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**COLLEGE OF HEALTH SCIENCES**

**SCHOOL OF MEDICAL SCIENCES  
DEPARTMENT OF CLINICAL MICROBIOLOGY**

**BACTERIAL CONTAMINATION OF INFANT FORMULA AND EXPRESSED  
BREAST MILK**

**A THESIS SUBMITTED TO THE DEPARTMENT OF CLINICAL MICROBIOLOGY,  
KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, IN PARTIAL  
FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE (CLINICAL MICROBIOLOGY)**

**BY**

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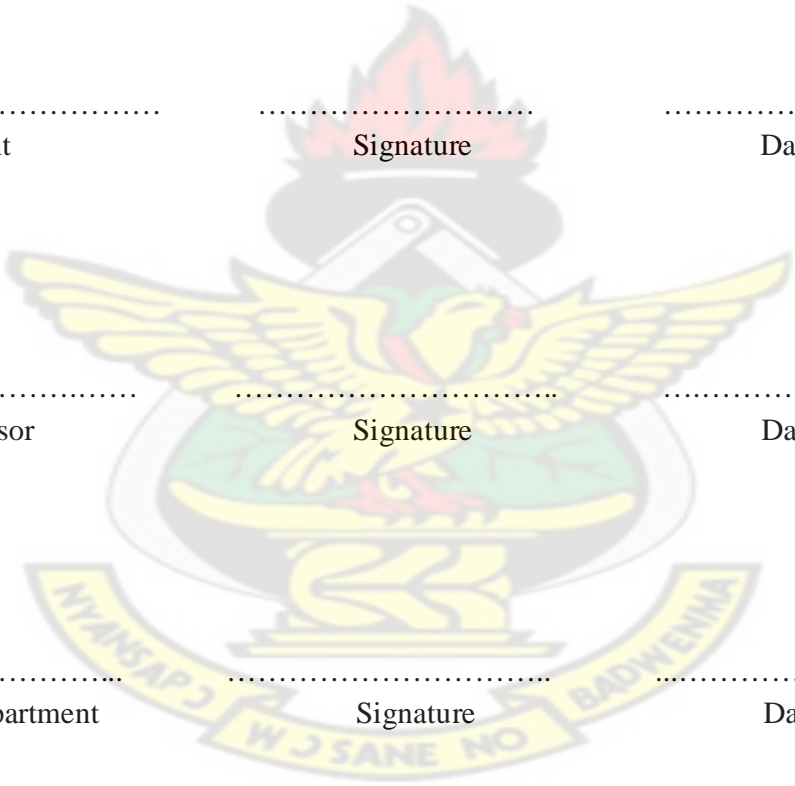
**AUGUST, 2011**

## DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgment has been made in the text.

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The logo of the University of KwaZulu-Natal (KNUST) is centered in the background. It features a yellow eagle with its wings spread, perched on a shield. Above the eagle is a red and orange flame. Below the eagle is a yellow banner with the text 'NYANSAPHO WJSANE NO BADWENNA' in black. The shield itself has a green base and a yellow top section with a red and white design.

## **DEDICATION**

This dissertation is dedicated to my Heavenly Father, whose amazing grace, abundant goodness and extravagant love has kept me through these years; and to my loving mother, Rev. Beatrice Osei, whose encouragement and love has been a burning blaze in my heart.

# KNUST



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## ABSTRACT

There is a high mortality of infants in Sub-Saharan Africa and diarrhoeal illnesses contribute significantly to this mortality. Cases of diarrhoeal infection in children have been epidemiologically linked to the consumption of contaminated feeds. The aim of this study was to investigate bacterial contamination of prepared formula and expressed breast milk. Seventy-two samples of prepared formulae on lying-in wards and nurseries, Mother and Baby Unit (MBU) and medical children's ward of Komfo Anokye Teaching Hospital and 34 samples of breast milk from lactating mothers on MBU were collected for the study. These milk samples were cultured for microbial contamination. Expressed breast milk samples (EBM) were stored at room temperature and cultured at zero ( $T_0$ ), three ( $T_3$ ), six ( $T_6$ ) and nine ( $T_9$ ) hours after collection to determine bacterial propagation over time. 85% (61/72) of the prepared formulae were contaminated primarily with *Acinetobacter* sp. (85.2%), *Klebsiella pneumoniae* (34.4%), *Enterococcus* sp. (18%) and *Staphylococcus epidermidis* (13.1%). Only 2 out of the 72 prepared formula had plate counts of bacteria greater than the maximum acceptable limit of  $1.0 \times 10^5$  CFU/ml. *Acinetobacter* sp., *Klebsiella pneumoniae*, *Enterococcus* sp. and *Bacillus* sp. were the isolates from these two samples. Bacterial contamination was found in 97.1% (33/34) of EBM. The dominant bacteria isolated from EBM were *Staphylococcus epidermidis* (82.4%), *Klebsiella pneumoniae* (20.6%), *Acinetobacter* sp. (14.7%) and *Staphylococcus aureus* (10%). The numbers of organisms in EBM during the 9-hour period of storage were consistently low. The majority of EBM (85.3%) had plate counts of bacteria less than  $1.0 \times 10^5$  CFU/ml. The findings indicate that control measures to eliminate microbial contamination of prepared formula on the wards were not adequate. However, safety of prepared formula could be assured as plate counts of organisms were less than the maximum acceptable limit of organisms. The results also demonstrate that it is possible to use EBM for baby's consumption up to 6 hours of storage at room temperature (mean = 27.9°C). It is recommended that stringent measures should be put in place to ensure that health staff on the pediatric wards comply with basic principles of good hygiene. Mothers should be educated on the possibility of using unprocessed breast milk to feed babies up to 6 hours after expression if access to refrigeration is unavailable.

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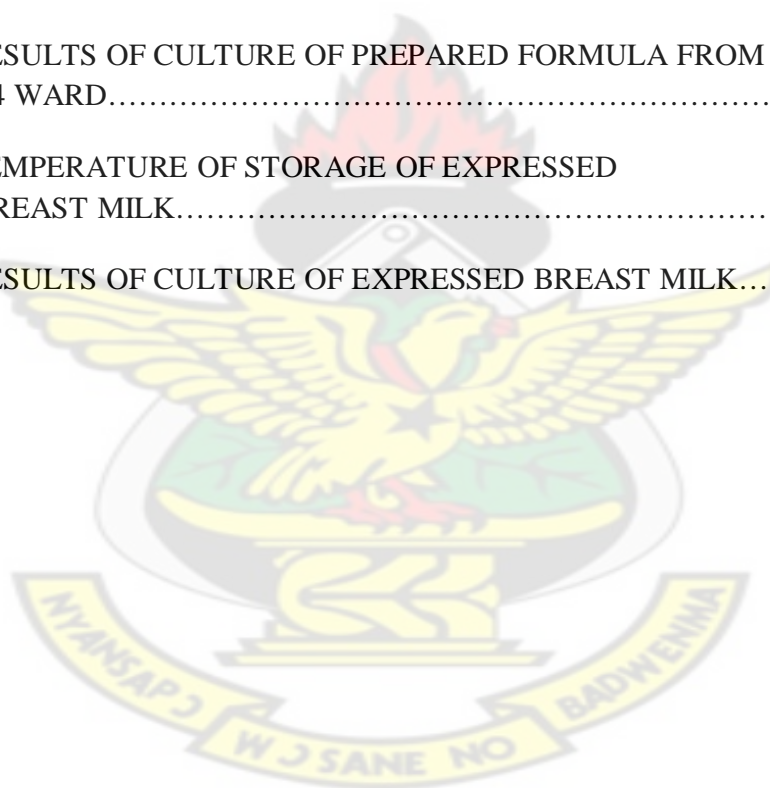
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## CHAPTER ONE

### INTRODUCTION

#### 1.1: BACKGROUND OF THE STUDY

Each year more than 10 million infants and young children in developing countries die before reaching age five (WHO, 2002). Of these 10 million, 70% of the children die of infections like acute respiratory infections, diarrhoea, measles and malaria or from malnutrition (WHO/UNICEF, 2001). Diarrhoea remains one of the commonest illnesses among infants, and is also one of the major causes of mortality in developing countries (Mihirshahi *et al.*, 2008). Infants who are not breast-fed exhibit a higher incidence and severity of diarrhoeal illnesses; demonstrating the connection between infant feeding practices and the risk of diarrhoea (Jason, 1984). About 55% of infant deaths yearly caused by diarrhoeal diseases and acute respiratory infections may be related to inappropriate feeding practices (WHO, 2002).

According to current United Nations' recommendations, infants should be exclusively breastfed for the first six months of life, and thereafter should receive appropriate complementary feeding with continued breastfeeding up to two years or beyond (WHO, 2004). Breast milk expression is a very common practice. It is associated most strongly with maternal employment, a recognized barrier to breastfeeding (Labiner-Wolfe *et al.*, 2008). Often, mothers must express their milk so it can be fed to their babies in their absence or at a later time (Tully, 2000). However, storage conditions are often not optimal (Hamosh *et al.*, 1996).

The protective effect of breast-feeding against infection has been recognized for many years, particularly in relation to enteric diseases (Jones *et al.*, 1979). Conserving the anti-infective properties of breast milk and providing milk that is not contaminated by pathogenic bacteria, however, pose practical problems of collection and storage.

Conversely, there are a number of infants who will not enjoy the benefits of breastfeeding in the early months of life or for whom breastfeeding will not occur or will stop before the recommended duration of two years or beyond and may have to depend on formula feeding (WHO, 2004). Infant formula-feeding involves many critical steps during handling, storage, preparation and cleaning, putting great demand upon good hygiene if contaminated and unsafe feeds are to be avoided (Henry *et al.*, 1990).

Marino *et al.* (2007) investigated the potential for microbial contamination during the in-hospital preparation of powdered infant feed and found that there was significant contamination even when preparation occurred in a controlled environment. Several studies have shown high bacterial contamination of milk feeds prepared for infants, especially in feeding bottles (Morais *et al.*, 2005) and contamination is often associated with poor hygiene, unclean feeding utensils and prolonged periods of storage (Imong *et al.*, 1995). *Enterobacter sakazakii* and *Salmonella* sp. have been reported in infant formula and the former has been implicated in several cases of infection and illness (FAO/WHO, 2004).

Consumption of contaminated powdered infant formula has been epidemiologically linked to cases of infection (Mullane *et al.*, 2007). Neonates up to four weeks of age and pre-term, low birth weight or immuno-compromised infants are at greatest risk (Mullane

*et al.*, 2007). Documented cases of illness have occurred in both the inpatient and outpatient settings (WHO/FAO, 2007). However, there is grave concern regarding the safety and efficacy of preparing infant formula, especially in a hospital setting as resultant bacterial contamination cause enteric infections, especially in premature, immuno-compromised and sick infants (Marino *et al.*, 2007).

Milk and milk products are excellent media for multiplication of potentially pathogenic bacteria (Agostoni *et al.*, 2004) and so may represent an important source of infectious agents to infants. Infant formula is not sterile, even prior to breaking the manufacturers' seal due to manufacturing processes and constraints (Drudy *et al.*, 2006). Therefore, inadequate conditions of preparation and handling of infant formula could pose a considerable risk to infants. Moreover, expressed breast milk may constitute a threat to the health of infants if unacceptable levels of microorganisms are present on storage.

The United Nations' Millennium Development Goal (MDG) 4 is aimed at reducing mortality of children less than 5 years by two-thirds between 1990 and 2015 (Black *et al.*, 2003). According to UNICEF, Ghana has seen a slow progress of reduction in mortality of children; recording an infant mortality rate (under 1) of 47 per 1,000 live births in 2009 compared with 76 per 1,000 live births in 1990 and an under -5 mortality rate of 69 per 1,000 live births in 2009 compared with 120 per 1,000 live births in 1990 (UNICEF, 2011). These marginal improvements with this MDG in Ghana has made research into problems associated with feeding of infants, such as microbial contamination of infant food, very crucial as these directly affect child health and survival.



## **1.2: PROBLEM STATEMENT**

There is a high mortality of infants in Sub-Saharan Africa and diarrhoeal illnesses contribute significantly to infant mortality (Black *et al.*, 2003). Most illnesses from bacterial infections have been linked to microbial contamination of infant foods. Faecal bacteria in infant food are well known as a major cause of illness in infants (Motarjemi *et al.*, 1993). Breast milk, the recommended food for infants, may pose a threat to their health if pathogenic bacteria or unacceptable levels of microorganisms are present on storage. Furthermore, contamination of infant formula by microorganisms has become a problem in developing countries. Inappropriate feeding practices are major contributing factors to high morbidity and mortality among infants and young children in developing countries as some of these practices facilitate contamination of foods by microorganisms (WHO, 2002).

## **1.3: JUSTIFICATION FOR THE STUDY**

Ghana's progress in achieving a reduction in the mortality of children less than 5 years by two-thirds between 1990 and 2015 as part of United Nations' MDGs, though encouraging, is worrying. Ghana is one of the countries with the highest rate of under-5 mortality in the world. For every 100 children born in Ghana, 12 die before their fifth birthday (Ackon, 2010).

Infectious diseases such as diarrhoea and acute respiratory infection continue to be the main cause of mortality and morbidity in infants aged less than one year in developing countries (Mihirshahi *et al.*, 2008). With increasing practice of breast milk expression, there is concern over safety of expressed milk kept for feeding babies by caregivers in the absence of lactating mothers.

At the Komfo Anokye Teaching Hospital (KATH), there is no information on the prevalence of microbial contamination and safety of formula feed prepared on the wards. These issues necessitated this research into infant formula-feeding and safety of expressed breast milk since consumption of unsafe foods has been epidemiologically linked with cases of diarrhoeal infection.

#### **1.4: RESEARCH OBJECTIVES**

##### **1.4.1: General Objective**

To investigate bacterial contamination of prepared infant formula and expressed breast milk.

##### **1.4.2: Specific Objectives**

- ☐ To identify microorganisms associated with bacterial contamination and determine prevalence of bacterial contamination of prepared infant formula and expressed breast milk
- ☐ To determine the length of storage within which expressed breast milk is safe for consumption by baby
- ☐ To describe handling and storage of prepared infant formula and offer suggestions on handling and storage that could reduce bacterial contamination of the formula and expressed breast milk if necessary

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1: INFANT BREASTFEEDING

Breastfeeding is the normal way of providing young infants with the nutrients they need for healthy growth and development. Virtually all mothers can breastfeed, provided they have accurate information, and the support of their family, the health care system and society at large. Breastfeeding is a major contributor to child health by protecting the infant and child against infections and death (WHO, 2011).

Exclusive breastfeeding, which means feeding an infant with only breast milk with no additional foods or liquids, is recommended up to 6 months of age, with continued breastfeeding along with appropriate complementary foods up to two years of age or beyond (WHO, 2011).

##### 2.1.1: BENEFITS OF BREASTFEEDING

###### 2.1.1.1: Protective factors and flora

In a breastfed infant, *Lactobacillus bifidus* is the normal flora of the intestinal tract while the number of other flora like Gram negative bacteria is small (Yoshioka *et al.*, 1983). The *Bifidobacterium* and *Lactobacillus* in the breastfed infants' gastrointestinal tract produce acetate and lactate which lowers the pH. The lower pH and other substances excreted by the bacteria inhibit some Gram positive and Gram negative bacteria. These bacteria also detoxify ammonia and other amines and activate the immune system. This helps fight bacteria that cause disease (Chierici, 2003). However, the protective factors

in breast milk vary depending on the pathogens the infant is exposed to and on the chronological age and maturity of the infant (Lewis-Jones *et al.*, 1985).

#### **2.1.1.2: Reduced infectious diseases and death**

It was noted in the early 1970's that infants in the developing world who were fed formula had a much higher death rate than infants who were fed breast milk (Arifeen, 2001). This was due to malnutrition and recurrent infectious diseases. In the mid 1990's, exclusive breastfeeding was linked to decreased death rates due to acute respiratory infections and diarrhea in infants aged 1 to 11 months in Bangladesh when compared to infants who were partially breastfed (breastfed with the addition of other milks, and/or supplementary foods) (Arifeen, 2001).

In a study designed to evaluate whether the time of breastfeeding initiation and the type of breastfeeding practiced were associated with risk of neonatal mortality in Ghana, the risk of neonatal death was fourfold higher in infants given milk-based fluids or solids in addition to breast milk. There was a marked increasing risk of neonatal mortality with delayed breastfeeding initiation from hour 1 to day 7. Initiation after day 1 was associated with a 2.4 fold increase in mortality risk. The study concluded that 16% of neonatal deaths can be prevented if all infants are breastfed from day 1 and 22% can be prevented if breastfeeding is initiated during the first hour (Edmond *et al.*, 2006).

