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COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Microbiological Safety of Powdered Infant Formula for Infants below Six Months of Age in Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Waktole Gobena Sima, entitled: ***Microbiological safety of powdered infant formula for infants below six months of age in Addis Ababa, Ethiopia*** and submitted in partial fulfillment of the requirements for a Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards for originality and quality.

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Abbreviations

AMR: Antimicrobial Resistance

ACC: Aerobic Colony count

CAC: Codex Alimentarius Commission

CLSI: Clinical and Laboratory Standards Institute

FDA: United States Food and Drug Administration

ICU: Intensive Care Unite

ISO: International Organization for Standardization

NEC: Necrotizing Enterocolitis

NMKL: Nordic Committee on Food Analysis

PIF: Powdered Infant Formula

TSA: Tryptone Soy Agar

UNICEF: United Nations Children's Fund

VRBA: Violet Bile Lactose Agar

WHO: World Health Organization

Abstract

Background: In terms of food safety infants are considered to be one of the highest risk groups because their resistantancy system is not wholly developed. Powdered infant formula issued in instances when breastfeeding is not possible or inadequate as a substitute but it is not a completely microbial free product and is sometimes a vehicle for certain pathogens like *Cronobacter* species and others.

Objective: the purpose of this work was to determine or evaluate the microbial quality of infant powdered formula milk for Infants which are lower than six months of age.

Methods: A cross sectional study was done on 106 Infant Powdered formula milk samples collected from Addis Ababa city between March and June 2020 to assess the microbial quality using mesophilic aerobic colony count, Enterobacteriaceae, coliforms, and pathogens *Salmonella* and *Cronobacter* species using standard ISO methods at EPHI and SPSS version 20 was used for analysis and mostly descriptive statistics were employed.

Results: Mesophilic aerobic counts were detected in 9.4% (n=20) of the samples. Enterobacteriaceae and total coliforms were detected in 9.4% (n=10) and 7.5% (n=8). *Cronobacter* species were also detected with a prevalence rate of 5.6% (n=6/106) and no *Salmonella* species was detected in this study.

Conclusion: This study shows that quality parameters and the incidence of *the Cronobacter* species were 5.6% (6/106). These findings are all out of the recommended international specifications by Codex of Alimentarius Commission.

Recommendation: The result of this study contributes to the knowledge used in monitoring the health problem of infants that come from PIF milk contamination. It will raise the producers and health regulatory body of Ethiopia since the consequence of these products could pose very great public damage as it is imported and consumed in mass to the infants in Ethiopia.

Keywords: Powdered Infant Formula, *Cronobacter* species, *Salmonella* species, Mesophilic Aerobic Colony Count, Enterobacteriaceae.

1 Introduction

1.1 Background

Globally attentions were given to the safety of feeds for infant children for the prevention of potentially severe infections (1). World Health's Organization (WHO) commends that infants are completely breastfed for up to the age of six months and thereafter follow to breastfed in mixing with appropriately healthful complementary foods for up to two years of age and above (2). Breast milk, a complex nutritional diet containing all necessary nutritional requirements like hormones, long-chain fatty acids, and antibodies to prevent infection, is important for infants in general (3). Hence, the mother's nutritious milk is widely accepted as an organic natural fluid necessary for maximum infant nutritive development and all side growth (3).

The selection of breastfeeding is mostly mother based or personal and is sometimes influenced by many factors. Under certain instances, it can be interrupted by a situation like inadequacy of mother's milk or if it is not possible for the mom to breastfed or the mother may go for work daily which doesn't enable her to breastfeed. Infant formula powders are hence of choice during this time as a second option to provide the necessary infant nutrition (4). Infant Powdered Formula (PIF) is provided to low and very-low-birth-weight newborns, newborns/infants with special nutritional needs, infants born from mothers with nutritional or health problems (e.g., HIV positive), in general, infants under six months (4, 5, 6). Though they are rated as options to mother's milk for infant growth, they are not always sterile (5). This makes PIF an excellent medium for microorganisms to replicate and because of their immunity; newborn babies are extremely sensitive to infections (6, 7). Maintaining the microbial quality of PIF is the total sum of the processes during production, preparation, and mixing of the components considering that the excellence of the raw ingredients and hygienic conditions of the production site (8).

