactating women groups. Firmicutes phylum percentage also increased in overweight (36%) lactating women' of breast milk microbiota compared with normal-weight (13%). Bacteroidetes and Actinobacteria bacterial phyla appeared same percentage in both groups of lactating women' breast milk. Although, there were no significant statistical relationship in dominate breast milk bacterial phyla ($p > 0.05$) between groups of normal-weight and overweight lactating women.

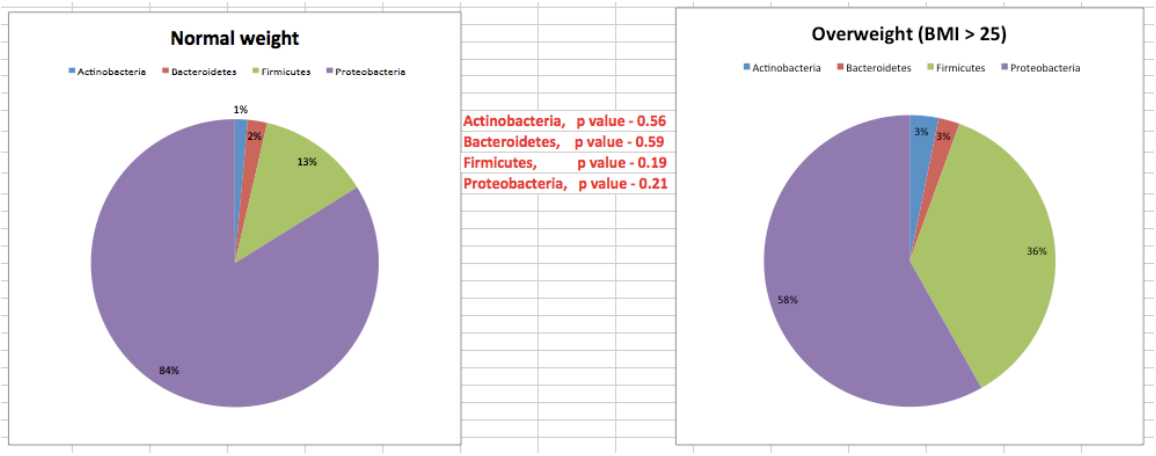

Figure 5. Pie charts for predominant bacterial phyla in breast milk microbiota of normal-weight and overweight lactating women.

It was observed that Actinobacteria was the predominant phyla present in all subjects of infants' gut microbiota (Figures 6). The Actinobacteria phylum percentage increased in overweight lactating women' infants gut microbiota (49%) compared with normal-weight (46%) but Proteobacterial percentage was less in overweight group (11%) that compared with normal-weight group (23%) infants' gut microbiota. Fermicutes and Bacteroidetes phyla were high (26% and 14% respectively) in overweight lactating women' infants gut microbiota compared with normal-weight women' infants' gut microbiota (23% and 8%). However, there were no significant statistical differences in major gut bacterial phyla ($p > 0.05$) between groups of normal-weight and overweight lactating women' respective infants.