In a pooled analysis of data from Brazil, Pakistan and the Philippines done by WHO's Collaborative Study Team, the effect of not breastfeeding on the risk of death due to infectious diseases was evaluated. The results showed that protection provided by breast milk steadily declined with age. (WHO, 2000). In a population-based case-control study

of infant mortality in Brazil, results showed that completely weaned (no breast milk) infants had 14.2 times a greater risk of death from diarrhoeal diseases than infants not receiving any supplementation with replacement milk (Victoria *et al.*, 1987).

In a longitudinal study of feeding practices and morbidity from infectious diseases among infants in Peru, the incidence and prevalence rate of diarrhoea among infants aged less than 6 months given nothing else than breast milk, were less than among infants given replacement milk and other fluids in addition to breast milk (Brown *et al.*, 1989).

Another longitudinal study in the Philippines examined the relationship between breastfeeding and diarrhoeal morbidity and age-specific effects of infant feeding patterns. Supplements of water, teas and other fluids not containing nutrients in addition to breast milk increased the risk of diarrhoea two or three times compared to exclusive breastfeeding. (Popkin *et al.*, 1990).

A meta-analysis of full term infants in 18 prospective cohort studies showed that children with a family history of atopic disease who were exclusively breastfed for at least 3 months had a 42% reduction in the risk of atopic dermatitis compared to those breastfed for less than 3 months (Ip *et al.*, 2007 ).

Exclusive breastfeeding up to 4 months of age was associated with a reduced risk of asthma diagnosed by a physician (with an odds ratio of 1.25); a reduced risk of wheezing at 5 years of age (OR: 1.31); and a reduced risk of sleep disturbance due to wheezing at 5 years of age (OR: 1.42) (Oddy *et al.*, 1999 ).

### **2.1.1.3: Breastfeeding and Premature Infants**

Premature infants fed with their mother's milk have been found to have decreased incidences of sepsis, meningitis, and necrotizing enterocolitis compared to premature infants fed formula. These infants are discharged earlier than formula fed infants (Schanler, 1995; Hylander *et al*, 1998).

### **2.1.1.4: Nutrients and energy**

Due to its exclusive composition including nutrients, non-nutrient growth factors, immune factors, hormones and other bioactive components, breast milk is well acknowledged to be the ideal food in providing the needs of the infants during the first six months of life (Oddy, 2002). It can provide the total needs for energy and nutrients for the first six months life, and often continues to provide half of all requirements during the following six months and one-third during the child's second year (WHO, 2001).

### **2.1.2: GLOBAL BREASTFEEDING INITIATIVES**

The World Health Organization (WHO) and UNICEF have for many years emphasized the importance of maintaining the practice of breastfeeding, and of reviving the practice where it is in decline, as a way to improve the health and nutrition of infants and young children (WHO, 2011). The WHO through partner Health Sector Organizations and Ministries of Health of member nations has developed strategies to encourage breastfeeding worldwide. Such strategies include:

- ☐ Implementing and Monitoring the International Code of Marketing of Breast milk substitutes. The 27th World Health Assembly in 1974 noted the general



decline in breastfeeding related to different factors including the production of manufactured breast-milk substitutes and urged Member countries to review sales promotion activities on baby foods and to introduce appropriate remedial measures, including advertisement codes and legislation where necessary. In May 1981 the Health Assembly debated and adopted the International Code of Marketing of Breast-Milk Substitutes. The International Code aims to contribute to providing safe and adequate nutrition for infants by protection and promoting breastfeeding.

- ☐ Adoption of health policies on breastfeeding. For example, the Innocenti Declaration was adopted in 1990 and was subsequently endorsed by the World Health Assembly and UNICEF's Executive Board. The aim was to create an environment globally that empowers women to breastfeed exclusively for the first six months and continue to breastfeed for two years or more (UNICEF, 2011).
- ☐ Breastfeeding promotion campaigns. Health sector ministries of member countries provide and support educational campaigns needed to encourage breastfeeding in their individual countries.

### **2.1.3: STORAGE OF BREAST MILK**

Breastfeeding mothers may encounter unforeseen reasons for separation from their infants, but more often women express and store milk for planned events and lifestyle flexibility. Labiner-Wolfe *et al.* (2008) conducted a study on the prevalence of breast milk expression in US. Of the 1,564 mothers who breastfed their infant in the 1.5 to 4.5 month age group, 68% had expressed milk, with 43% having done so occasionally and

25% on a regular schedule. The results of the study suggest that breast milk expression has become a very common practice as it seeks to address the problem of breast feeding among working mothers, a recognized barrier to breastfeeding.

Stored human milk maintains its unique qualities such that it continues to be the gold standard for infant feeding, superior to artificial feeding (ABM Protocol, 2010). However, storage conditions are often not optimal (Hamosh *et al.*, 1996). In a study conducted by Jones *et al.* (1979) of breast milk collected into sterile bottles rinsed in 1% hypochlorite solution, the hypochlorite solution adherent to the sides of the bottles apparently caused a large reduction in bacterial contamination of the milk (22-75%) after storage at 4°C for up to four hours. Heating expressed breast milk at 62.5°C for five minutes destroyed over 90% of the *Escherichia coli*, *Staphylococcus aureus*, and group B beta-haemolytic streptococci inoculated into the milk samples. The study revealed that rinsing collecting bottles with hypochlorite solution may be valuable in collecting milk with a low bacterial content for human-milk banks. However, the effect of heat on substances such as IgA and lactoferrin is still being considered since these substances need to be conserved.

According to Moulin *et al.* (1998), it is possible to use unprocessed breast milk for baby's consumption if it is stored at room temperatures (17 - 30.5°C) until nine hours after it has been collected. Several studies have demonstrated the safety of refrigerating human milk (4°C), either by evaluating the bactericidal capacity of stored milk as a marker for milk quality or by measuring bacterial growth in the stored milk samples. Bactericidal capacity of stored refrigerated human milk declines significantly by 48–72 hours (Silvestre *et al.*, 2006; Martí'nez-Costa *et al.*, 2007). However, studies of

expressed human milk with little contamination at the time of expression demonstrate safe, low levels of bacteria growth in milk at 72 hours (Igumbor *et al.*, 2000) and even after 4–8 days of refrigeration (Sosa *et al.*, 1987). The temperature and duration of storage of breast milk are very critical if microorganisms present are to be kept from reaching unacceptable levels due to multiplication (Igumbor *et al.*, 2000).

There is no agreed-upon definition of unsafe breast milk. Several studies describe the degree of milk contamination over a period of time under certain temperature and storage time conditions, typically described as the number of colony forming units per milliliter. There is no accepted limit at which point breast milk should not be consumed, although  $1.0 \times 10^4$  colony forming units/ml has been suggested (ABM Protocol, 2010).

## **2.2: INFANT FORMULA FEEDING**

Infant formula feeding is recommended only under exceptional circumstances and in a few medical conditions that make breastfeeding impossible or not suitable (WHO, 2002). The most common breast milk substitutes are formula feeds manufactured as dried powders, reconstituted by adding water.

### **2.2.1: RISKS OF INFANT FORMULA FEEDING**

#### **2.2.1.1: Increased Risk of Infections and Mortality**

Reconstituted baby foods are considered to be a high-risk food because of the susceptibility of the consumer population to enteric pathogens (Rowan *et al.*, 1997).

Seven hundred and seventy-six infants from New Brunswick, Canada were assessed for the relationship between respiratory and gastrointestinal illnesses and breastfeeding during the first six months of life. Although the rates of exclusive breastfeeding were

low, the results showed a significant protective effect against total illness during the first six months of life. For those breastfed, the incidence of gastrointestinal infections was 47% lower; the rate of respiratory disease was 34% lower than those who were not breastfed (Beaudry *et al.*, 1995).

A nested case-controlled study by Cesar *et al.* (1999) revealed the Brazilian children not breastfed were 16.7 times more likely to be diagnosed with pneumonia than children who had received only breast milk as infants. A comparison between infants who received primarily breast milk during the first 12 months of life to infants who were exclusively formula-fed or who were breastfed for three months or less found that diarrheal disease was twice as high for the formula-fed infants than for those who were breastfed (Dewey *et al.*, 1995).

The number of episodes of acute otitis media increased significantly with decreased duration and exclusivity of breastfeeding. US infants who were exclusively breastfed for four months or more had a 50 per cent reduction of episodes compared to infants who were not breastfed. A 40 per cent reduction of episodes was reported for breastfeeding infants who were supplemented before four months of age (Duncan *et al.*, 1993).

Compared with exclusive breastfeeding, children who were partially breastfed had a 4.2 times increased risk of death due to diarrheal disease. Not breastfeeding was associated with a 14.2 times increased risk for death due to diarrheal disease in Brazilian children (Victora *et al.*, 1989).

Infants in Bangladesh who were partially breastfed or not breastfed had a risk of acute respiratory infection death 2.4 times greater than exclusively breastfed infants. If

children were predominantly breastfed the risk of death due to acute respiratory infection was similar to that of exclusively breastfed children (Arifeen *et al.*, 2001).

The researchers examined 1204 infants who died between 28 days and 1 year from causes other than congenital anomaly or malignant tumor and 7740 children who were still alive at 1 year to calculate mortality and whether or not the infant was breastfed as well as the duration–response effects. Children who were never breastfed had a 21% greater risk of dying in the post-neonatal period than those who were breastfed. Longer breastfeeding was associated with lower risk. Promoting breastfeeding has the potential to save ~720 post-neonatal deaths in the United States each year (Chen *et al.*, 2004)

According to Lucas and Cole (1990), if all the preterm babies in British neonatal units were fed breast milk rather than formula, 100 deaths a year from necrotising enterocolitis would be prevented.

#### **2.2.1.2: Increased Risk of Chronic Diseases**

A review of infant feeding practices and childhood chronic diseases showed increased risk for Type I diabetes, celiac disease, some childhood cancers, and inflammatory bowel disease associated with artificial infant feeding (Davis, 2001).

Celiac disease may be triggered by an autoimmune response when an infant is exposed to a food containing gluten proteins. Ivarsson (2002) and her team of researchers looked at the breastfeeding patterns of 627 children with celiac disease and 1254 healthy children to determine the effect of breastfeeding during the time of introduction of gluten-containing foods on the outcome of the development of celiac disease. An astounding 40 per cent risk reduction was reported for the development of celiac disease



in children at two years of age or younger for those who were breastfed when dietary gluten was introduced. The effect was even more pronounced in infants who continued to be breastfed after dietary gluten was introduced, the researchers noted.

Inflammatory bowel disease and Crohn's disease are chronic gastrointestinal conditions that are more frequent for those who are formula-fed. A meta-analysis on 17 relevant studies supported the hypothesis that breastfeeding is linked with lower risks of Crohn's disease and ulcerative colitis (Klement *et al.*, 2004).

#### **2.2.1.3: Increased Risk of Diabetes**

Early introduction of infant formula, solids and cow's milk are factors shown to increase the incidence of Type I diabetes later in life. Swedish (517) and Lithuanian (286) children aged 0 to 15 years who were diagnosed with Type I diabetes were compared to non-diabetic controls. The results showed that exclusive breastfeeding for five months and total breastfeeding for longer than seven or nine months are protective against diabetes (Sadauskaite-Kuehne *et al.*, 2004).

In a case-controlled study, 46 native Canadian Type II diabetes patients were matched with 92 controls. Pre- and postnatal risk factors were compared. Breastfeeding was found to reduce the risk of Type II diabetes (Young *et al.*, 2002).

#### **2.2.1.4: Reduced Cognitive Development**

To determine the impact of exclusive breastfeeding on cognitive development for infants born small for gestational age, a US based study evaluated 220 infants, using the Bayley Scale of Infant Development at 13 months and the Wechler Preschool and Primary



Scales of Intelligence at five years. The researchers concluded that exclusively breastfed (without supplements) small for gestational age infants had a significant advantage in cognitive development without compromising growth (Rao *et al.*, 2002).

A total of 3880 Australian children were followed from birth to determine breastfeeding patterns and later cognitive development. Children breastfed for six months or more scored 8.2 points higher for females and 5.8 points higher for males in vocabulary tests over those who had never been breastfed (Quinn *et al.*, 2001).

#### **2.2.1.5: Other Threats**

Other risks of infant formula feeding includes: threats of nutritional deficiencies, increased risk of childhood cancers and obesity (Bener *et al.*, 2001; Frye *et al.*, 2003).

#### **2.2.2: BACTERIAL CONTAMINATION OF INFANT FORMULA**

Powdered infant formula products are not sterile and may be colonized with bacterial organisms (Agostoni *et al.*, 2004). During the last 20 years, there have been multiple reports associating bacterial colonization of dried formula with infection among infants who have been fed with these products. Contamination of nutritional formula has been implicated in the etiology of nosocomial infections, especially when administered to immunocompromised patients. The association with gastrointestinal symptoms or other infectious consequences such as bacteremia has also been demonstrated (Patchel *et al.*, 1994). There is evidence showing phenotypic and genotypic similarities between isolates from human infections and those recovered from food and nutritional formula given to hospitalized patients (Navajas *et al.*, 1992). *Salmonella* species and other members of the family *Enterobacteriaceae* have been identified in most of these events (Estrada, 2002).

Two FAO/WHO meetings of experts on the microbiological safety of powdered infant formula considered cases of illnesses in infants associated with prepared formula consumption either epidemiologically or microbiologically (FAO/WHO, 2004; FAO/WHO, 2006). They identified three categories of microorganisms based on the strength of evidence of a causal association between their presence in prepared formula and illness in infants:

- ☐ microorganisms with a clear evidence of causality, namely, *Salmonella enterica* and *Enterobacter sakazakii*
- ☐ microorganisms for which the causality is plausible but not yet demonstrated, i.e., they are well-established causes of illness in infants and have been found in prepared formula, but contaminated formula has not been convincingly shown, either epidemiologically or microbiologically, to be the vehicle and source of infection, e.g., other *Enterobacteriaceae*
- ☐ microorganisms for which causality is less plausible or not yet demonstrated, including microorganisms which despite causing illness in infants, have not been identified in prepared formula, or microorganisms which have been identified in prepared formula but have not been implicated as causing such illness in infants, including *Bacillus cereus*, *Clostridium botulinum*, *C. difficile*, *C. perfringens*, *Listeria monocytogenes* and *Staphylococcus aureus*.

Salmonellosis caused by ingestion of contaminated powdered infant formula has been reported in the United States and other countries. In 1993, infection caused by *Salmonella* serotype Tennessee among infants in Canada and the United States was linked to the ingestion of a powdered milk product, which was recalled after cases were

identified (Centers for Disease Control and Prevention, 1993). Similarly, during 1994 in Spain, 48 cases of salmonellosis among infants younger than 12 months were linked to ingestion of formula contaminated with *Salmonella virchow* (Usera *et al.*, 1998). In a study at Children's Hospital and Regional Medical Center to describe a nosocomial outbreak of *Salmonella* serotype *Saintpaul* gastroenteritis and to explore risk factors for infection, formula mixed by the hospital appeared to have been the source of *Salmonella* outbreak (Bornemann *et al.*, 2002).

In addition to *Salmonella* species, other *Enterobacteriaceae* are known to be able to colonize powdered infant formula. In a study performed by Muytjens and collaborators (Muytjens *et al.*, 1988), 141 powdered human milk substitutes obtained from 35 different countries were analyzed for the presence of *Enterobacteriaceae*. These investigators were able to isolate bacterial organisms from 52.5% of the products evaluated. The species most frequently isolated included *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter sakazakii*, and *Klebsiella pneumoniae*. However, none of the formula tested in this study had bacterial concentrations higher than 3 colony-forming units/g; therefore these formulae met the requirement for bacterial count of coliform organisms in infant formula recommended by the Food and Agricultural Organization of the United Nations (Muytjens *et al.*, 1988).

Contamination of dried milk products with *E. sakazakii* frequently has been associated with development of disease among infants. The spectrum of infection caused by these organisms includes septicemia, meningitis, and urinary tract infection (Simmons *et al.*, 1989; Bar-Oz *et al.*, 2001). In addition, a significant association between infant formula colonization with *E. sakazakii* and the development of necrotizing enterocolitis was

reported among infants in a neonatal ICU in Belgium (van Acker *et al.*, 2001). It has been suggested that increased resistance to heat by *E. sakazakii* may play a role in its ability to colonize dried milk.

In a study conducted to evaluate the prevalence of bacterial contamination of prepared infant formula given to infants at Red Cross War Memorial Children's Hospital, Cape Town, Marino *et al.* (2007) demonstrated that even when milk is prepared in a controlled environment, there is significant bacterial contamination of prepared infant formula post production. The study found that 30% of prepared infant formula studied, including those that were newly prepared, were heavily contaminated with bacteria. Opportunities for contamination arise during mixing and addition of additives, and when prepared food is allowed to stand in the milk kitchen or the ward.