On some occasions, PIFs were contaminated with dangerous bacteria like *salmonella, Cronobacter* species which are implicated as the cause of infection in infants (9). These infection-causing pathogenic microorganisms are rated as the supreme hazard for infants and children less than one year. According to FAO/WHO, 2004 reported for the first time, these organisms have clear shreds of evidence of causalities with illness (10).

Cronobacter species are oxidase-positive, facultative anaerobe, Gram-negative, motile, non-spore-forming peritrichous flagella (have flagella covering the surface of the cell), and rod-shaped, belonging to the Enterobacteriaceae family. At present, the genus comprises eight species which *C.sakazakii, C. malonaticus, C. muytjensii, C. turicenseis, C. condiment, C. dublinensis,* and *C. universalis*. *Cronobacter* is ubiquitous and they were identified from a varied kinds of food categories, domestic and hospital environments, and also from animal and human sources. *Cronobacter* spp. can grow at a range of temperatures between 5-47ºC and PH as low as 3(9, 10). Although rare, a Cronobacter infection signifies a severe health threat in highly susceptible neonates (<28 days), infants immunocompromised, especially those of lower birth-weight (<2500g), and elderly (8). In neonates, death has been continuously reported by different organs to occur from 40% to 80% of cases (11). The microorganism has been implicated in neonatal infections leading to meningitides, necrotizing type enterocolitis (NEC), septicemia, chronic sequelae, and possible death (12, 13).

The other pathogenic organisms rated as the greatest risky in PIF are *Salmonella* species; which can be isolated from dried infant food including infant formula powder, with different serotypes (14). Sometimes intrinsic contamination of PIF with non-typhoidal fever from *Salmonella enterica* is an imperative source of infection and illness in infants who consume reconstituted PIF in place of breast milk (15). Non- typhoidal *Salmonella* causes diarrhea, vomiting, and fever but in certain instances may worsen to more serious circumstances such as bacteremia, septic arthritis, pneumonia, and meningitis (16, 17). Due to their not fully formed immune-system, premature infants diseased by non-typhoidal *Salmonella* are most probable to grieve this devastating potentially lethal illness (17, 18).

There are also many other Enterobacteriaceae and other bacteria that can reside in a PIF and may pose risks to infants (19). *S.aureus, Streptococcus species, Pseudomonas species, Proteus species,* and *Bacillus cerus* are some of the most common microorganisms isolated from PIF (18).

Therefore, Information about infant formula and microbial quality is scarce in Ethiopia and we planned to assess the situation and the finding will be used for intervention measures in general.

1.2 Statement of the Problem

Infant Powdered feeding formulae are mostly not a type of sterile foods or products (19). It has been associated with very serious and severe illness and death to infants who feed on it due to infection with dangerous microorganisms like *the Cronobacter* species. During production, that can most of the time easily be microbially contaminated by harmful microorganisms like *C. sakazakii* (19, 20).

By the current production expertise, it is not promising to produce sterility assured products. Contamination can easily happen from chemicals and/or additives for nutrition and from the production surroundings. By creating biofilm on the production equipment severe pathogens like *C. sakazakii* and *salmonella enterica* cause recontamination during the manufacturing course of the product. *C. sakazakii* is a microorganism or type of opportunistic bacteria that can easily found in this environment by resisting a highly dry and desiccated environment. These microbes can also reside in dry and desiccated foods, such as powdered infant formula. Any individual can get sick from *Cronobacter*, but the infection get worsen most of the time in premature infants and immunocompromised ones (21).