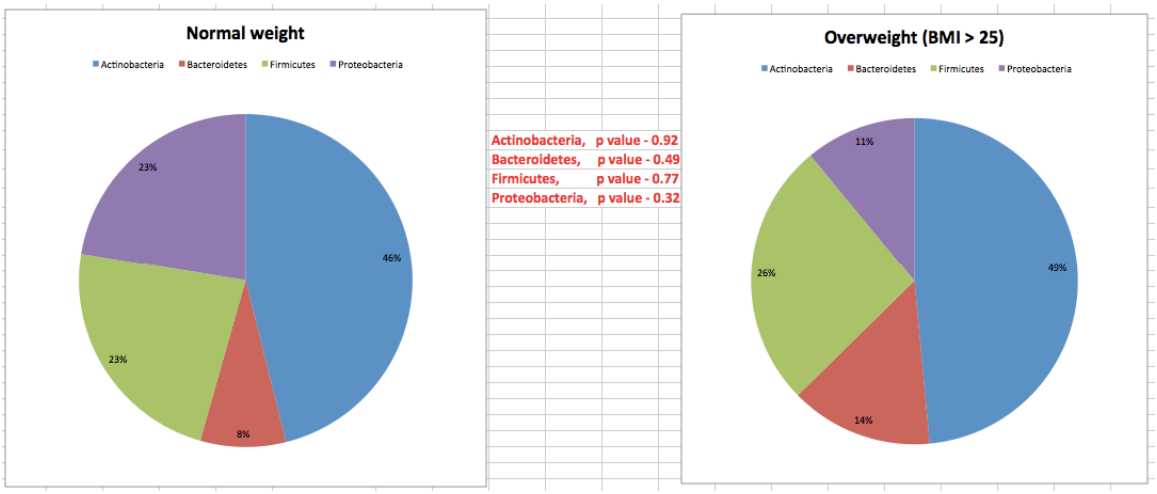

Figure 6. Pie charts for predominant bacterial phyla in infant gut microbiota of normal-weight and overweight lactating women.

Extraction and purification of bacterial DNA from breast milk was not a simple task. Removing fat layer from breast milk, protein precipitation and bacterial cell wall lysis are critical steps, which interfere the bacterial DNA concentration in the breast milk. Existing methods of commercially available 3 bacterial DNA extracting kits (QlAamp DNA stool kit, Zymo DNA Purification kit and MasterPure Purification kit) were given poor DNA concentration. Therefore, methodology of all 3 kits were modified as well as combined kits together and tested. Relatively, high bacterial DNA concentration was obtained from MasterPure Purification kit. Therefore, initially selected 33 breast milk samples were undergone by modified method of MasterPure Purification kit (Table 8). However, there were only 16 breast milk samples that provided enough bacterial DNA concentrations to carried out further downstream investigation such as PCR and sequencing.

13. Discussion

This pilot study observed that Actinobacteria and Proteobacteria were predominant bacterial phyla in all, including normal-weight and overweight mothers of infants gut microbiota and their breast milk microbiota respectively. This result matched with previous studies related to infant gut microbiota (231) although this study observed that there were no significant relationships among infant of normal-weight and overweight groups as well as groups of normal-weight and overweight lactating women. It has been evidenced that percentage of Fermicutes phyla increased in overweight group of infants gut microbiota as well as overweight group of lactating women' breast milk microbiota. Bacteriodetes phylum percentage was high only in infant gut microbiota. These obesogenic bacteria are involving increasing the energy recovery from the diet, increasing fermentation of indigestible complex plant polysaccharides, changing gut transit time, modulating host signalling pathways involved in lipogenesis, energy storage, and inflammation as well as enhancing gut ecosystem favour to phylum Bacteroidetes and dropping in the content of genus Bifidobacterium (193). Therefore, the abundance Fermicutes and Bacteroidetes phyla may be associated with pre-pregnancy overweight and this obesogenic bacterial profile may then be introduced into their infants gut through the breast milk. However, there were no significant statistical relationship that relative abundance of Fermicutes and Bacteroidetes phyla may introduce into their infants gut through the breast milk within 6 months of lactating period.

In addition, this pilot study was not able to analyze statistical relationship between normal-weight and overweight lactating women' breast milk microbiota with their

respective infants' gut microbiota because there was no previous studies explained that breast milk and infant gut microbiota's relationship statistically to follow. Therefore, there is no definitive statistical method to analyze relationship. Furthermore, hypothesized such changes in the microbial environment of an infant's gut and the introduction of specific obesogenic bacterial strains may not support a theory that children can be predisposed to conditions of overweight and obesity which they may carry on into adulthood. In other words, this pilot study is impossible to answer the main objective; it is to understand whether any significant relationships exist between the human breast milk and the infant gut microbiota, and whether obesity is an intervening variable.