### **2.3: OUTBREAKS OF FOOD-BORNE INFECTIONS IN HOSPITALS**

Contaminated milk is frequently implicated in hospital food-related outbreaks of gastroenteritis, which is especially worrisome due to the wide distribution of milk in the hospital (American Hospital Association, 1983). Most hospital outbreaks of food-borne infections are caused by foods prepared by the health workers (Bryan, 1990). Nosocomial outbreaks as a result of infant formula contamination have been reported worldwide with special reference to Gram-negative bacilli such as *Klebsiella* spp., *Enterobacter* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Salmonella* spp. (Patchel *et al.*, 1994). Additionally, *Enterobacter sakazakii* has recently been identified as a pathogenic contaminant of milk (WHO/FAO, 2004). It is possible that health staff are asymptomatic carriers of pathogenic microorganisms and thus represent a continuous source of contamination (Vanetti, 2004).

In a cross-sectional study conducted by Cairo and collaborators (Cairo *et al.*, 2008), 91 workers from the 20 milk kitchens of all the public and private hospitals in Salvador, Brazil, were screened to describe their bacterial microflora profile using hand swabs, pharynx swabs and stool samples. Samples of the milk and formula delivered by the kitchens were also collected for microbiological analysis. The most common bacteria isolated from hand samples were coagulase-negative *Staphylococcus* spp. (69.2%), *Bacillus* spp. (25.3%), *Pseudomonas* spp. (14.3%) and *Micrococcus* spp. (11.0%). Pathogenic bacteria (*Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella oxytoca*, *Serratia* spp. and *Citrobacter freundii*) were isolated from 20 (22.0%) hand samples. No pathogenic bacteria were isolated from stool samples. The bacteria isolated from milk and formula samples were most often *Bacillus* spp. (30.8%), coagulase-negative *Staphylococcus* spp. (13.2%) and *Pseudomonas* spp. (7.7%). However, pathogenic bacteria (*Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia* spp., *Citrobacter freundii*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*) were found in 17 (18.7%) samples of milk/formula. The results appeared to demonstrate a link between bacterial contamination of the milk/formula and the carrier profile of the health workers.

Outbreaks of *E. sakazakii* infections have been linked with prepared formula, especially in the context of neonatal intensive care setting. *E. sakazakii* is known to be present at low concentration in a proportion of prepared formula. While the microorganism has been detected in other types of food and environmental settings, only prepared formula has been linked to outbreaks of disease (CAC/RCP 66 – 2008).



There are four routes by which *E. sakazakii* and *Salmonella* can enter prepared formula:

- ☐ through the ingredients added in dry mixing operations during the manufacturing of prepared formula
- ☐ through contamination of the formula from the processing environment in the steps during or following the drying
- ☐ through contamination of the prepared formula after the package is opened, and
- ☐ through contamination during or after reconstitution by the caregiver prior to feeding. *E. sakazakii* may be found in many environments such as food factories, hospitals, institutions, day-care facilities and homes.

## **2.4: MICROBIOLOGICAL CRITERIA FOR INFANT FORMULA**

### **2.4.1: Microbiological Criterion**

A microbiological criterion for food defines the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot (CAC/GL 21 – 1997).

A microbiological criterion consists of:

- ☐ A statement of the microorganisms of concern and/or their toxins/metabolites
- ☐ The analytical methods for their detection and/or quantification
- ☐ A plan defining the number of field samples to be taken and the size of the analytical unit
- ☐ Microbiological limits considered appropriate to the food at the specified point(s) of the food chain



- ☐ The number of analytical units that should conform to these limits

Generally, microbiological criteria may be applied to define the distinction between acceptable and unacceptable raw materials, ingredients, products, lots, by regulatory authorities and/or food business operators. Microbiological criteria may also be used to determine that processes are consistent with the General Principles of Food Hygiene (CAC/RCP 1-1969).

#### 2.4.2: Microbiological Requirements for Infant Formula

The microbial load of milk should be as low as achievable. Tables 2.1 and 2.2 are FAO/WHO microbiological criteria in accordance with the Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-97). Two sets of criteria are provided below, one for pathogens and a second for process hygiene indicators.

Table 2.1: Criteria for pathogenic microorganisms

Microorganisms	n	c	m	Class Plan
<i>Enterobacter sakazakii</i> ( <i>Cronobacter</i> species)*	30	0	0/10 g	2
<i>Salmonella</i> **	60	0	0/25 g	2

Where n = number of samples that must conform to the criteria:

c = the maximum allowable number of defective sample units in a 2-class plan.

m = a microbiological limit which, in a 2-class plan, separates good quality from defective quality.

\*The mean concentration detected is 1 cfu in 340g (if the assumed standard deviation is 0.8 and probability of detection is 95%) or 1 cfu in 100g (if the assumed standard deviation is 0.5 and probability of detection is 99%).

\*\*The mean concentration detected is 1 cfu in 526g (if the assumed standard deviation is 0.8 and probability of detection is 95%).

Table 2.2: Criteria for process hygiene

Microorganisms	n	c	m	M	Class Plan
Mesophilic Aerobic Bacteria*	5	2	500/g	5000/g	3
Enterobacteriaceae**	10	2	0/10 g	Not applicable	2

Where n = number of samples that must conform to the criteria

c = the maximum allowable number of defective sample units in a 2-class plan or marginally acceptable sample units in a 3-class plan

m = a microbiological limit which, in a 2-class plan, separates good quality from defective quality or, in a 3-class plan, separates good quality from marginally acceptable quality

M = a microbiological limit which, in a 3- class plan, separates marginally acceptable quality from defective quality.

\* The proposed criteria for mesophilic aerobic bacteria are reflective of good manufacturing practices and do not include microorganisms that may be intentionally added such as probiotics.

\*\* The mean concentration detected is 1 cfu in 16g (if the assumed standard deviation is 0.8 and probability of detection is 95%) or 1 cfu in 10g (if the assumed standard deviation is 0.5 and probability of detection is 99%).

#### **2.4.3: Ghana's Standard for Infant Formula**

Ghana subscribes to FAO/WHO microbiological limits for infant formula and also has the requirements in Table 2.3 (GS574:2003).

Table 2.3: Microbiological requirements for infant formula

Microorganism	Acceptable
Total Viable Count	$< 1.0 \times 10^5$ cfu/ml
Total Coliforms	$< 1.0 \times 10^2$ cfu/ml
Salmonella	Absent

## **CHAPTER THREE**

### **MATERIALS AND METHODS**

#### **3.1: STUDY SITE AND SAMPLING STRATEGY**

##### **3.1.1: Study Site**

The study was conducted at the Mother and Baby Unit (MBU), A2, A5, and B4 wards of the Pediatric Unit of Komfo Anokye Teaching Hospital (KATH), Kumasi. A2 and A5 wards are lying-in wards and nurseries for normal babies and their mothers whiles Mother and Baby Unit is a division for diseased babies and their mothers. B4 ward is the medical children's ward, a ward for seriously diseased children above 1 year; children with severe malaria, malnutrition, renal diseases or other threatening medical conditions are found on this ward.

Babies on A2, A5 and MBU wards are given infant formula within the first 6 hours of birth when mothers are indisposed to breastfeed their babies. Also, babies are formula fed when their mothers are not producing breast milk or have passed away during childbirth. At B4 ward, malnourished children are given formula feed.

Samples of the formula feeds were collected from these wards and analyzed at the Microbiology laboratory of KATH.

##### **3.1.2: Sampling Period**

The research was undertaken between July and December, 2010. Pre-sampling activities included the acquisition of ethical clearance from the Committee on Human Research,

Publications and Ethics (CHRPE) at the Komfo Anokye Teaching Hospital, and the School of Medical Sciences, KNUST (appendix 6).

### **3.1.3: Study Procedure**

The study population was breastfeeding mothers at the MBU ward and the health staff of KATH involved in preparing formula feed for children on the wards.

The study had 3 parts:

- ☐ Sampling and culture of microorganisms
- ☐ Observation of formula preparation
- ☐ Questionnaire administration and staff interview

### **3.1.4: Inclusion and Exclusion Criteria**

Inclusion Criteria:

1. Prepared infant formula on A2, A5, B4 and MBU wards
2. Unfinished portions of prepared formula available on the wards
3. Expressed breast milk of mothers
4. Staff of KATH involved with formula feeding

Exclusion Criteria:

1. Mothers who did not agree to express their breast milk

### **3.1.5: Sample Size**

A convenient size of 136 milk samples was used for the study. Seventy-two samples of prepared infant formula were taken from the wards and 34 samples of expressed milk were taken from lactating mothers on MBU ward. Thirty samples of unprepared infant formula from opened tins were also taken for the study.

### **3.1.6: Sample Collection, Labeling and Confidentiality**

The purpose of the study was explained to lactating mothers and willing mothers expressed 10 - 15 ml of breast milk into sterile collection bottles under the supervision of health staff. Infant formula prepared by health staff were obtained from A2, A5, MBU and B4 wards on unannounced dates for the study. Five to ten milliliters of prepared formula were collected into sterile collection bottles during sample collection. Samples of unprepared infant formula (2.2g) were collected from opened tins of formula at the wards for microbiological analysis.

All samples collected were identified only by ID-numbers. No personal details of mothers and health staff were recorded to ensure confidentiality.

### **3.1.7: Observation of formula preparation**

During sampling, the method of formula preparation, handling and storage of prepared formula were closely observed at the wards. Information from observations were also recorded without personal details of health staff.

### **3.1.8: Questionnaire Administration and Staff Interview**

A questionnaire (appendix 1) was used to collect some information on method of preparation, handling and storage of prepared formula. Information on the length of stay of prepared formula before sampling was given by health staff on the wards during sample collection. Information recorded had no personal details of health staff.

## **3.2: MICROBIOLOGICAL ANALYSIS**

Breast milk samples obtained from mothers were kept at room temperature and put on culture media at zero ( $T_0$ ), three ( $T_3$ ), six ( $T_6$ ) and nine ( $T_9$ ) hours after collection to determine bacterial propagation over time. Part of the breast milk obtained from mothers



were stored in the refrigerator and cultured at nine hours (FT<sub>9</sub>) after collection to determine the bacterial count at nine hours after sampling if refrigerated.

Aliquots of samples of prepared formula from the wards were inoculated onto culture media on reaching the laboratory. They were incubated for an hour at room temperature and inoculated again onto culture media.

Samples from opened tins of formula at the wards were reconstituted aseptically according to manufacturers' instructions. 2.2g of powdered formula were dissolved in 15ml of sterile water in sampling collecting bottles. Reconstituted formula were then inoculated onto culture media.

### **3.3: CULTURE OF MICROORGANISMS**

MacConkey agar and Campylobacter media were used for initial culture of organisms in all samples of expressed breast milk and infant formula. Kligler iron agar, Simmon's citrate agar and urea agar were biochemical media used to determine the biochemical profile of isolated bacteria.

Cultures on MacConkey agar were incubated aerobically at 37°C for 24 hours whereas cultures on Campylobacter media were incubated in a microaerophilic condition (10% carbon dioxide concentration) at 42°C for 48 hours. Negative cultures at 24 hours were re-incubated for another 24 hours. Culture of isolated bacteria on Kligler iron agar, Simmon's citrate agar and urea agar were incubated aerobically at 37°C for 24 hours.

Plate count agar was used for plate count of bacteria in all samples of expressed breast milk and infant formula. Cultures on plate count agar were incubated aerobically at 37°C for 24 hours.

### 3.4: PLATE COUNT OF BACTERIA

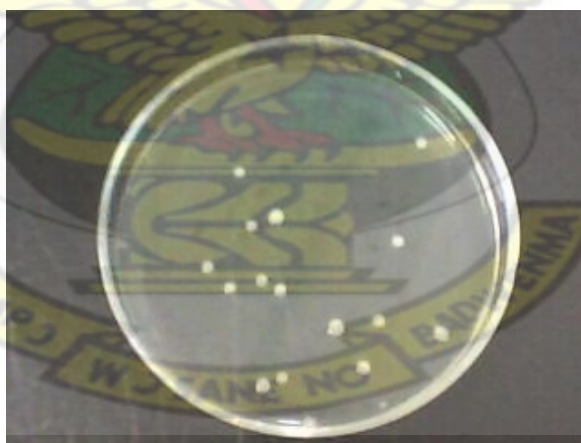
Plate count of bacteria was done using the spread-plate method to determine the number of colony-forming units of bacteria per ml of infant formula and expressed breast milk.

#### 3.4.1: Spread-Plate Method

Milk dilutions of 1:10, 1:100 and 1:1000 were prepared from samples of infant formula and expressed breast milk. One milliliter of each milk dilution was spread over the surface of plate count medium. Inoculated plates were incubated at 37°C. Cultured plates were examined for growth after 24 hours.

$$\text{Plate Count of bacteria (cfu/ml)} = \frac{\text{Number of colonies}}{\text{Test Volume (aliquot)}} \times \text{Dilution Factor}$$

**Figure 3.1: Growth of Organisms on Plate Count Medium**



### **3.5: IDENTIFICATION OF MICROORGANISMS**

All colonies on MacConkey agar and Campylobacter agar were identified with Gram staining and standard biochemical tests (appendix 3). Novobiocin test was also conducted for all coagulase negative staphylococci. Culture of bacteria on Kligler iron agar and Simmon's citrate agar together with urease and indole test were done to determine the biochemical profile of all Gram negative isolates.

### **3.6: DATA ANALYSIS**

Data analysis was done using GraphPad Prism 5.02 and MS Excel 2007 software. Data were analyzed for the relationship between length of storage of expressed breast milk and bacterial propagation over time. The relationships between bacteria isolated, bacterial propagation over time in expressed breast milk and variations in room temperature were explored using T-tests and Analysis of Variance (ANOVA). The associations between duration of stay of prepared infant formula on the wards before sample collection and extent of contamination were based on plate counts of colony-forming units of bacteria per ml of infant formula.

## **CHAPTER FOUR**

### **RESULTS**

#### **4.1: PREPARATION, HANDLING AND STORAGE OF PREPARED FORMULA**

A total of 72 prepared samples of infant formula were collected from the MBU, A2, A5 and B4 wards of KATH. Of these, 42 were reconstituted infant formula from Lactogen, Nan, Nan1 and SME powdered formula and were manually prepared by nurses at the wards. The other 30 samples of formula were Formula 100 (F100) and Formula 75 (F75) and were collected from B4 ward. The F100 and F75 were made from powdered milk (Nido), oil, sugar and combined minerals and vitamins (CMV). These were reconstituted manually by nutritionists at B4 ward. F75 is a starter formula for all malnourished children. It has low protein content and 75 kilocalories of energy whereas F100 is a catch-up formula given to children after their condition have been stabilized with F75. F100 has high protein content and contains 100 kilocalories of energy.

At MBU, A2 and A5 wards, infant formula is reconstituted and served to babies whose mothers were not producing breast milk or have passed away during childbirth and those who couldn't enjoy breast milk because their mothers were indisposed to breastfeed during the first 6 hours of delivery. These babies were served 4 times in a day. Leftovers of prepared formula at these wards were not expected to stay on the wards after children have been fed. Prepared formula on the B4 ward were also delivered 4 times in a day but leftovers on this ward may be stored and delivered to children at timely interval (3 hours).

#### **4.1.1: Hand washing and Sterilization of Vessels for Preparation of Reconstituted Infant formula**

The hands of health staff were thoroughly washed with soap and water. Hands were allowed to air-dry before reconstitution of formula was done. Vessels were washed with soap and water and then immersed in boiling water or hot water with salt before vessels were used for reconstitution of infant formula. Standard Operating Procedures (SOPs) for hand washing, sterilization of vessels and reconstitution of formula were not found on the wards. Figure 4.1 and 4.2 show some vessels used for reconstitution of formula on the wards.

**Figure 4.1: Vessels used for the preparation of PIF at MBU ward**





A photograph of a laboratory setup for measuring the heat of solution. The setup includes a blue bucket, a pink basin containing several small colored cubes, a blue basin with a purple cup inside it, and various containers and equipment on a table. A large, semi-transparent watermark reading "KNUST" is overlaid across the center of the image.

Water used for the preparation of PIF was boiled tap water stored in thermos flasks. Sachet water or bottled water was used by health staff when water from taps did not flow. Preparation of PIF was done following manufacturers' outlined instructions for reconstitution of the various formulae. PIF were prepared in a pool and given to children in the warm state (Figure 4.3).