Cronobacter infection is rare, but it can be fatal in newborn infants. Infection in the case of infants usually may occur for the 1st days of life or weeks of age. *Cronobacter* species scan causes dangerous blood sepsis or infection of the membrane rounding the brain and meninges (meningitis). Infants who get born prematurely and infants with lower immunity or lower capability to fight germs and sickness due to diseases (such as HIV) or medicinal management (such as chemotherapy for cancer) are also more easily likely to get sick (22, 23,24, 25, 26).

In Europe and most western countries, the risk for infants from PIF milk has given increased attention in recent years due to increased knowledge about *Cronobacter* and *Salmonella* species in PIF. It is suggested that occurrence of the infection may be under-reported in developing, possibly due to absence of medical microbiology analysis facilities which are resulted from a knowledge gap (27).

Furthermore, no studies are available on the contamination levels of PIF available to the public utilizing these products. Therefore this study will provide data for the micro-parametric profile and quality status of PIF.

1.3 Significance of the study

The final results of this study will have a great use to individuals who are consumers of PIF by providing the microbial quality status of the product used for infants. It provides mature information for health care providers who prescribe PIF for babies and infants who are below their six months of age and prematurely born infants which are admitted to hospital and are on powdered infant formula. The study also provides good information to the scientific community about the quality status of the PIF. Finally, the controlling body of the Ethiopian ministry of health and other concerned body will utilize the results of the study to plan on how to make the market safe for consumers.

2 Literature Review

As dehydrated products, it's not usually possible using currently available technology to manufacture formulae which are free of or low numbers of organisms, i.e., the products may not easily be fully sterilized (27, 28). Thus, its microbiological safety requires strict devotion to good hygienic practice during both manufacture and utilization (27). Two FAO/WHO conferences by experts on the microbial quality parameters and safety issues of PIF well-thought-out cases of illnesses in infants due to PIF consumption either epidemiologically or microbiologically. These experts recognized three groups of microorganisms basing on the power of confirmation of a connecting relationship between their occurrence in PIF milk products and illness of infants: 1) microorganisms with apparent facts of causality, like *Salmonella enterica* and *Enterobacter sakazakii*; 2) microorganisms with clear cases of disease and found in PIF, but not been convincing means of transportation and source of infection. 3) Micro-organism that has been isolated in PIF but hasn't remained associated as causing illness (29).

Salmonella is familiar with long-standing food-borne human pathogens (30). The incidences of Salmonellosis among infants, originating from different sources, in the USA the incidences of salmonellosis were reported to be more than eight times than all ages. Infants, especially the immunocompromised, are mostly and particularly susceptible to this infection severely. From the most reported 6 outbreaks of Salmonellosis infections connecting about 287 infants were related with PIF from 1985 to 2005(20-23).

***Cronobacter* species** has newly emerged as a pathogen of infants in recent times. FAO/WHO professional conference has identified all babies of infant type (<12 months age) as a group at a specifically proximate to risk for *C.sakazakii*. Infections from *Cronobacter* group of organism have mostly been entirely recognized as both sporadic cases and outbreaks. Even though the frequency of *Cronobacter* species infections in infants seems to be low, the consequences of the illness can be severe. Infants with immunocompromising circumstances have developed bacteremia at 10 months of age and formerly healthy infants have also developed invasive disease outside the neonatal period. Infections have also happened in both hospital and outpatient department settings and also at individuals' homes (31, 32, 33, 34).

Aerobic colony counts (ACC) is a sign of the number of the microbial population in a sample of interest. The idea for ACC was developed basing on the hypothesis that each microbial cell will grow to a single apparent colony after cultured media containing suitable basic

nutrients for non-selective growth of all available microorganisms. ACC is not a total microbial population rather it is a nonspecific measure for microorganisms that will grow aerobically at 25 to 40°C. ACC does not distinguish types of bacteria.