One of objective of this study is develop a good method for extraction and purification of DNA from breast milk because all other steps until identifying the bacterial strains depend on quality bacterial DNA. For this study, we used QlAamp DNA Stool kit, Zymo Fecal DNA Mini kit and MasterPure Gram Positive DNA Purification kit for milk DNA extraction and purifications. However, QlAamp DNA Stool kit and Zymo Fecal DNA Mini kit were not able to give enough bacterial DNA concentration even the protocols were modified (Table 8). When we adhered to the protocol of MasterPure Gram Positive DNA Purification kit we could not extract high DNA concentrations. Therefore, we modified the methodology such as removed fat layer until sample is cleared, increased incubating temperature up to 95°C, increased Proteinase K up to 1.5µl and MPC Protein Precipitating Reagent up to 200µl. Removing fat layer is a critical step as lipid concentration in breast milk is variable from woman to woman. Therefore, fat layer removing cycle was changed base on lipid concentration of milk.

14. Conclusion

This research modified existing methodology to extract bacterial DNA from human breast milk microbiota. This method enabled a comprehensive observation of the entire microbiota of human breast milk samples rather than singular bacterial strains. By now being able to look at the entire bacterial profile of human breast milk and infant fecal samples, similarities and differences in bacteria were more easily identifiable.

Interestingly, this study observed that there was no significant statistical relationship of breast milk microbiota in between normal-weight and overweight lactating women' group. In addition, there was no significant statistical relationship of gut microbiota among normal-weight and overweight lactating women' respective infants.

Overall, this pilot study was not able to make a meaningful conclusion due to small sample size and difficulties to exclude other causative factors, which intervene obesity such as genetic and epigenetic as well as over-nutrition status of lactating women.

15. Future Perspectives

Some external factors of pregnant women such as medications, diseases, stress and exposure to heavy metals may be important factors that may influence infant's physical and mental growth and development in later life. A few studies have focused on how these maternal factors impact the gut microbiota of the infant. In animal studies, stressed mother monkeys delivered infant monkeys that had lower number of Bifidobacterium and Lactobacillus than control infants, born from non-stressed mothers (232). Therefore, a new field of research may be to examine how mental health factors of lactating women

can impact and alter the infant gut microbiota.

Further development of sequencing technologies will open the fields for scientists to understand the human microbiota and its influences for neurological disorders. For example, liver failure may change intestine microbiota and cause gut inflammation due to barrier dysfunction of the intestine, which can then lead to hepatic encephalopathy (192).

Gut microbiota diversity and composition are associated with developing obesity as well as alteration of maternal gut microbiota is strongly depended on pre-pregnancy BMI and pre-pregnancy BMI is positively correlated with childhood obesity (10). Therefore, modification of maternal gut microbiota during the prenatal and natal periods may reach new possibilities and directions for the prevention, treatment and management of childhood obesity and further avoid complications related to obesity in children later in life.

16. Limitation

There were no previous studies, which observed relationship between breast milk and infant gut microbiota. Therefore, this pilot study was impossible to find statistical relationships among breast milk and infant gut microbiota.

Removing the fat layer from breast milk samples is a critical first step in bacterial DNA extraction and purification process. Removing the fat layer of centrifuged breast milk samples was completely proved to be a difficult task even after three attempts. Lipid concentration of breast milk is different from participant to participant and it is therefore subjective. Protein precipitation is also an important step as presence of protein also

affects the purity of the DNA. Bacterial cell lysing is another important step. Therefore, the development of a more workable method for protein precipitation from samples and a step of bacteria lyse are needed.

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18. Appendix

Table.1 Pre-pregnancy BMI and breast milk DNA concentrations of participated of lactating women.