#### **4.1.3: Storage of Prepared Formula**

Infant formula reconstituted at MBU, A2 and A5 wards were not supposed to be stored or left on the wards for later use. In spite of this, some PIF were found to have been kept for later use, following no standard storage procedure.

Prepared formula at B4 ward are stored in thermos flasks (figure 4.4) immediately after preparation and served to the children from the thermos flasks afterwards. The next batches of prepared formula were made after the complete consumption of the latter by the children. There was no stipulated length of storage for these prepared formula at B4 ward.

**Figure 4.4: Thermos flasks used at B4 ward**



#### **4.2: LENGTH OF STAY OF PREPARED INFANT FORMULA BEFORE SAMPLE COLLECTION**

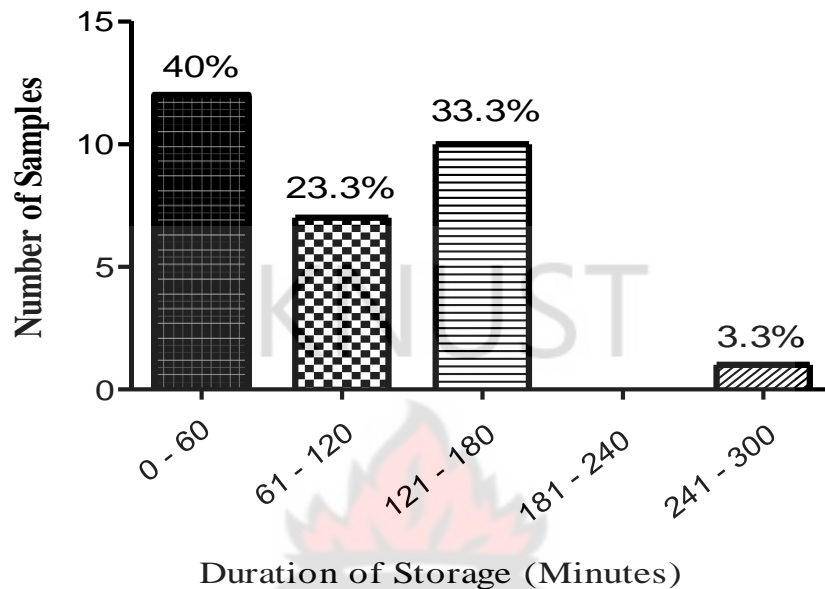
Thirty-six out of 42 (85.7%) PIF samples collected at MBU, A2 and A5 wards had stayed for at most an hour after their preparation before the unannounced sampling. The range of length of stay of PIF before sample collection was 3 minutes to 3 hours.

However, the mean length of stay was 44 minutes (95% CI = 29 - 59). Figure 4.5 shows the distribution of the length of stay of the PIF before sample collection.

Twelve out of 30 (40%) prepared formula collected at B4 ward had been stored for at most an hour after their preparation. The range of duration of storage of the prepared formula on this ward was 5 minutes to 5 hours. But the mean duration of storage was 108 minutes (95% CI = 79 – 137). Figure 4.6 shows the duration of storage of the prepared formula collected from B4 ward.



Figure 4.6: Duration of Storage of Prepared Formula at B4 Ward before Sampling



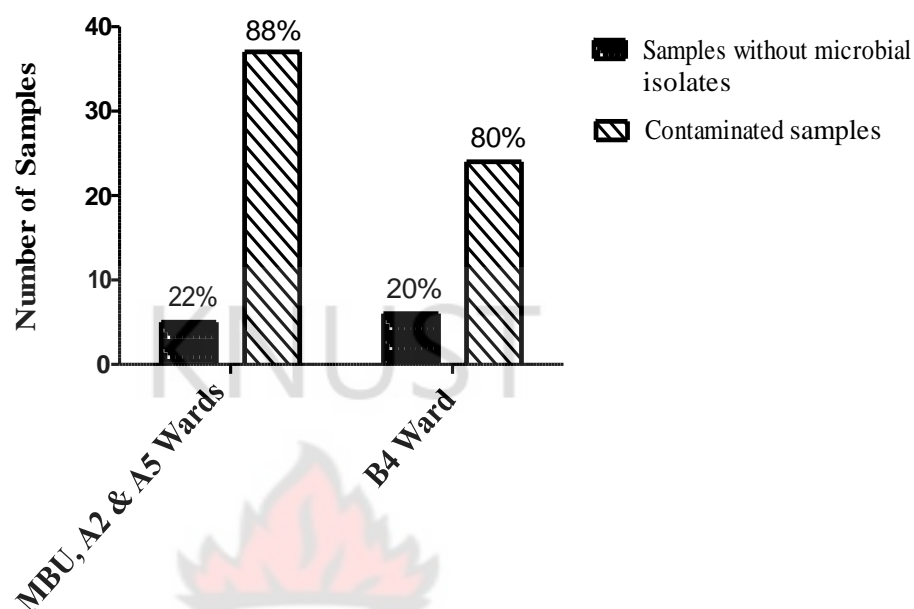
#### 4.3: RESULTS OF CULTURE OF UNPREPARED FORMULA

Thirty samples of unprepared formula (powdered state) were collected from MBU, A2, A5 and B4 wards and were reconstituted according to manufacturers' instructions under aseptic conditions. From these 30 samples, no organisms were isolated.

#### 4.4: PREVALENCE OF BACTERIAL CONTAMINATION OF PREPARED INFANT FORMULA

Of the 72 samples of prepared formula collected from the MBU, A2, A5 and B4 wards, 85% (n=61) had microbial isolates. 88% (n=37) of the PIF collected from MBU, A2 and A5 (n=42) were contaminated with bacterial organisms whereas prepared formula samples from B4 had 80% (n=24) of them being contaminated. The figure below (figure 4.7) shows the prevalence of bacterial contamination of PIF obtained from the wards.

**Figure 4.7: Prevalence of Bacterial Contamination of PIF from the wards**



#### **4.5: MICROBIAL QUALITY OF PREPARED INFANT FORMULA**

The most common bacteria isolated were *Acinetobacter* sp. (52/61; 85.2%), *Klebsiella pneumoniae* (21/61; 34.4%), *Enterococcus* sp. (11/61; 18.0%) and *Staphylococcus epidermidis* (8/61; 13.1%). *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus aureus* were also isolated with a combined prevalence of 4.8% in the PIF obtained from the wards. *Candida* yeast was isolated from one of the samples (1.6%). Interestingly, all 11 (18%) samples from which *Enterococcus* sp. were isolated came from the B4 ward. No *Campylobacter* sp. was isolated. Microbial isolates from PIF collected from MBU, A2, A5 and B4 wards are shown in Table 4.1.

**TABLE 4.1: MICROORGANISMS ISOLATED FROM PIF COLLECTED FROM THE WARDS**

ORGANISMS	MBU, A2 AND A5 WARDS		B4 WARD		ALL WARDS	
	N	%	N	%	N	%
<i>Acinetobacter</i> sp.	36	97.3	16	66.7	52	85.2
<i>Staphylococcus epidermidis</i>	3	8.1	5	20.8	8	13.1
<i>Staphylococcus aureus</i>	1	2.7	-	-	1	1.6
<i>Klebsiella pneumoniae</i>	10	27	11	45.8	21	34.4
<i>Enterococcus</i> sp.	-	-	11	45.8	11	18.0
<i>Bacillus</i> sp.	1	2.7	-	-	1	1.6
<i>Pseudomonas</i> sp.	1	2.7	-	-	1	1.6
Candida yeast	-	-	1	4.2	1	1.6

#### 4.6: PLATE COUNT OF BACTERIA IN PREPARED FORMULA

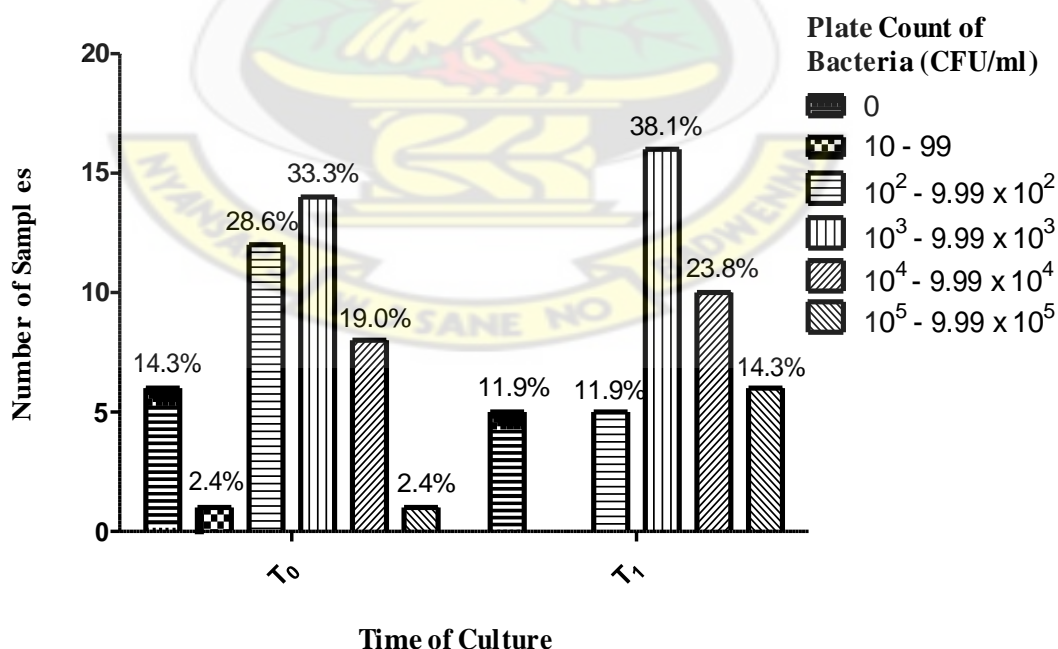
##### 4.6.1: Plate Count of Bacteria in PIF at Collection Time ( $T_0$ ) Versus End of an Hour of Incubation ( $T_1$ )

The Total Viable Count (TVC) of bacteria in PIF from A2, A5 and MBU wards that were contaminated at  $T_0$  ranged from  $7.5 \times 10^2$  CFU/ml to  $2.86 \times 10^5$  (25% percentile =  $1.68 \times 10^2$  CFU/ml; 75% percentile =  $6.22 \times 10^3$  CFU/ml). The differences between the TVC of the PIF at  $T_0$  were not significant ( $p = 0.0725$ ). At  $T_0$ , 97.6% of PIF cultured had TVC less than the standard acceptable limit of  $1.0 \times 10^5$  CFU/ml with the majority between  $1.0 \times 10^2$  CFU/ml and  $9.99 \times 10^3$  CFU/ml (36/42; 61.9%).

However, the TVC of PIF cultured after an hour of incubation at room temperature ( $T_1$ ) spanned  $1.0 \times 10^2$  to  $6.60 \times 10^5$  CFU/ml (25% percentile =  $9.28 \times 10^2$  cfu/ml; 75% percentile =  $1.91 \times 10^4$  CFU/ml) with significant differences between the plate counts of bacteria ( $p = 0.0204$ ). At  $T_1$ , 76.2% (32/42) of PIF had plate counts of bacteria within  $1.0 \times 10^3$  and  $9.99 \times 10^5$  CFU/ml. 14.3% (6/42) of the PIF had significant contamination (TVC  $\geq 1.0 \times 10^5$  CFU/ml) with *Acinetobacter* sp., *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Bacillus* sp., exceeding the acceptable limit of bacteria in milk.

The 1-hour incubation of the PIF at room temperature (mean =  $27.8^\circ\text{C}$ ) had a significant effect on the plate count of bacteria in PIF at  $T_1$ . It accounted for 2.93% of total variance in the plate counts of bacteria with a significant  $p$ -value of 0.0125. Figure 4.8 shows plate counts of bacteria in PIF at  $T_0$  and  $T_1$ .

**Figure 4.8: Plate Count of Bacteria in PIF at  $T_0$  and  $T_1$**





#### 4.6.2: Variation of Plate Count of Bacteria in PIF with the Length of Stay before Sample Collection

There was no significant difference between the mean plate counts of bacteria in PIF at different lengths of stay of PIF on the wards (A2, A5 and MBU) before sampling ( $p = 0.3180$ ). The plate counts of bacteria in the greater number of PIF (36/42) that had stayed for at most 60 minutes differed less significantly with duration of stay ( $p = 0.3660$ ). Figure 4.9 represents the variation of plate counts of bacteria in PIF with length of stay of PIF before sample collection.



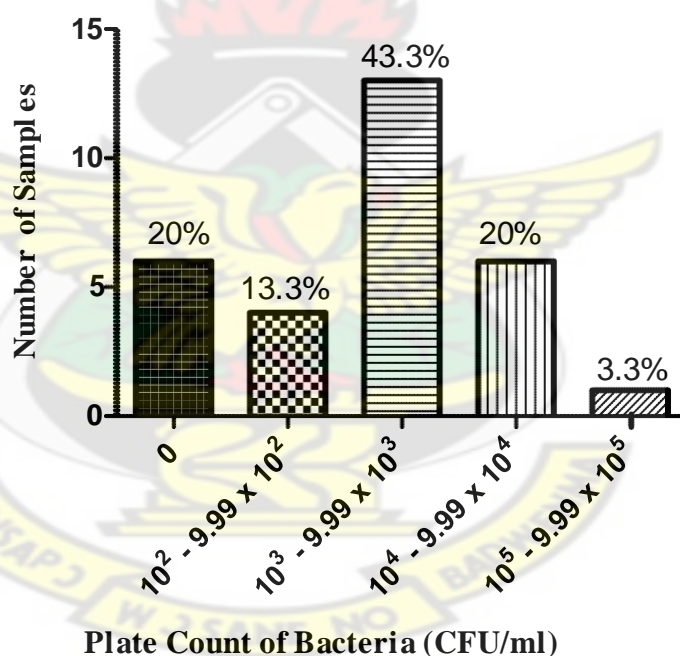
#### 4.6.3: Plate Count of Bacteria in Prepared Formula from B4 Ward

The Total Viable Count (TVC) of bacteria in contaminated prepared formula from B4 ward ranged from  $3.0 \times 10^2$  CFU/ml to  $1.01 \times 10^5$  (25% percentile =  $3.75 \times 10^2$ ; 75% percentile =  $8.73 \times 10^3$  CFU/ml), with significant differences in the plate counts of

bacteria ( $p = 0.0239$ ) . Of the 24 contaminated samples, 19 (79%) had TVC of bacteria within  $1.0 \times 10^3$  CFU/ml –  $9.99 \times 10^4$  CFU/ml (Figure 4.10).

Only 1 sample (1/30) had bacterial contamination beyond the maximum acceptable limit of organisms in formula feed (TVC =  $1.01 \times 10^5$  CFU/ml). *Acinetobacter* sp., *Klebsiella pneumoniae* and *Enterococcus* sp. were the contaminants in the sample. Thus 96.7% of prepared formula from B4 ward had TVC of bacteria within the limit of acceptance for consumption.

**Figure 4:10 : Plate Count of Bacteria in Prepared Formula from B4 ward**

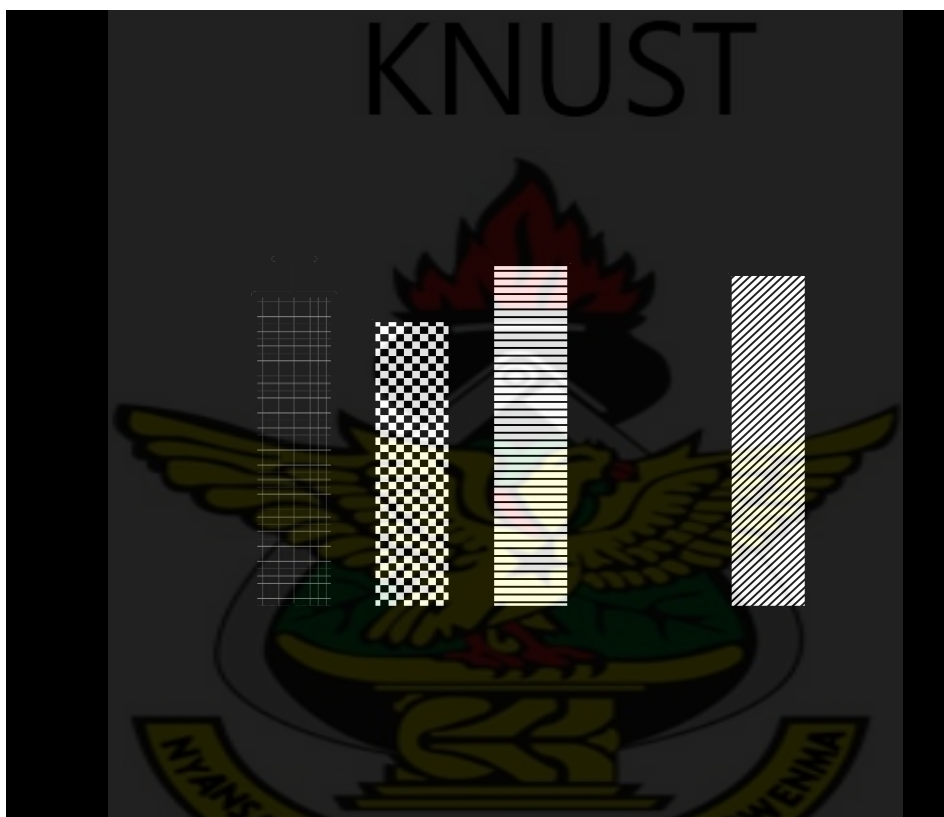


#### **4.6.4: Variation of Plate Count of Bacteria with Length of Storage of Prepared Formula before Sample Collection in B4 ward**

The Total Viable Count of prepared formula at different lengths of storage did not vary much. Figure 4.11 is a plot of the mean TVC of bacteria in the prepared formula from

B4 ward with the length of storage before sample collection. The duration of storage of the prepared formula accounted for 7.71% of the total variance.