ACC is used to measure hygienic quality, organoleptic suitability, adherence to the good practices manufacturing (GMP), and lesser extent, as an indicator of quality and safety (2, 15, and 28). ACC may also offer information concerning shelf-life or impending organoleptic alteration in a food. ACCs are not good indicators of safety on most occasions, since it does not directly correlate to the presence of pathogens or toxins. A low ACC result is not to say the PIF is pathogen-free. However, products and some ingredients of these products having very high or remarkably high ACCs may practically be expected to be possible health threats, giving pathogen screening results (28, 35).

Safety guiding principles are regularly functional to raw ingredients and end products. Using ACC for ingredients cannot be or can be suitable as a quality measure. A food producer's decision to apply ACC guidelines on ingredients must be based on the ingredient's consequence on the final product. For instance, in dried foods like PIF, ACC may be used as a means of assessing the sufficiency of moisture control during the drying process. Finally, ACC can also be utilized to evaluate sanitary conditions of equipment and utensils mostly used in the production of PIF (2, 15, 28, and 35).

3 Objectives

3.1 General Objectives:

- To determine microbial safety and quality of infant powdered formula milk products for infants less age than six months of life in Addis Ababa, Ethiopia.

3.2 Specific Objectives:

1. To assess microbial quality (ACC, Enterobacteriaceae, total coliforms, thermotolerant coliforms, and *E.coli*) of PIF milk for infants less-than the first six months in the stated study area,
2. To determine the prevalence of pathogens *Cronobacter* and *Salmonella* species in PIF milk for infants less-than the first six months.

4 Hypothesis

Ho: Powdered infant formulas available in the Ethiopian market are not contaminated by pathogens, safe, and do not pose any health risks to the public utilizing these products.

5 Materials and Methods

5.1 Study Area

This study was currently conducted some selected areas in Addis Ababa city. Addis Ababa is an administrative and commercial capital in Ethiopia and home to most businesses including PIF retails. These PIF products are imported from abroad. Despite their economic, knowledge and attitude variations, most populations of the city which are unable to access breast milk utilize PIF by purchasing it from the supermarkets and pharmacies.

5.2 Study Design and Period

A cross sectional study was done from March to December 2020 to assess the microbial quality of PIF milk for infants less than the first six months. This work was done in some purposively selected supermarkets and pharmacies of Addis Ababa, where most of the retail outlets for PIF products are frequently available.

5.3 Source powdered Infant Formula

The samples were bought from one of the following: supermarkets, shops, and pharmacies which were found in (the stated study area).

5.3.1 Powdered Infant formula for the Study

The study of powdered infant formula food includes all powdered formula for infants who are below the first six months,54 found in Addis Ababa city administration, that ful-fill the inclusion and exclusion.

5.4 Inclusion and exclusion criteria

5.4.1 Inclusion criteria

The samples include the following:

- Dried or powdered infant formulae intended for infants below 6 months of age(level 1)

5.4.2 Exclusion criteria

- Other baby food types (e.g. tinned and jarred baby food, and cereals.)
- Expired PIF
- Opened PIF
- Not clearly labeled

5.5 Measurement and Data collection

5.5.1 Sample size calculation and sampling method

During sample collection, there were five brands of PIF in Addis Ababa markets of which were listed as brand A, brand B, brand C, brand D, brand E. Sample collection was done using the *convenience sampling technique* from the sample source. Samples of PIF that fit the inclusion criteria and easily accessible, readily available during the sample collection time was purchased according to *American Theoretical and Applied Statistics* (33). To get the most desirable results the sampling was done from different locations. As a result, 106 PIF were *purposively* collected. All the available brands of PIF products were *proportionally* purchased during sample collection.

5.5.2 Data Collection Procedure

Sampling procedures were conducted by purchasing powdered infant formulas during the period of sample collection. From every sample source, five samples, according to International code microbiological sampling for food (ICMSF), which have a similar batch number and properly sealed was sampled (purchased). The sample collectors have filled the appropriate sections of the associated questionnaire (Appendix A) at the time of sampling.