No.	Participant number	Age of mother	Mothers categorized on pre-pregnancy BMI	Gestational weeks	Birth Length cm	Birth Weight g
1.	03	22	Normal Weight (18.5 - 24.9)	41	46	2750
2.	08	40		40	58	3995
3.	12	33		38	52	2680
4.	21	28		40	57	4672
5.	28	32		39	53	3492
6.	34	33		39	50	3175
7.	37	29		39	51	3628
8.	41	37		42	51	3600
9.	19	27	Over Weight (25.0 – 29.9)	41	53	4626
10.	22	27		39	54.5	3683
11.	26	31		39	52.5	3174
12.	33	38		40	50	3685
13.	35	34		39	53	3628
14.	32	29	Obesity I (30.0 – 34.9)	40	51	3515
15.	51	32		38	53	3600
16.	30	30	Obesity II (35.0 – 39.9)	40	49.5	3345

Table.2 Anthropometric measurements of participated respective infants

No.	Participant Number	BMI	Pre-pregnancy Weight on BMI	DNA ng/µl	260/280	260/230
1.	03	22.00	Normal Weight (18.5 - 24.9)	15.5	1.81	0.26
2.	08	22.00		591.2	1.84	1.06
3.	12	22.10		47.2	1.67	0.40
4.	21	19.20		25.6	1.67	0.32
5.	28	24.40		44.4	1.74	0.39
6.	34	21.60		25.6	1.81	0.33
7.	37	21.70		73.2	1.66	0.49
8.	41	22.80		49.1	1.76	0.33
9.	19	29.60	Over Weight (25.0 – 29.9)	50.1	1.59	0.31
10.	22	25.80		23.5	1.69	0.30
11.	26	26.20		30.9	1.71	0.32
12.	33	25.90		37.0	1.75	0.33
13.	35	28.10		80.3	1.78	0.56
14.	32	32.30	Obesity I (30.0 – 34.9)	390.4	1.77	1.10
15.	51	31.00		71.5	1.65	0.44
16.	30	36.50	Obesity II (35.0 – 39.9)	30.6	1.58	0.30

Table 3. Customized sequencing primers added to Miseq V3 reagent kits to support paired-end sequencing reactions.

Primer name	**Primer sequence (5′→3′)**	**Cartridge's well #**	**Volume (µl)**	**Concentration (µM)**
Read 1 (Forward)	TATGGTAATTGTACTCCTACGGGAGGCAG	12	4	100
Read 2 (Reverse)	AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT	14	4	100
Index	ATTAGAWACCCBDGTAGTCCGGCTGACTGACT	13	4	100

Table 4. The rarefaction graph showing information on the richness of the microbial communities of breast milk microbiota of normal-weight, overweight and obese lactating women as well as providing an overview on the sequencing depth and coverage.

Rarefaction graph: Chao1 estimates of species richness (milk microbial communities)

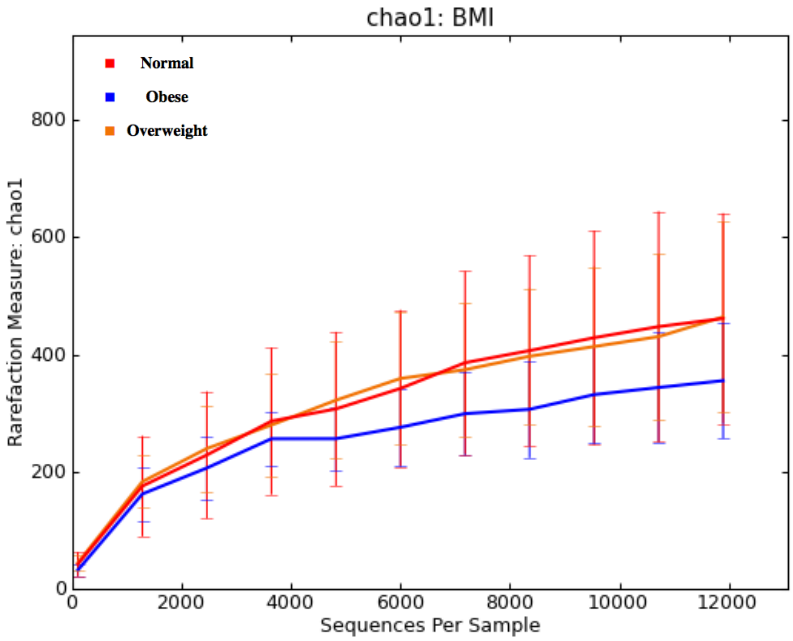

Table 5. The rarefaction graph showing information on the richness of the microbial communities of infant gut microbiota of normal-weight, overweight and obese lactating women as well as providing an overview on the sequencing depth and coverage