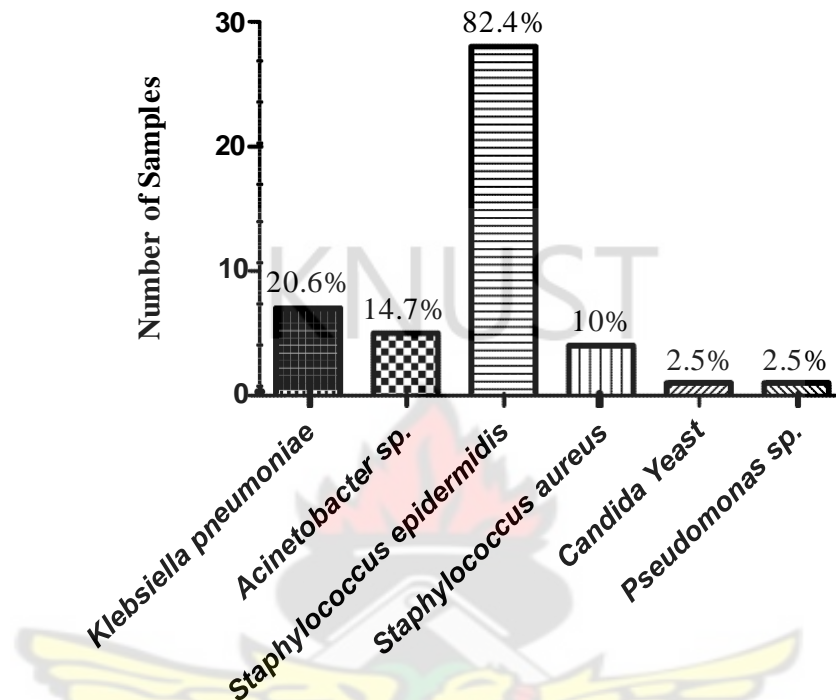
The effect of the duration of storage on the plate count of bacteria was not significant as the TVC of bacteria in the prepared formula stored for different periods before collection did not have relevant statistic differences ( $p = 0.5207$ ).



#### **4.7: PREVALENCE OF MICROBIAL CONTAMINATION IN EXPRESSED BREAST MILK**

Thirty-three out of 34 (97.1%) samples of the freshly expressed breast milk were contaminated, mostly with the normal skin flora, *Staphylococcus epidermidis* (28/34; 82.4%). *Klebsiella pneumoniae*, *Acinetobacter* sp., *Staphylococcus aureus*, *Candida* yeast and *Pseudomonas* sp. were the other contaminants of the expressed breast milk (Figure 4.12).

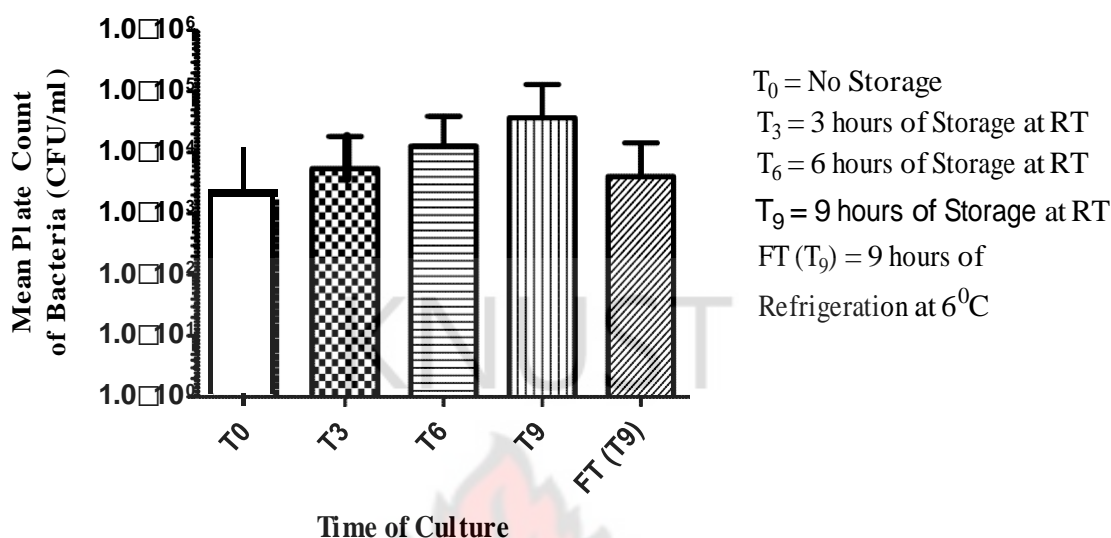
**Figure 4.12: Microorganisms Isolated from the Expressed Breast Milk**



#### **4.8: PLATE COUNT OF BACTERIA IN EXPRESSED BREAST MILK**

The plate count of bacteria in contaminated expressed breast milk (EBM) over the nine-hour storage period, at room temperature (25 – 29°C; mean = 27.5°C) and refrigeration temperature (6°C), ranged from  $1.0 \times 10$  CFU/ml at  $T_0$  to a maximum of  $5.0 \times 10^5$  CFU/ml at  $T_9$  (Figure 4.13). The total viable counts of bacteria in the EBM cultured at  $T_0$  (without storage) were not significantly different from each other ( $p = 0.0527$ ). However, all cultures of EBM stored for 3 hours ( $T_3$ ), 6 hours ( $T_6$ ) and 9 hours ( $T_9$ ) had significant differences between the plate count of bacteria that had contaminated them ( $p = 0.0183, 0.0089$  and  $0.0262$  for  $T_3, T_6$  and  $T_9$  respectively).

**Figure 4.13: Plate Count of Bacteria in Expressed Breast Milk**

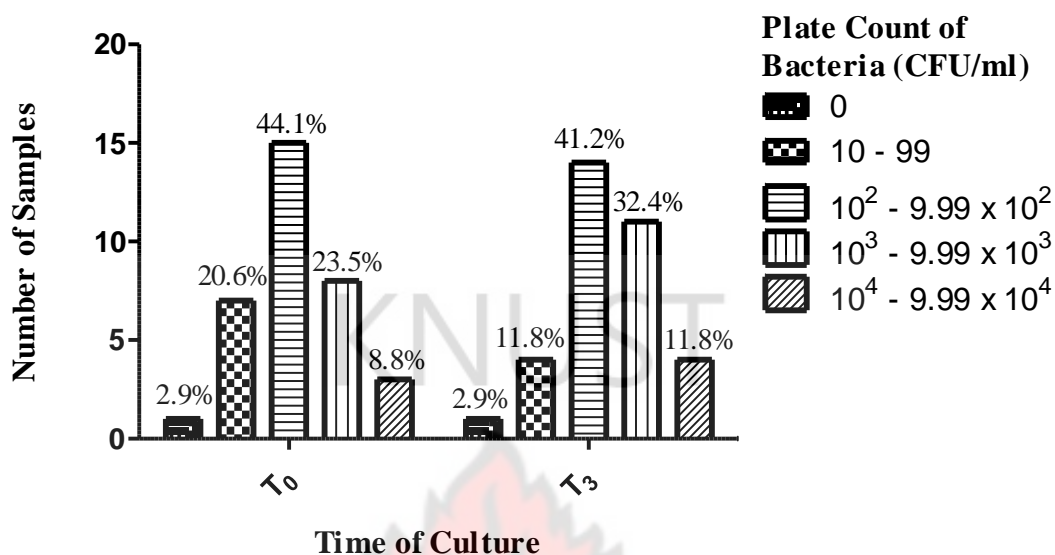


#### 4.9: VARIATION OF PLATE COUNT OF BACTERIA IN EBM WITH TIME OF STORAGE

The freshly expressed breast milk from mothers did not have significant bacteria contamination as all samples had total viable counts (TVC) of bacteria less than  $1.0 \times 10^5$  CFU/ml ( $TVC < 1.0 \times 10^5$  CFU/ml) at  $T_0$  (mean  $TVC = 3.0 \times 10^3$  CFU/ml). 67.6% (23/34) of EBM had total viable counts of bacteria within  $0 - 9.99 \times 10^2$  CFU/ml (mean =  $2.25 \times 10^2$  CFU/ml) with not much differences in plate count of bacteria (Figure 4.14).

After 3 hours of storage ( $T_3$ ) at room temperature (mean =  $28.9^\circ\text{C}$ ), the plate count of bacteria in all EBM yielded total viable counts of bacteria less than  $1.0 \times 10^5$  CFU (Figure 4.14) though the differences between the plate counts of bacteria at  $T_0$  and  $T_3$  were statistically significant (Mean  $TVC = 5.49 \times 10^3$  CFU/ml,  $p = 0.0183$ ). 55.9% (19/34) of the EBM had total viable counts of bacteria within  $0 - 9.99 \times 10^2$  CFU/ml (Mean  $TVC = 2.42 \times 10^2$  CFU/ml).

**Figure 4.14: Plate Count of Bacteria in EBM at T<sub>0</sub> and T<sub>3</sub>**



However after 6 hours of storage at a mean temperature of 28.1°C (T<sub>6</sub>), the majority of EBM (25/34; 73.5%) had total viable counts of bacteria within  $1.0 \times 10^2 - 9.99 \times 10^2$  CFU/ml (Mean TVC =  $2.12 \times 10^3$  CFU/ml). 2 (5.9%) of the samples had significant levels of contamination at T<sub>6</sub> as their total viable counts were not less than  $1.0 \times 10^5$  CFU/ml (Figure 4.15). These two were EBM with the highest TVC of bacteria at T<sub>0</sub> with a mean plate count of  $2.75 \times 10^4$  CFU/ml at T<sub>0</sub>.

There was a steady increase in the number of bacteria in EBM up to 9 hours of storage at room temperature (Figure 4.15). At T<sub>9</sub> (mean temperature = 26.6°C), 55.9% (19/34) of EBM had  $1.0 \times 10^3 - 9.99 \times 10^5$  CFU/ml of bacteria present (Mean TVC =  $6.57 \times 10^4$  CFU/ml). Unacceptable numbers of microbial contaminants (TVC  $\geq 1.0 \times 10^5$  CFU/ml) were found in 5 (14.7%) of the breast milk samples having been stored for 9 hours (Mean TVC =  $2.0 \times 10^5$  CFU/ml).



**Figure 4.15: Plate Count of Bacteria in Expressed Breast Milk at T<sub>6</sub> and T<sub>9</sub>**

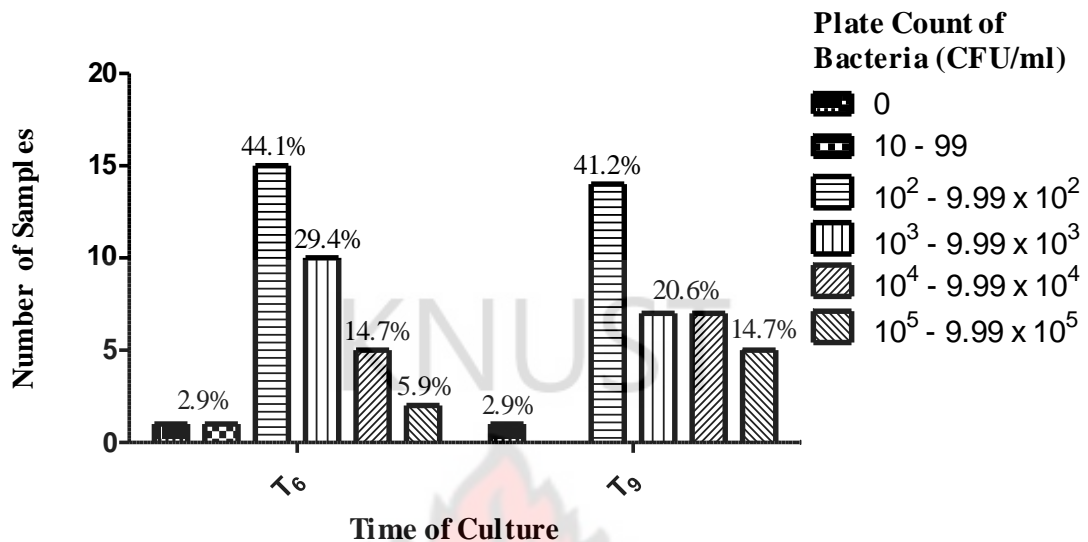


Plate count of bacteria in EBM kept for 9 hours of refrigeration at 6°C [FT(T<sub>9</sub>)] were not much different from cultures of EBM at T<sub>0</sub> ( $p$ -value of mean TVC at T<sub>0</sub> and FT(T<sub>9</sub>) = 0.0939) (Figure 4.13). 58.9% (20/34) of samples had total viable count of bacteria within 0 – 9.99 x 10<sup>2</sup> CFU/ml. None had unacceptable number of microorganisms as all plate counts of bacteria were less than 1.0 x 10<sup>5</sup> CFU/ml.

The effect of length of storage of EBM at room temperature on the plate count of bacteria was significant statistically ( $p$  = 0.0028) and accounted for 7.25% of total variance. However, the mean plate count of bacteria in EBM at T<sub>0</sub>, T<sub>3</sub>, T<sub>6</sub> and T<sub>9</sub> did not have relevant statistic difference ( $p$  = 0.1577).

## CHAPTER FIVE

### DISCUSSION

#### 5.1: PREVALENCE OF MICROBIAL CONTAMINATION OF PREPARED FORMULA ON A2, A5, MBU and B4 WARDS

Infant formula feeding involves many critical steps during handling, storage, preparation and cleaning, putting great demands upon good hygiene if contaminated and unsafe feeds are to be avoided (Agostoni *et al.*, 2004). The study shows that there is a high prevalence of bacterial contamination of prepared formula on the wards. This is consistent with results of a study conducted by Marino *et al.* (2007) to investigate the potential for microbial contamination during in-hospital preparation of infant formula. Their study found that 30% of prepared infant formula (PIF) studied, including those that were newly prepared, were heavily contaminated with bacteria. Marino and his collaborators concluded that, even when milk is prepared in a controlled environment, there is significant bacterial contamination of PIF post production.

Eighty-five percent of PIF sampled from the wards were contaminated. *Acinetobacter* sp. was the most common bacterial isolate (85.2%). *Acinetobacter* sp. is widely distributed in nature. It has been isolated from soil, seawater, freshwater, estuaries, sewage, contaminated food and mucosal and outer surfaces of animals and humans (Percival *et al.*, 2004). Also included in this list is the clinical environment where *Acinetobacter* sp. has been isolated in hospital sink traps, hospital floor swab cultures and in air samples in wards (Percival *et al.*, 2004). The ability of *Acinetobacter* sp. to survive on various surfaces in the hospital environment makes it a major source of

infection in debilitated patients (Bergogne-Berezin *et al.*, 1996). *Klebsiella pneumoniae* was the other high-yield contaminant in the PIF. It is a member of the *Enterobacteriaceae*, a group widely known to contaminate PIF (Patchel *et al.*, 1994). Also, it is part of the transient microflora of the hands (Cairo *et al.*, 2008). *Staphylococcus aureus* and *Staphylococcus epidermidis* are components of normal flora of the skin. These organisms could contaminate prepared formula if present on hands whilst handling; so are *Bacillus* sp., *Pseudomonas* sp. and *Candida* yeast which are transient flora of the hands and are eliminated through hand washing (Cairo *et al.*, 2008).