5.6 Microbiological Analysis

Sample analysis: Each five bottles of PIF (of the same batch of production) from each brand was analyzed for: *Cronobacter* species, *Salmonella* species, Aerobic colony count, Total-coliforms, fecal-coliforms, and *E.coli* and Enterobacteriaceae using the following methods.

A method used for detection of *C. sakazakii* in milk products (ISO 22964:2006). During this procedure, 25g of PIF was mixed with 225ml of peptone buffered water (Oxoid UK) for non-selective enrichment. After 24 hrs incubation at 36±1 0c 1ml of the culture from the buffered peptone water was transferred to a selective enrichment broth Vancomycine modified leuren triptone broth (Van mLTB) (fluka) and incubated at 42^0c for the next 24hrs and then loop full of the culture was transferred to Cronobacter sakazakii chromogenic agar (fluka). The isolated organism was reported in 25g as present or absent (34).

Aerobic colony Counts at 30^oc for 48 hours using (NMKL, No 86, 2006). It was done by dissolving 25g of PIF in 225ml of buffered peptone water. After homogenization, the samples were serial diluted using 1% normal saline peptone water and inoculating 1ml by pour plate

method into a Petri dish with plate count agar (oxoid) and incubating at 30^0c for 48-72 hours. During this procedure, all mesophilic microorganisms that grow with any colony characteristics were counted, enumerated, and reported as cfu/g (35).

Salmonella spp were detected by using a detection method (NMKL, No 125, 2005). The samples were non-selectively enriched using buffered peptone water (BPW). After 24 hrs of incubation at 36 ± 1^0c again selectively enriched using selenite cysteine broth and Rappaport vassilaids enrichment broths and incubated at 35 ± 1^0c. After purification and biochemical identification qualitative results were reported as present absent (36).

Isolation of Enterobacteriaceae was done by using a version of (*NMKL No 144, 2005).* A total of 25g of infant powder formula was mixed with 225 ml of diluents of buffered peptone water. Then serial dilution was carried out using 9ml of normal saline. For primary isolation of Enterobacteriaceae, an overlay method was carried out where 1ml of each dilution was pipetted into a Petri dish and then mixed with 10-15ml of Violet Red Lactose agar(VRBLA) after which about 10ml of VRBLA was poured on to the solidified medium. The plate was then be incubated at 37^oc for 24h. After incubation five suspected colonies were subcultured from VRBLA to tryptone soya agar (TSA).TSA was incubated at 37^oc for 24h ±4h (36) and then checked for oxidase. Finally, oxidase negative results were considered as Enterobacteriaceae (37).

For differentiation of Enterobacteriaceae Laboratory standard methods were employed (37). Discrete colonies of bacteria were subcultured from plates having appropriate colonies using the streaking method. This takes place several times until a separate colony was identified. The separated single colony was transferred to Tryptone soy agar for biochemical tests(38, 39).

5.7 Data Quality Assurance

A new batch of media was incubated with known organisms *(C.sakazakii, salmonella, E.coli)* in each analysis. Universal safety precautions for microbiology laboratory were strictly followed. A new batch of media was incubated with known organisms *Cronobacter sakazakii ATCC* 29544 Salmonella enterica ATCC 14028 and *E.coli* 25922 were used as a quality control protocols.

5.8 Data Analysis and Interpretation

Data entry and analysis: all data from the different microbiological quality analyses were cleaned and processed by using SPSS (version 20). Statistical bias during analysis was reduced by using values different from zero (0.5) for results with no growth or values. Mainly descriptive statistics mainly measures of central tenancy and dispersion were employed for quantitative variables. For qualitative variables percentage and absolute frequencies were used. For comparison purposes, the Kruskal-Wallis tests were used with SPSS statistical software at the significance level of α=0.05. The findings were compared with international standard of codex Alimentarius commission.