Rarefaction graph: Chao1 estimates of species richness (fecal microbial communities)

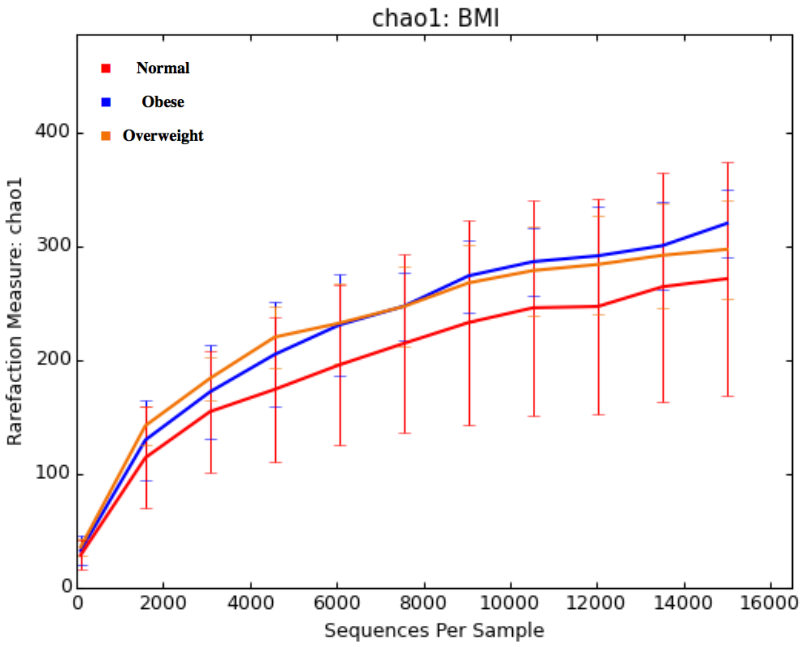

Table 6. Stack Bar chart representing the Operational Taxonomic Units (OTUs) of breast milk bacterial populations of normal-weight, obese and overweight lactating women.