In a study in Brazil, *Bacillus* spp., coagulase-negative staphylococci, *Pseudomonas* sp., *Klebsiella pneumoniae*, *Enterococcus* sp., *Staphylococcus aureus*, *Enterobacter aerogenes* and *Enterobacter cloacae*, among other bacteria were isolated by Cairo and collaborators from milk and formula delivered by milk kitchens in public and private hospitals in Salvador (Cairo *et al.*, 2008).

The isolation of *Acinetobacter* sp., *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and the other microbial contaminants suggests the ineffectiveness of hand washing and sterilization of vessels for formula preparation done by health staff on the wards. These organisms are rapidly removed during hand washing and proper sterilization of vessels (Cairo *et al.*, 2008) and for that reason should not be present on hands and vessels during reconstitution of formula.

*Enterococcus* sp., a contaminant of 18% of prepared formula, is an indicator for faecal contamination. Therefore, the isolation of *Enterococcus* sp. from formula samples from B4 ward appears to suggest a problem with sanitary quality on the ward. Detection of

*Enterococcus* sp. in the prepared formula suggests recent contamination of hands with faecal material, indicating poor compliance with basic principles of hygiene such as hand washing or the use of water contaminated with faecal matter which indicates inadequate sterilization procedures on the B4 ward.

## **5.2: CULTURE OF UNPREPARED FORMULA**

Thirty samples of unprepared formula collected from the wards and reconstituted at the laboratory to investigate bacterial contamination were sterile. A significant body of international data indicates that powdered infant formula, prior to breaking of manufacturers' seal, are not sterile and may contain some organisms (Estrada, 2002; Drudy *et al.*, 2006). Muytjens and collaborators isolated bacterial organisms from 52.5% of powdered infant formula evaluated from 35 different countries (Muytjens *et al.*, 1988). Oonaka *et al.* (2010) also isolated *Enterobacteriaceae* from 36 (24%) of 149 powdered infant formula sampled in Japan.

The absence of organisms in the powdered samples of formula assures safety of powdered infant formula used by the Pediatric Unit of KATH. Also, the absence of organisms in the powdered samples clearly indicates that hand-washing, sterilization of vessels and or handling of prepared formula on the wards were not adequate. As a result, opportunities for contamination arose during reconstitution and when prepared foods were allowed to stay on the wards.

## **5.3: PLATE COUNT OF BACTERIA IN PREPARED FORMULA**

In spite of the high prevalence of microbial contamination of prepared formula on the wards, the quantum of microbial contamination was low; 97.6% of PIF from A2, A5 and

MBU had TVC less than the maximum locally acceptable limit of  $1.0 \times 10^5$  CFU/ml (GS574:2003) and had the majority (61.9%) between  $1.0 \times 10^2$  CFU/ml and  $9.99 \times 10^3$  CFU/ml. Also, only 1 prepared formula from B4 ward had bacterial contamination beyond the maximum acceptable limit (TVC =  $1.01 \times 10^5$  CFU/ml).

The relatively low levels of contamination of prepared formula on all wards indicate that control measures to prevent microbial contamination were considerably effective but not wholly adequate and as a result, low numbers of bacteria contaminated the formula during reconstitution.

#### **5.4: SAFETY OF PREPARED FORMULA ON THE WARDS**

The acceptable numbers of organisms that were present in the prepared formula assures the safety of formula prepared on the wards of KATH though prevalence of microbial contamination is high. However, there is concern over the microorganisms that were found in the 2 samples that contained unacceptable TVC of bacteria.

*Acinetobacter* sp., *Klebsiella pneumoniae* and *Enterococcus* sp. are notable opportunistic pathogens that could cause severe, life-threatening infections in compromised children (Percival *et al.*, 2004; Ryan *et al.*, 2004). Moreover, these 3 organisms are known to exhibit various degrees of antibiotic resistance which makes them a threat to hospitals (Sirot *et al.*, 1991; Gales *et al.*, 2001; Ryan *et al.*, 2004).

In a study conducted by Carneiro *et al.* to investigate antimicrobial resistance in Gram-negative bacilli isolated from infant formula, antimicrobial susceptibility testing showed significant resistance rates, particularly to amoxicillin/clavulanic acid, cefoxitin, cephalotin or ampicillin. One extended-spectrum  $\beta$ -lactamase-producing *K. pneumoniae*



strain was also recovered from their study (Carneiro *et al.*, 2003). Therefore, the need to control the numbers of these organisms in prepared food is imperative.

The isolation of the faecal indicator, *Enterococcus* sp., in some prepared formula is also a matter of concern. This is because obligate pathogenic enteric organisms like *Shigella* and *Salmonella* could have contaminated the prepared formula if the vehicle through which microbial contamination took place had been exposed to them since their mode of spread is faeco-oral.

### **5.5: STORAGE OF PREPARED FORMULA ON THE WARDS**

The significant effect of the 1-hour incubation of the PIF at room temperature (mean = 27.8°C) on TVC of bacteria at T<sub>1</sub> was expected ( $p = 0.0125$ ) and confirms to what has been demonstrated in a significant body of international data on microbial propagation in infant formula. Bacteria rapidly multiply in milk kept at or above room temperature and so it has been recommended that milk foods prepared should not be kept at room temperature (Agostoni *et al.*, 2004).

There were no significant differences between the mean plate counts of bacteria in PIF at different lengths of stay on the A2, A5 and MBU wards. The majority of PIF that had stayed for at most 60 minutes differed less significantly with duration of stay ( $p = 0.3660$ ). This implies that the length of stay of prepared formula on these wards did not have a significant influence on the rate of propagation of bacteria in the contaminated feeds.

Also, samples of prepared formula from B4 ward exhibited the same pattern of variation. The effect of the duration of storage on the plate count of bacteria was not significant as



the TVC of bacteria in the prepared formula stored for different periods of time before sampling did not have relevant statistic differences ( $p = 0.5207$ ). This is contrary to a known fact that bacteria greatly multiply in milk with time.

This result may have been influenced by the temperature of the prepared feeds. *Enterobacter* spp. cultivated by Nazarowec and Farber (1997) from infant formula did not grow at temperatures below 5.5°C but began to multiply at temperatures between 5.5°C and 8°C. Average generation times were 5 hours at 10°C and only 40 minutes at 23°C (Nazarowec *et al.*, 1997).

The prepared formula on B4 ward were kept in thermos flasks whereas most of the samples from the other wards were still warm at the time of sampling. Therefore, high temperature of the formula may have influenced the rate of microbial propagation and prevented organisms from multiplying greatly with time as was evident in the samples incubated at room temperature in the laboratory. This finding indicates that storage of the prepared formula in vacuum flasks could control rapid multiplication of microbial contaminants.

#### **5.6: PREVALENCE OF MICROBIAL CONTAMINATION IN EXPRESSED BREAST MILK**

Often, mothers must express their milk so it can be fed to their babies in their absence or at a later time (Tully, 2000). Breast milk expression may help mothers to overcome some obstacles to successful breastfeeding and, therefore, increase breastfeeding duration (Win *et al.*, 2006).

The results of this study indicated a high prevalence (97.1%) of microbial contamination of expressed breast milk (EBM). This is consistent with results of studies on microbial contamination of EBM. Ajusi *et al.* (1989) investigated bacterial contamination of unheated expressed breast milk stored at room temperature and found that, all 30 EBM sampled contained bacteria even though bacterial colony counts were consistently low. A bacteriological screening of expressed breast milk by Ng *et al.* (2009) revealed a high rate of bacterial contamination of EBM in Chinese women. Of 59 samples they took from 23 mothers, 63% were contaminated.

82.4% of the EBM were contaminated with *Staphylococcus epidermidis*, a major component of the skin flora. The other microbial contaminants, *Klebsiella pneumoniae* (20.6%), *Acinetobacter* sp. (14.7%), *Staphylococcus aureus* (10%), *Candida* yeast (2.5%) and *Pseudomonas* sp. (2.5%), are mostly transient flora of the skin. In a study to investigate bacteriological quality of raw human milk, Olowe and his collaborators isolated coagulase-negative *Staphylococcus* (64%) and *Streptococcus viridans* (1.1%) from 87 expressed breast milk obtained from 63 mothers on the neonatal intensive care unit of the Lagos University Teaching Hospital. The other isolates in their study were *Staphylococcus aureus* (2.3%) and *Klebsiella* (28.3%) (Olowe *et al.*, 1987). Ajusi *et al.* (1989) also isolated *Staphylococcus epidermidis* (76.6%), *Streptococcus viridans* (40%), *Escherichia coli* (2.6%), *Enterococcus faecalis* (13.6%) and *Staphylococcus aureus* (6.7%) from EBM they sampled for bacterial contamination.

The unduly high prevalence of microbial contamination may be because opportunities for contamination are readily available during expression and so these normal and transient flora of the skin may have easily contaminated the expressed milk. This finding

was probable since the lactating mothers at the MBU ward were not given rigid hygienic precautions for breast milk expression during sampling. Consequently, these organisms may have easily gained access to the milk during expression.

In recommendations for handling of mothers' own milk, Pittard *et al.* (1991) found that careful hand washing before pumping or expressing milk and using pump pieces and containers that have been thoroughly washed with soapy water and rinsed well were sufficient to control microbial contamination of expressed breast milk. Cleaning the breast and nipples or expressing and discarding the first few drops of milk were unnecessary (Pittard *et al.*, 1985; Thompson *et al.*, 1997). The findings of Thompson *et al.* (1997) in a study of contamination in expressed breast milk following breast cleansing revealed that chemical interventions may not be effective at rendering breast milk free from pathogenic bacteria. More research is needed to determine the optimal cleansing protocol to achieve bacterial decontamination of breast milk.

#### **5.7: PLATE COUNT OF BACTERIA IN EXPRESSED BREAST MILK**

The freshly expressed milk did not have significant numbers of bacteria, in spite of the high prevalence of bacterial contamination. 67.6% of EBM had TVC of bacteria within  $0 - 9.99 \times 10^2$  CFU/ml with not much differences in plate count of bacteria at  $T_0$ . However, the effect of length of storage of EBM at room temperature (mean = 27.9°C) on microbial propagation with time was significant ( $p = 0.0028$ ). This was apparent since microbial propagation with time was imminent.

Milk and milk products are excellent media for multiplication of pathogenic bacteria (Agostoni *et al.*, 2004). They have a highly nutritious composition (Talaro *et al.*, 2002).

Human milk and infant formula contain lactose, as their primary carbohydrate, minerals and vitamins (Schmidl *et al.*, 2000). These constituents make them good media for growth of most food-borne organisms, especially members of the *Enterobacteriaceae* (Goff, 2009). Also at room temperature, which falls within the mesophilic range, most of these microbial contaminant grow rapidly.

Although the microbial contaminants increased with time, the rate of their propagation was not enormous in the EBM. This may be due to inhibition of microbial growth by immune substances (phagocytes, lysozymes, lactoferrin and the others) found in human breast milk (Schmidl *et al.*, 2000). As a result, microorganisms were kept from multiplying greatly in EBM over the 9-hour storage period as compared with microbial growth in PIF, in which organisms multiplied greatly within 1-hour incubation period in the laboratory.

#### **5.8: VARIATION OF PLATE COUNT OF BACTERIA IN EBM WITH DURATION OF STORAGE**

The numbers of microorganisms in EBM at each time during the 9-hour storage period at room temperature (mean = 27.9°C) were consistently low, with TVC of bacteria in the majority of EBM (85.3%) less than the maximum acceptable limit of organisms in infant milk. Only 2 and 5 out of 34 samples of EBM exceeded the acceptable limit ( $1.0 \times 10^5$  CFU/ml) at 6 hours and 9 hours of storage respectively. Worthy of note is the fact that, the 5 samples of EBM with TVC of bacteria that exceeded the acceptable limit at T<sub>9</sub> had more isolates of organisms and relatively higher TVC in the freshly expressed milk at T<sub>0</sub>. This finding demonstrates the fact that, the type and number of organisms present at the time of breast milk expression influences the microbial quality of EBM on storage.

Storage of EBM at refrigeration temperature still remains the gold standard for keeping EBM for later use (ABM Protocol, 2010). This was demonstrated as all EBM kept at 6°C had TVC of bacteria less than the maximum acceptable limit of organisms in infant milk at T<sub>9</sub>. Also the TVC of bacteria in these refrigerated samples at T<sub>9</sub> did not differ significantly from those at T<sub>0</sub> ( $p = 0.0939$ ).

These results indicate that EBM could be kept safely at room temperature for 3 hours and could also be kept up to 9 nine hours at room temperature (mean = 27.9°C) if microbial contamination is limited. This may be done through hygienic precautions during breast milk expression as these could control the number of microorganisms that could gain access to the milk at the time of expression. Pittard *et al.* (1991) found that careful hand washing before pumping or expressing milk and using pump pieces and containers that have been thoroughly washed with soapy water and rinsed well were sufficient to control microbial contamination of expressed breast milk.

## **5.9: CONCLUSION**

It has been found that provision of germ-free reconstituted formula for children on A2, A5, MBU and B4 wards of KATH is a problem. Microbial contaminants revealed by the study suggest the existence of inadequate methods of hand washing, sterilization of vessels for reconstitution and/or handling of prepared formula by health staff on the wards. The isolation of *Enterococcus* sp., a faecal indicator, in some PIF is also a matter of grave concern. Therefore, there is the need to enact stringent measures to ensure compliance with basic techniques of good hygiene on the part of health staff on the wards.



In spite of this problem, the prepared formula on the wards have acceptable number of organisms, so are safe for consumption. The results also point out that storage of prepared formula in vacuum flasks could limit the rate of microbial propagation with time. Many studies have shown that powdered infant formula are not sterile, and may be contaminated with pathogenic organisms like *Enterobacter sakazakii* (Oonaka *et al.*, 2010). However, the absence of organisms in powdered infant formula from tins on the wards of KATH is commendable and assures the safety of PIF fed to the children.

Research has shown that human milk is remarkably good at keeping its properties for long if good food-handling techniques are used (Tully, 2000). This study demonstrates that it is possible to use unprocessed breast milk for baby's consumption if it is stored at room temperatures until 6 hours after it has been collected, in case access to refrigeration is not available. However, mothers have to be advised on the need to exercise good hygienic precautions during breast milk expression to keep the number of microbial contaminants low.

#### **5.10: RECOMMENDATIONS**

It is recommended that Standard Operating Procedures (SOPs) for hand washing, sterilization of vessels and handling of formula should be provided on the wards. In addition, stringent measures should be put in place to ensure that health staff on the pediatric wards comply with the SOPs and basic principles of good hygiene. This could guarantee adequate procedures to eliminate microbial contaminants during reconstitution of formula. Furthermore, the health staff are advised to improve upon general hygiene within the wards in order to control the number of potential microbial contaminants. Also, mothers and caregivers could be educated on the possibility of using unprocessed



breast **milk** to feed babies up to 9 hours after expression if access to refrigeration is not available.

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## **APPENDICES**

### **APPENDIX 1: QUESTIONNAIRE FOR HEALTH STAFF**

#### **BACTERIAL CONTAMINATION OF INFANT FORMULA**

**PURPOSE:** The questionnaire is to collect data for a research exercise only. Data provided shall be treated confidentially.

**INSTRUCTION:** Please tick [✓] the appropriate box where necessary and provide brief answers where required. The researcher will be very grateful if you can help provide a candid response to the following questions. Thank you.

Ward:.....

Date of sampling: .....

Time of collection of sample: .....

Sample ID. Number: .....

Name of infant formula (e.g. Cerelac, Lactogen, etc.): .....

How long has the tin of formula been opened?

☐ Within a day ☐

☐ 1 - 3 days ☐

☐ 3 - 7 days ☐

☐ More than a week ☐

How was hand washing done before infant formula was prepared:

.....  
.....  
.....

.....  
.....  
.....

How was sterilization of vessels for the preparation of infant formula done:

.....  
.....  
.....  
.....  
.....  
.....

Source of water used for preparing infant formula: .....

Temperature of water used for preparation of infant formula:

- ☐ Cold      ☐  
☐ Hot      ☐

Procedure for preparation by health staff:

.....  
.....  
.....  
.....  
.....  
.....  
.....  
.....

In what state is prepared infant formula before given to baby?

- ☐ Cold      ☐  
☐ Warm      ☐  
☐ Hot      ☐

What becomes of leftovers?