5.9 Ethical considerations

As the samples were purchased from supermarkets, shops, pharmacies and Hospitals; each of the samples were represented with laboratory codes to prevent the undesirable effect of the research findings. Final report writing was utilized these codes until publication and no brand or generic name of the PIF was included. Ethical clearance was collected from Addis Ababa University School of medical laboratory science and Ethiopian Public health Inastitute.

5.10 Operational Definitions

Microbial Food quality indicators: reflect the microbial quality of foods relative to product shelf life or their safety from foodborne pathogens.

Mesophilic Aerobic counts or Aerobic Colony Count (ACC): the load or the number of microorganisms that grow using basic and general-purpose medias in a food and feed or product obtained under optimum circumstances of culture conditions.

Total coliforms: is a hygienic status indicator and its high level counts generally indicates an unhygienic condition and/or poor sanitation practices through food production and are expected to be null in the case of PIF.

Fecal coliforms: The groups of fecal coliforms have a higher probability to contain organisms of fecal origin animals or birds and hence indicating fecal origin contamination and are thermotolerant organisms.

Powdered Infant formula: breast milk desired to substitute or manufactured to fulfill the nutritional necessities of children up to the introduction of suitable complementary and balanced feeding.

Microbial safety: is the status of PIF to contain pathogens like *Cronobacter* spp., *Salmonella* spp., drug-resistant microrganisms.

6 Results and Discussions

6.1 Results

A total of 106 PIF milk samples of five different brands imported from abroad were selected for this study. The products were usually purchased by entirely at different market cites of Addis Ababa and evaluated for the mesophilic microbial count, Enterobacteriaceae, total coliforms, and *Cronobacter* species and they were detected in (n= 20; 18.8%, n=10; 9.4%, n=8; 7.5%), of the samples respectively.

From the total analyzed 106 PIF milk samples 20 had ACC (n=20; 18.8%). When the samples were analyzed for ACC in each of the PIF milk brands no growth was found in brands A, B, and E but brand C had growth in (n= 10/24; 41%) and brand D had growth in (n=10/24: 41%). In brand C 9(n=9) samples have ACCs above 5×10^2 cfu/g. Again brand C has the highest ACC from all brands with counts between 2.8×10^2 - 5.52×10^3 cfu/g. The other brand labeled D which contains few Aerobic colony counts of 4×10^1cfu to 1×10^2 cfu/g with most counts below 1×10^2cfu/g (Table 1).

Table 1. Aerobic plate counts of different brands of infant formulae samples (cfu/g).

brands		**Aerobic colony counts (cfu/g)**					
		$<1\times10^1$	1-10	11-10^2	101-10^3	$>10^3$	Total
Brand	Brand A	24	0	0	0	0	24
	brand B	24	0	0	0	0	24
	brand C	14	0	0	1	9	24
	brand D	14	0	9	1	0	24
	Brand E	11	0	0	0	0	11
Total		87	0	9	5	5	106

Table 2. Aerobic colony counts (ACC), Enterobacteriaceae (ENT), and Total coliform count for PIF brands and country of origin of manufacturers.

	Counts in cfu/g		
PIF brands	ACC (min-maximum)	Ent (min-maximum)	Total coliforms (min-maximum)
A	<1	<1	<1
B	<1	<1	<1
C	$0\text{-}5\times10^3$	$0\text{-}1.5\times10^3$	$0\text{-}1.7\times10^2$
D	$0\text{-}1\times10^2$	<1	<1
E	<1	<1	<1
α=0.05			
Manufactures and country of origin			
1, Switzerland	<1	<1	<1
2, Germen	<1	<1	<1
3, USA	$0\text{-}5\times10^3$	$0\text{-}1.5\times10^3$	$0\text{-}1.7\times10^2$
4, France	<1	<1	<1
5, UK	<1	<1	<1
α=0.05			

Note: <1= no growth

Positivity for Enterobacteriaceae (ENT) was found in one product of the evaluated five brands of PIF milk products at 9.4% (n=10/106). It was found in only brand C with minimum counts of 3.8×10^1 cfu/g to maximum 3.88×10^3 cfu/g. Brands A, B, D, and E were free from Enterobacteriaceae. The statistical difference between the Enterobacteriaceae counts and the brands of PIF was (α=0.02). For the PIF manufacturers, only country 3 has Enterobacteriaceae highest counts of $3.88x10^3$ CFU/ g and Enterobacteriaceae were found in (n=10/24, 33.3%) of brand C but null in other brands.