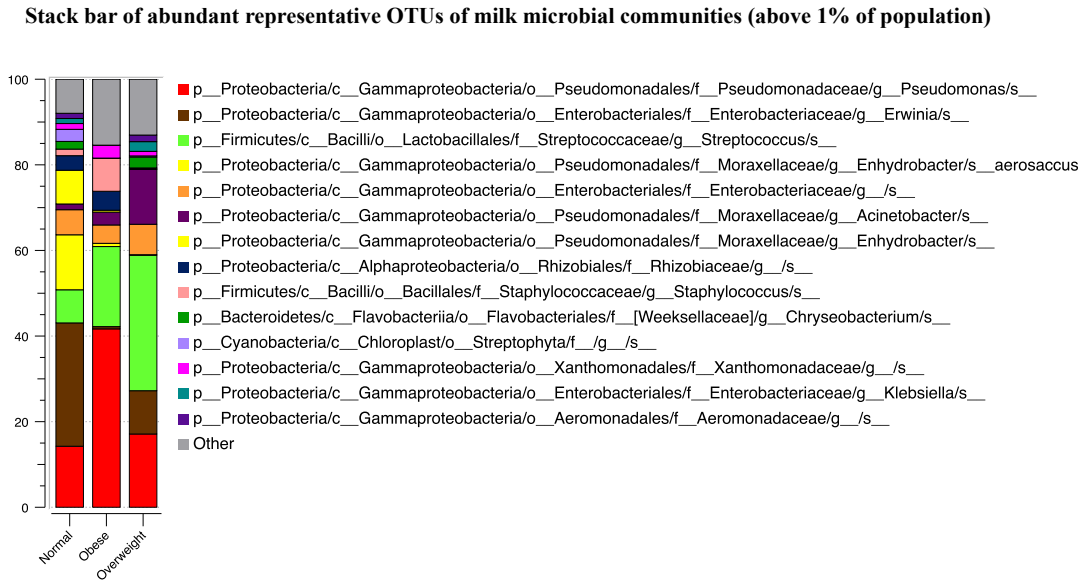

Table 7. Stack bar chart representing the Operational Taxonomic Units (OTUs) of infant fecal bacterial populations associated with the infants of normal-weight, obese and overweight lactating women.

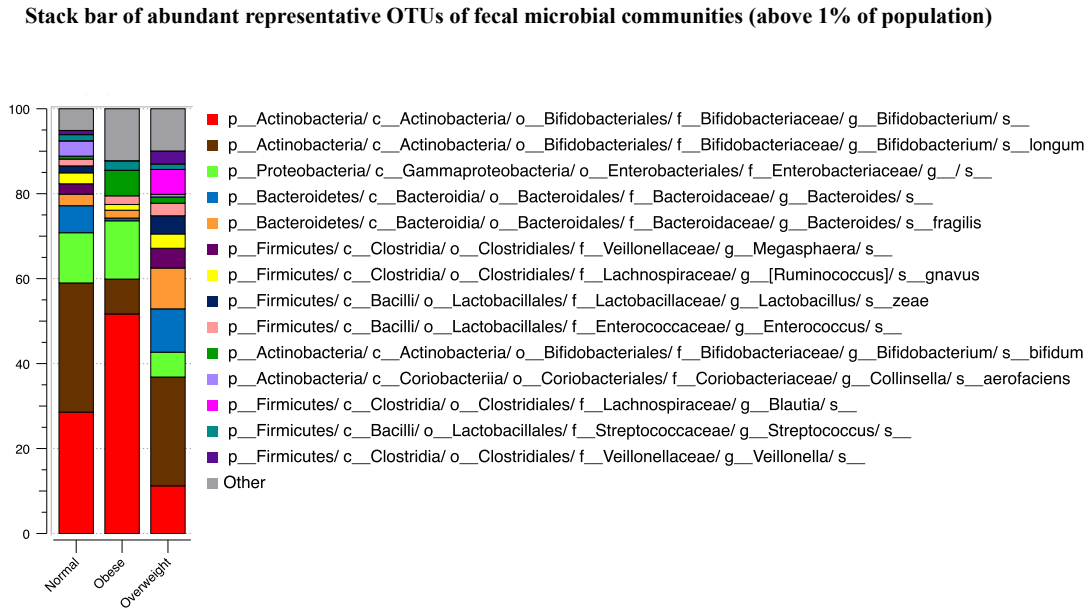

Table 8. Chart explains the changed methodology of existing method of MasterPure Purification kit.

Name of Kits	Modified Methodology	How	Results
➢ QIAamp DNA Stool kit ➢ Zymo Fecal DNA Mini kit ➢ MasterPure Gram Positive DNA Purification kit	Increased sample volume	0.5, 1, 1.5 and 2µl	Failed
	Increased incubating temperature	70°C —> 95°C	
	Increased centrifugation speed & doubled the time	5000 —> 10000rpm	
	Increased Proteinase K	0.5, 1, 1.5, 2µl	
	Increased all other reagents proportionately to the increased volume of sample		
	Added bead beater steps		
➢ MasterPure Gram Positive DNA Purification kit	Best sample volume	1ml	Developed the method
	Best incubating temperature	95°C	
	Best centrifugation speed	10000rpm	
	Best Proteinase K volume	1.5µl	
	Best MPC Protein Precipitating Reagent	200µl	
	Fat layer removing	3- 5 times	