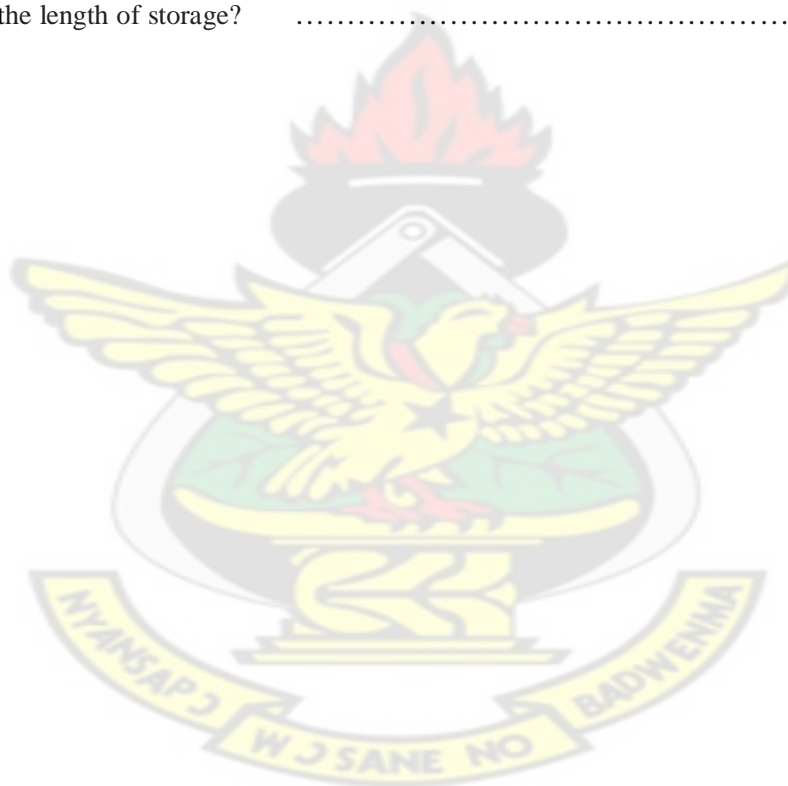
- |   |                          |
|---|--------------------------|
| <input type="checkbox"/> Discarded            | <input type="checkbox"/> |
| <input type="checkbox"/> Stored for next feed | <input type="checkbox"/> |

If stored, how stored?

.....  
.....  
.....

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What is the length of storage? .....



## **APPENDIX 2: STERILIZATION OF MATERIALS AND MEDIA PREPARATION**

### **2.1: HOT-AIR OVEN**

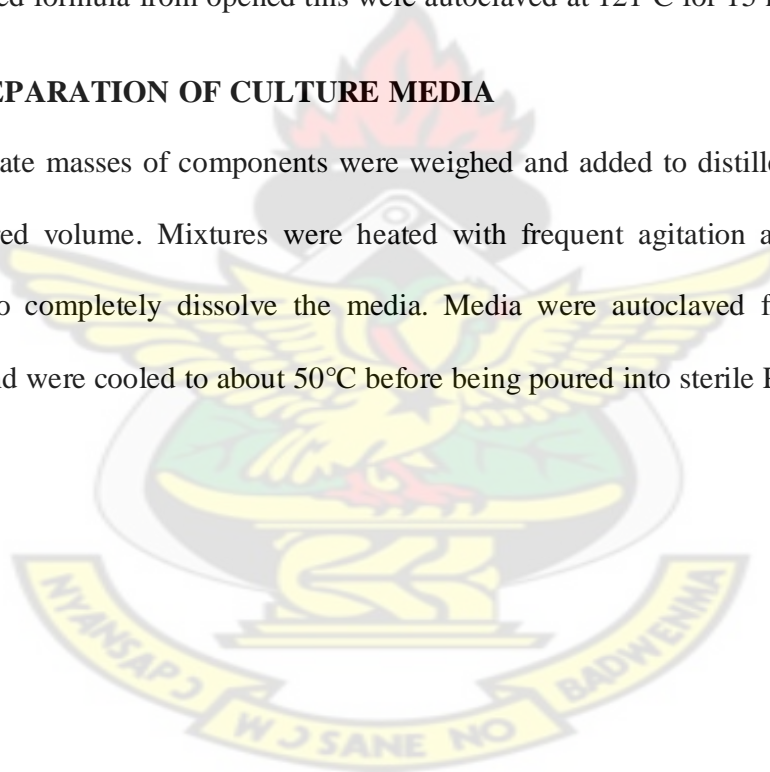
Petri dishes and sample collection bottles were sterilized by dry heat at a temperature of 160°C held for 60 minutes to kill all microorganisms and bacterial endospores.

### **2.2: AUTOCLAVING**

Media for bacterial cultures and distilled water for reconstitution of samples of unprepared formula from opened tins were autoclaved at 121°C for 15 minutes.

### **2.3: PREPARATION OF CULTURE MEDIA**

Appropriate masses of components were weighed and added to distilled/deionized water of required volume. Mixtures were heated with frequent agitation and boiled for one minute to completely dissolve the media. Media were autoclaved for 15 minutes at 121°C and were cooled to about 50°C before being poured into sterile Petri dishes.



## **APPENDIX 3: LABORATORY TESTS**

### **3.1: GRAM TECHNIQUE**

#### **Method:**

1. Dried smears were fixed with heat.
2. Fixed smears covered with crystal violet for 60 seconds.
3. Clean water was used to wash off the stain
4. Water was tipped off and the smears were covered with Lugol's iodine for 60 seconds.
5. Clean water was used to wash off the iodine.
6. Smears were rapidly decolorize (5 seconds) with acetone and washed immediately with clean water.
7. The smears were covered with neutral red stain for 2 minutes and washed off with clean water.
8. Blotting paper was used to gently blot away water from the Gram stained smears.
9. Gram stained smears were examined microscopically with the oil immersion objective to report the bacteria.

### **3.2: BIOCHEMICAL TESTS TO IDENTIFY BACTERIA**

#### **3.2.1: Catalase Test**

#### **Method:**

1. 2-3 ml of hydrogen peroxide (3%  $\text{H}_2\text{O}_2$ ) was pour into a test tube.
2. Using a sterile glass applicator, several colonies of the test organism were removed and immerse in the hydrogen peroxide solution.

3. Active bubbling indicated a positive catalase test whilst catalase negative test produced no bubbles.

**Controls:**

- ☐ Positive catalase control: *Staphylococcus aureus* ATCC 25923
- ☐ Negative catalase control: Known and characterized *Streptococcus pneumoniae* from KATH

**3.2.2: Coagulase Test**

**Method:**

1. A drop of distilled water was placed on two separate slides.
2. A colony of the test organism (previously checked by Gram staining) was emulsified in each of the drops to form thick suspensions.
3. A loopful of plasma was added to one of the suspensions and mixed gently.
4. The suspension with the plasma was carefully observed for clumping of the organisms within 10 seconds.
5. Clumping of organisms indicated that the test organism was *Staphylococcus aureus* whilst no clumping of organisms indicated the absence of bound coagulase.

**Controls:**

- ☐ Positive coagulase control: *Staphylococcus aureus* ATCC 25923
- ☐ Negative coagulase control: Known and characterized *Staphylococcus epidermidis* from KATH



### 3.2.3: Oxidase Test

#### Method:

1. 3 drops of oxidase reagent was added to a piece of filter paper placed in a clean Petri dish.
2. Using a glass applicator, a colony of the test organism was removed and smeared on the filter paper.
3. Observation was made for development of a blue-purple colour within 10 seconds.
4. Development of a blue-purple colour indicated a positive test whilst negative test produced no colour change.

#### Controls:

Positive oxidase control: *Pseudomonas aeruginosa* ATCC 27923

Negative oxidase control: *Escherichia coli* ATCC 25922

### 3.2.4: Indole Test

#### Method:

1. Test organism was inoculated into bijoux bottles with 5 ml of peptone water.
2. Inoculated bottle was incubated at 37°C for 24 hours.
3. 0.5 ml of Kovac's reagent was added and agitated gently.
4. The mixture was examined for formation of a red colour in the surface layer within 10 minutes.
5. A positive test developed red coloured surface layer whereas there was no colour change for a negative test.

**Controls:**

Positive indole control: *Escherichia coli* ATCC 25922

Negative indole control: *Klebsiella pneumoniae* ATCC 33495

**3.2.5: Urease Test****Method:**

1. The test organism was inoculated into test tube with sterile urea agar using a sterile straight wire loop.
2. Inoculated medium was incubated at 37°C for 24 hours.
3. Observation was made for colour change of medium. Pink colouration indicated a positive test whereas no change of colour of medium signified a negative test.

**Controls:**

Positive urease control: *Proteus mirabilis* ATCC 49565

Negative urease control: *Escherichia coli* ATCC 25922

**3.2.6: Citrate Utilization Test****Method:**

1. The test organism was inoculated into test tube with sterile Simmon's citrate agar using a sterile straight wire loop.
2. Inoculated medium was incubated at 37°C for 24 hours.

3. Observation was made for colour change of medium. Blue colouration indicated a positive test whereas no change of colour of medium signified a negative test.

**Controls:**

Positive urease control: *Klebsiella pneumoniae* ATCC 33495

Negative urease control: *Escherichia coli* ATCC 25922

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#### APPENDIX 4: RESULTS OF CULTURE OF PREPARED FORMULA

**TABLE 4.1A: RESULTS OF CULTURE OF PREPARED INFANT FORMULA FROM A2, A5 AND MBU WARDS**

NO.	SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub> (CFU/ml)	PLATE COUNT AT T <sub>1</sub> (CFU/ml)	ROOM TEMPERATURE AT T <sub>1</sub> (°C)	DURATION OF STAY BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
1	PFA21	$2.5 \times 10^3$	$1.0 \times 10^4$	28	2 hrs	<i>Acinetobacter</i> sp.
2	PFA51	$7.5 \times 10$	$2.5 \times 10^2$	29	30 min	<i>Acinetobacter</i> sp.
3	PFM1	$3.4 \times 10^3$	$1.0 \times 10^4$	29	1 hr	<i>Klebsiella pneumoniae</i>
4	PFA22	$5.0 \times 10^2$	$2.5 \times 10^3$	28.5	30 min	<i>Acinetobacter</i> sp.
5	PFA23	$2.36 \times 10^4$	$1.0 \times 10^5$	29	1 hr	<i>Acinetobacter</i> sp.
6	PFM2	$2.09 \times 10^4$	$1.0 \times 10^5$	29	1 hr	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.
7	PFA24	0	0	29	10 min	-
8	PFA25	$6.0 \times 10^4$	$1.6 \times 10^5$	27	1 hr	<i>Acinetobacter</i> sp.
9	PFA52	0	0	28	5 min	-

NO.	SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub> (CFU/ml)	PLATE COUNT AT T <sub>1</sub> (CFU/ml)	ROOM TEMPERATURE AT T <sub>1</sub> (°C)	DURATION OF STAY BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
10	PFA53	$2.35 \times 10^4$	$1.1 \times 10^5$	28	1 hr	<i>Staphylococcus epidermidis</i> , <i>Acinetobacter</i> sp.
11	PFA26	$1.9 \times 10^2$	$7.1 \times 10^2$	27	30 min	<i>Staphylococcus epidermidis</i> , <i>Acinetobacter</i> sp.
12	PFM3	$3.8 \times 10^3$	$1.64 \times 10^4$	28	1 hr	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>
13	PFM4	$1.9 \times 10^2$	$3.0 \times 10^2$	29	20 min	<i>Acinetobacter</i> sp.
14	PFA28	0	$1.0 \times 10^2$	28	15 min	<i>Acinetobacter</i> sp.
15	PFM5	$1.17 \times 10^4$	$3.8 \times 10^4$	28	25min	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>
16	PFA29	$9.9 \times 10^3$	$2.7 \times 10^4$	29	2 hrs	<i>Acinetobacter</i> sp.
17	PFA210	$1.0 \times 10^3$	$4.5 \times 10^3$	29	30 min	<i>Acinetobacter</i> sp.

NO.	SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub> (CFU/ml)	PLATE COUNT AT T <sub>1</sub> (CFU/ml)	ROOM TEMPERATURE AT T <sub>1</sub> (°C)	DURATION OF STAY BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
18	PFM6	$2.86 \times 10^5$	$6.60 \times 10^5$	28	1 hr. 30 min.	<i>Klebsiella pneumoniae</i> , <i>Bacillus</i> sp., <i>Acinetobacter</i> sp.
19	PFA27	$2.0 \times 10^2$	$1.1 \times 10^3$	29	30 min	<i>Acinetobacter</i> sp.
20	PFA211	$1.0 \times 10^2$	$1.1 \times 10^3$	27	5 mins	<i>Acinetobacter</i> sp.
21	PFA212	$3.0 \times 10^2$	$4.6 \times 10^3$	28	3 hrs	<i>Acinetobacter</i> sp.
22	PFM7	$1.4 \times 10^3$	$3.9 \times 10^3$	28	1 hr.	<i>Staphylococcus aureus</i> , <i>Acinetobacter</i> sp.
23	PFA213	$4.5 \times 10^3$	$8.8 \times 10^3$	26	45 mins	<i>Acinetobacter</i> sp.
24	PFM8	$4.0 \times 10^2$	$1.0 \times 10^3$	26	3 hrs	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i> , <i>Pseudomonas</i> sp.
25	PFM9	$3.09 \times 10^3$	$7.0 \times 10^3$	28	5 mins	<i>Acinetobacter</i> sp.
26	PFA214	$6.0 \times 10^2$	$1.9 \times 10^3$	28	45 mins	<i>Acinetobacter</i> sp.



NO.	SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub> (CFU/ml)	PLATE COUNT AT T <sub>1</sub> (CFU/ml)	ROOM TEMPERATURE AT T <sub>1</sub> (°C)	DURATION OF STAY BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
27	PFA215	$6.0 \times 10^2$	$1.6 \times 10^3$	27	5 mins	<i>Acinetobacter</i> sp.
28	PFM10	$3.1 \times 10^4$	$3.18 \times 10^5$	28	3 hrs	<i>Acinetobacter</i> sp.
29	PFM11	$1.12 \times 10^4$	$5.0 \times 10^4$	28	5 mins	<i>Acinetobacter</i> sp.
30	PFM12	0	0	28	30 mins.	-
31	PFM13	$2.5 \times 10^3$	$7.5 \times 10^3$	27	5 mins	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>
32	PFA216	$1.0 \times 10^2$	$4.5 \times 10^2$	27	10 mins.	<i>Acinetobacter</i> sp.
33	PFM14	0	0	27	45 mins	-
34	PFM15	$3.5 \times 10^3$	$1.10 \times 10^4$	28	5 mins	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.
35	PFA217	$1.5 \times 10^4$	$8.0 \times 10^4$	28	5 mins	<i>Acinetobacter</i> sp.
36	PFA218	0	0	27	5 mins.	-

NO.	SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub> (CFU/ml)	PLATE COUNT AT T <sub>1</sub> (CFU/ml)	ROOM TEMPERATURE AT T <sub>1</sub> (°C)	DURATION OF STAY BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
37	PFA219	$1.0 \times 10^2$	$1.1 \times 10^3$	27	1 hr.	<i>Acinetobacter</i> sp.
38	PFM16	$1.80 \times 10^3$	$1.1 \times 10^4$	27	5 mins.	<i>Acinetobacter</i> sp., <i>Staphylococcus epidermidis</i>
39	PFM17	$3.10 \times 10^3$	$6.60 \times 10^3$	27	5 mins	<i>Acinetobacter</i> sp.
40	PFM18	$5.0 \times 10^3$	$1.10 \times 10^4$	27	3 mins	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>
41	PFA220	$1.1 \times 10^3$	$8.0 \times 10^3$	28	30 mins	<i>Acinetobacter</i> sp.
42	PFM19	$8.50 \times 10^2$	$5.0 \times 10^3$	28	35 mins	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>

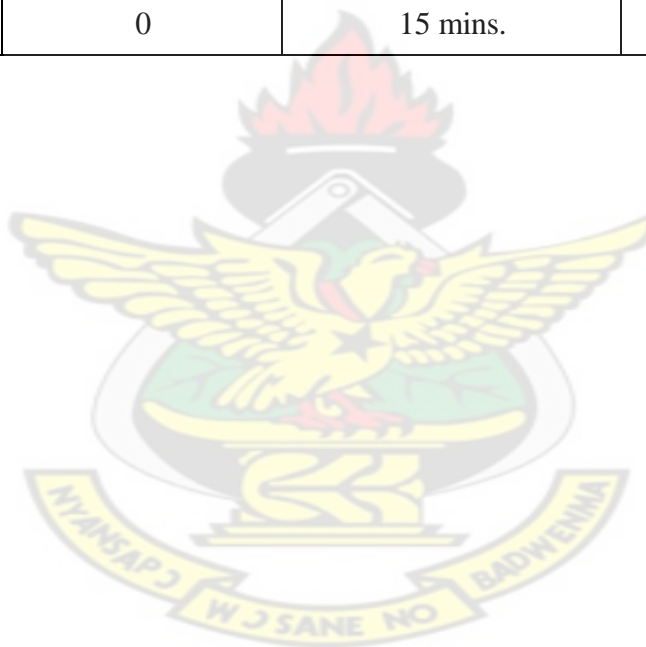
**TABLE 4.2A: RESULTS OF CULTURE OF PREPARED FORMULA FROM B4 WARD**

NO.	SAMPLE ID. NO.	PLATE COUNT (CFU/ml)	LENGTH OF STORAGE BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
1	PFB41	$5.1 \times 10^3$	1 hr.	<i>Staphylococcus epidermidis</i>
2	PFB42	$1.0 \times 10^4$	1 hr.	<i>Enterococcus</i> sp., <i>Candida</i> yeast
3	PFB43	$1.2 \times 10^3$	2 hrs.	<i>Klebsiella pneumoniae</i>
4	PFB44	0	2 hrs.	-
5	PFB45	$1.98 \times 10^3$	3 hrs.	<i>Staphylococcus epidermidis</i> , <i>Acinetobacter</i> sp.
6	PFB46	$2.2 \times 10^4$	3 hrs.	<i>Enterococcus</i> sp.
7	PFB47	$3.2 \times 10^3$	3 hrs.	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i> , <i>Enterococcus</i> sp.
8	PFB48	$4.0 \times 10^2$	3 hrs.	<i>Enterococcus</i> sp.
9	PFB49	$1.6 \times 10^3$	2 hrs.	<i>Klebsiella pneumoniae</i>
10	PFB410	$4.6 \times 10^2$	2 hrs.	<i>Staphylococcus epidermidis</i>

NO.	SAMPLE ID. NO.	PLATE COUNT (CFU/ml)	LENGTH OF STORAGE BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
11	PFB411	$1.0 \times 10^4$	2 hrs.	<i>Acinetobacter</i> sp. , <i>Enterococcus</i> sp.
12	PFB412	$1.1 \times 10^4$	5 hrs.	<i>Klebsiella pneumoniae</i> , <i>Enterococcus</i> sp., <i>Acinetobacter</i> sp.
13	PFB413	0	3 hrs.	-
14	PFB414	$8.3 \times 10^3$	3 hrs.	<i>Acinetobacter</i> sp., <i>Enterococcus</i> sp.
15	PFB415	$3.0 \times 10^2$	3 hrs.	<i>Acinetobacter</i> sp.
16	PFB416	$6.0 \times 10^2$	2 hrs.	<i>Acinetobacter</i> sp.
17	PFB417	$3.6 \times 10^4$	20 mins.	<i>Enterococcus</i> sp., <i>Acinetobacter</i> sp.
18	PFB418	$5.0 \times 10^3$	3 hrs.	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp., <i>Enterococcus</i> sp.
19	PFB419	$2.2 \times 10^3$	3 hrs.	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.

NO.	SAMPLE ID. NO.	PLATE COUNT (CFU/ml)	LENGTH OF STORAGE BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
20	PFB420	$1.01 \times 10^5$	3 hrs	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i> , <i>Enterococcus</i> sp.
21	PFB421	$3.9 \times 10^3$	5 mins	<i>Enterococcus</i> sp.
22	PFB422	$5.0 \times 10^3$	1 hr.	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.
23	PFB423	0	1 hr.	-
24	PFB424	$1.4 \times 10^3$	10 mins.	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>
25	PFB425	$1.0 \times 10^4$	10 mins.	<i>Klebsiella pneumoniae</i> , <i>Staphylococcus epidermidis</i> , <i>Acinetobacter</i> sp.
26	PFB426	$7.5 \times 10^3$	2 hrs	<i>Acinetobacter</i> sp., <i>Staphylococcus epidermidis</i>
27	PFB427	$1.10 \times 10^3$	15 mins.	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.
28	PFB428	0	5 mins.	-

NO.	SAMPLE ID. NO.	PLATE COUNT (CFU/ml)	LENGTH OF STORAGE BEFORE SAMPLE COLLECTION	ORGANISMS ISOLATED
29	PFB429	0	5 mins	-
30	PFB430	0	15 mins.	-





## APPENDIX 5: RESULTS OF CULTURE OF EXPRESSED BREAST MILK

**TABLE 5.1A: TEMPERATURE OF STORAGE OF EXPRESSED BREAST MILK**

SAMPLE ID. NO.	ROOM TEMPERATURE AT T <sub>0</sub> (°C)	ROOM TEMPERATURE AT T <sub>3</sub> (°C)	ROOM TEMPERATURE AT T <sub>6</sub> (°C)	ROOM TEMPERATURE AT T <sub>9</sub> (°C)	REFRIGERATION TEMPERATURE (°C)
BM1	29	28	26	25	6
BM2	29	28	26	25	6
BM3	29	28	26	25	6
BM4	28	29	27	25	6
BM5	28	29	27	25	6
BM6	28	29	27	25	6
BM7	26	29	28	27	6
BM8	26	29	28	27	6
BM9	26	29	28	27	6
BM10	26	29	28	27	6

SAMPLE ID. NO.	ROOM TEMPERATURE AT T <sub>0</sub> (°C)	ROOM TEMPERATURE AT T <sub>3</sub> (°C)	ROOM TEMPERATURE AT T <sub>6</sub> (°C)	ROOM TEMPERATURE AT T <sub>9</sub> (°C)	REFRIGERATION TEMPERATURE (°C)
BM11	25	29	29	27	6
BM12	25	29	29	27	6
BM13	25	29	29	27	6
BM14	25	29	29	27	6
BM15	25	29	29	27	6
BM16	26	29	28	26	6
BM17	26	29	28	26	6
BM18	26	29	28	26	6
BM19	26	29	28	26	6
BM20	26	29	28	26	6
BM21	26	29	29	27	6
BM22	26	29	29	27	6

SAMPLE ID. NO.	ROOM TEMPERATURE AT T <sub>0</sub> (°C)	ROOM TEMPERATURE AT T <sub>3</sub> (°C)	ROOM TEMPERATURE AT T <sub>6</sub> (°C)	ROOM TEMPERATURE AT T <sub>9</sub> (°C)	REFRIGERATION TEMPERATURE (°C)
BM23	26	29	29	27	6
BM24	26	29	29	27	6
BM25	26	29	29	27	6
BM26	27	29	28	27	6
BM27	27	29	28	27	6
BM28	27	29	28	27	6
BM29	27	29	28	27	6
BM30	27	29	28	27	6
BM31	27	29	28.5	28	6
BM32	27	29	28.5	28	6
BM33	27	29	28.5	28	6
BM34	27	29	28.5	28	6

**TABLE 5.2A: RESULTS OF CULTURE OF EXPRESSED BREAST MILK**

SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub>	PLATE COUNT AT T <sub>3</sub> (RT*)	PLATE COUNT AT T <sub>6</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (FT*)	ORGANISMS ISOLATED
BM1	$1.5 \times 10^2$	$2.0 \times 10^2$	$3.1 \times 10^2$	$3.2 \times 10^2$	$1.1 \times 10^2$	<i>Staphylococcus aureus</i>
BM2	$1.0 \times 10^3$	$1.7 \times 10^3$	$2.0 \times 10^3$	$3.0 \times 10^3$	$2.0 \times 10^3$	<i>Staphylococcus aureus</i>
BM3	$1.1 \times 10^2$	$1.0 \times 10^2$	$1.5 \times 10^2$	$2.0 \times 10^2$	$1.0 \times 10^2$	<i>Staphylococcus aureus</i>
BM4	$4.5 \times 10^4$	$5.0 \times 10^4$	$1.0 \times 10^5$	$5.0 \times 10^5$	$5.0 \times 10^4$	<i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.
BM5	$2.5 \times 10^4$	$3.5 \times 10^4$	$4.0 \times 10^4$	$1.0 \times 10^5$	$3.2 \times 10^4$	<i>Staphylococcus epidermidis</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter</i> sp.
BM6	$1.0 \times 10^4$	$5.0 \times 10^4$	$1.0 \times 10^5$	$2.0 \times 10^5$	$2.0 \times 10^4$	<i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i> , <i>Staphylococcus aureus</i>
BM7	$1.0 \times 10$	$5.0 \times 10$	$3.0 \times 10^2$	$5.0 \times 10^2$	$2.0 \times 10$	<i>Pseudomonas</i> sp., <i>Staphylococcus epidermidis</i>

SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub>	PLATE COUNT AT T <sub>3</sub> (RT)	PLATE COUNT AT T <sub>6</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (FT)	ORGANISMS ISOLATED
BM8	$1.0 \times 10$	$4.0 \times 10$	$1.0 \times 10^2$	$5.0 \times 10^2$	$7.0 \times 10$	<i>Staphylococcus epidermidis</i>
BM9	$1.6 \times 10^2$	$3.4 \times 10^2$	$5.0 \times 10^2$	$2.8 \times 10^3$	$3.2 \times 10^2$	<i>Staphylococcus epidermidis</i>
BM10	$1.5 \times 10^2$	$2.7 \times 10^2$	$7.0 \times 10^3$	$2.0 \times 10^4$	$2.3 \times 10^2$	<i>Staphylococcus epidermidis, Klebsiella pneumoniae</i>
BM11	$8.0 \times 10^2$	$3.11 \times 10^3$	$2.79 \times 10^4$	$5.0 \times 10^4$	$1.29 \times 10^3$	<i>Staphylococcus epidermidis, Acinetobacter sp.</i>
BM12	$2.2 \times 10$	$7.0 \times 10$	$1.0 \times 10^2$	$2.5 \times 10^2$	$2.3 \times 10$	<i>Staphylococcus epidermidis, Candida Yeast</i>
BM13	$2.0 \times 10^2$	$4.9 \times 10^2$	$8.0 \times 10^2$	$1.5 \times 10^3$	$1.5 \times 10^2$	<i>Staphylococcus epidermidis</i>
BM14	$2.0 \times 10^2$	$2.6 \times 10^2$	$4.0 \times 10^2$	$7.0 \times 10^2$	$2.2 \times 10^2$	<i>Staphylococcus epidermidis</i>

SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub>	PLATE COUNT AT T <sub>3</sub> (RT)	PLATE COUNT AT T <sub>6</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (FT)	ORGANISMS ISOLATED
BM15	$7.8 \times 10$	$1.8 \times 10^2$	$4.0 \times 10^2$	$8.0 \times 10^2$	$5.0 \times 10$	<i>Staphylococcus epidermidis</i>
BM16	$1.18 \times 10^3$	$4.20 \times 10^3$	$1.09 \times 10^4$	$3.09 \times 10^4$	$1.87 \times 10^3$	<i>Staphylococcus epidermidis</i>
BM17	$3.7 \times 10^2$	$4.2 \times 10^2$	$5.0 \times 10^2$	$7.0 \times 10^2$	$4.0 \times 10^2$	<i>Staphylococcus epidermidis</i>
BM18	$2.20 \times 10^3$	$2.64 \times 10^3$	$3.0 \times 10^3$	$5.30 \times 10^3$	$1.95 \times 10^3$	<i>Staphylococcus epidermidis</i>
BM19	$2.0 \times 10$	$4.0 \times 10$	$6.0 \times 10$	$1.0 \times 10^2$	$1.8 \times 10$	<i>Staphylococcus epidermidis</i>
BM20	$1.92 \times 10^3$	$2.82 \times 10^3$	$4.2 \times 10^3$	$6.5 \times 10^3$	$2.0 \times 10^3$	<i>Staphylococcus epidermidis</i>
BM21	0	0	0	0	0	-
BM22	$5.0 \times 10$	$1.0 \times 10^2$	$2.5 \times 10^2$	$6.0 \times 10^2$	$4.3 \times 10$	<i>Staphylococcus epidermidis</i>
BM23	$4.0 \times 10$	$1.5 \times 10^2$	$3.0 \times 10^2$	$5.5 \times 10^2$	$4.4 \times 10$	<i>Staphylococcus epidermidis</i>

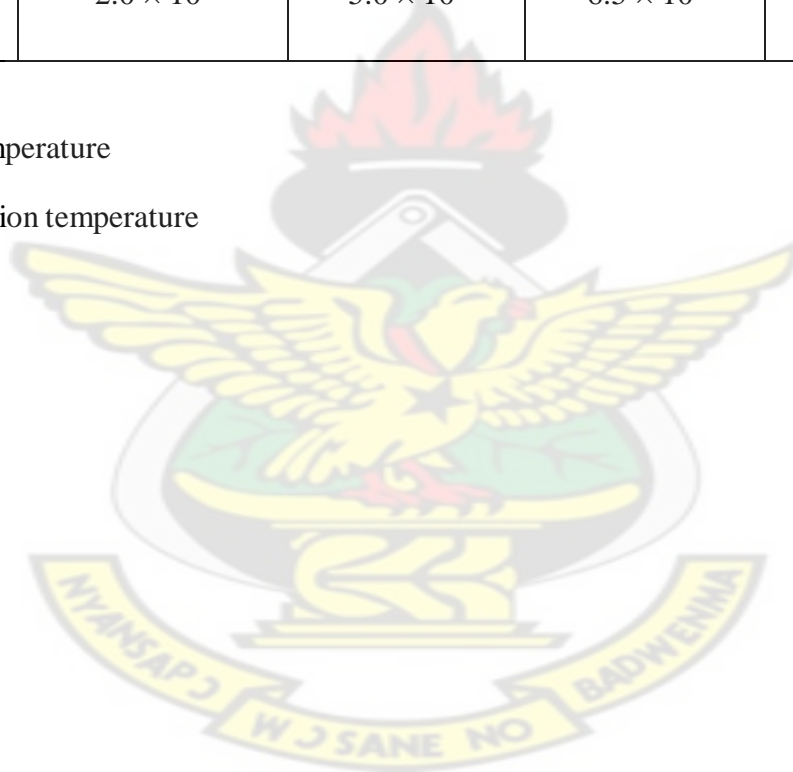


SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub>	PLATE COUNT AT T <sub>3</sub> (RT)	PLATE COUNT AT T <sub>6</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (FT)	ORGANISMS ISOLATED
BM24	$6.6 \times 10^2$	$9.7 \times 10^2$	$1.2 \times 10^3$	$3.5 \times 10^3$	$7.0 \times 10^2$	<i>Staphylococcus epidermidis</i>
BM25	$1.5 \times 10^2$	$2.4 \times 10^2$	$4.0 \times 10^2$	$7.0 \times 10^2$	$1.7 \times 10^2$	<i>Staphylococcus epidermidis</i>
BM26	$8.6 \times 10^2$	$1.26 \times 10^3$	$4.4 \times 10^3$	$8.0 \times 10^3$	$1.13 \times 10^3$	<i>Staphylococcus epidermidis</i>
BM27	$4.4 \times 10^2$	$1.1 \times 10^3$	$6.2 \times 10^3$	$3.12 \times 10^4$	$1.34 \times 10^3$	<i>Staphylococcus epidermidis</i> , <i>Klebsiella pneumoniae</i>
BM28	$5.2 \times 10^2$	$1.56 \times 10^3$	$3.5 \times 10^3$	$2.48 \times 10^4$	$7.1 \times 10^2$	<i>Staphylococcus epidermidis</i>
BM29	$2.01 \times 10^3$	$7.5 \times 10^3$	$3.0 \times 10^4$	$1.0 \times 10^5$	$5.0 \times 10^3$	<i>Staphylococcus epidermidis</i> , <i>Acinetobacter</i> sp., <i>Klebsiella pneumoniae</i>
BM30	$1.25 \times 10^3$	$6.0 \times 10^3$	$9.7 \times 10^3$	$5.0 \times 10^4$	$4.5 \times 10^3$	<i>Acinetobacter</i> sp., <i>Staphylococcus epidermidis</i>
BM31	$2.1 \times 10^2$	$4.7 \times 10^2$	$5.0 \times 10^2$	$7.8 \times 10^2$	$3.3 \times 10^2$	<i>Staphylococcus epidermidis</i>

SAMPLE ID. NO.	PLATE COUNT AT T <sub>0</sub>	PLATE COUNT AT T <sub>3</sub> (RT)	PLATE COUNT AT T <sub>6</sub> (RT)	PLATE COUNT AT T <sub>9</sub> (RT)	PLATE COUNT AT T <sub>12</sub> (FT)
BM32	$1.0 \times 10^2$	$2.1 \times 10^2$	$3.2 \times 10^2$	$6.5 \times 10^2$	$1.4 \times 10^3$
BM33	$5.0 \times 10^3$	$1.0 \times 10^4$	$7.0 \times 10^4$	$1.0 \times 10^5$	$5.5 \times 10^5$
BM34	$2.0 \times 10^3$	$5.0 \times 10^3$	$6.5 \times 10^3$	$1.0 \times 10^4$	$5.0 \times 10^4$

RT = Room temperature

FT = Refrigeration temperature



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