As shown in **table1** and **table 2** total coliforms were detected in one brand (n=8/24, 33.3% in brand C) in 7.5 %(n=8/106) of total PIF milk with the maximum number of 1.7×10^2 cfu/g. Only brand C has shown contamination with total coliforms. The other four brands of PIF A, B, D, and E do not show contamination with Total coliforms.

Regarding the source country (**table 2**) United States exhibited the highest ACC counts with a maximum count of 5.52×10^3 cfu/g, nine samples with counts above 5×10^2 cfu/g, and France (brand D) showed a low number of ACC but PIF formulas from other counties have no ACC counts at all. There is a significant difference existed between the source country and the ACC and Enterobacteriaceae counts of PIF milk products. For ACC also product from the United States has the highest counts up to 5.88×10^3 followed by a product from France which contains maximum ACC counts 1×10^2 cfu/g. products from other countries have no ACC, Enterobacteriaceae, and total coliforms at all. Total coliforms were also isolated from a product imported from the United States. It was detected in nine products of brand C ou of 24 bottles (9/24, 37.5%). PIF milk Products from other countries does not contain total coliforms (table2).

The spearman's rank-order correlations r (sig. at the 0.05 level, 2-tailed) were run to determine the relationship between ACC, Enterobacteriaceae and total coliforms as shown in table 3 and there was a strong positive correlation between them.

Table 3. Correlation of APC, Enterobacteriaceae, total coliforms, in PIF samples between March 2019 and October 2020.

Parameters	ACC	Total coliforms	Enterobacteriaceae
APC	1		
Total coliforms	0.551	1	
Enterobacteriaceae	0.741	0.740	1

From the total of 106 analyzed samples, six *Cronobacter* species were isolated from PIF using chromogenic agar whereas *Salmonella* species were not isolated from any one of the PIF milk products. Thermo-tolerant coliforms and E.coli were also not isolated from any of the PIF milk products which were analyzed during the study period.

6.2 Discussion

The microbiological parametric quality and safety of PIF for infants below or less than six months in some purposively selected products in Addis Ababa were evaluated using ACC, Enterobacteriaceae, Total coliforms, thermotolerant coliforms, and pathogens *Cronobacter* species and non-typhoidal *Salmonella* enterica. The PIF which are analyzed in this study is

products commercialized in most parts of Africa and the whole of Ethiopia. Therefore, determining the microbiological status and quality, situations of hygienic situations during manufacturing and also identifying the microbial risks in the product which may occur during reconstitution and inoculated into it during supplementing the product after pasteurization to supplement it with a suitable nutritional product is up to date information (40, 41 and 42).

For mesophilic Aerobic Colony Count (ACC) 91.6% of all the analyzed PIF samples had found results less than 5×10^2 cfu/g. There were 8.4% of the samples contained higher ACC results above $5x10^2$ cfu/g in brands imported from the United States and none of the powdered infant formulas analyzed were produced in Ethiopia. These ACC results are found within range with other studies (7, 8, and 43). In PIF milk it's a concern to find ACC numbers with a load greater than the maximum values recommended by codex Alimentarius commission (5×10^2 cfu/g CAC/2008); rather these findings are very high as compared to values reported in other findings (44). Also in a similar study done by Heperkan *et al* (45) found results similar to the range of values found in this study. Mesophilic aerobic microbial loads in PIF milk products are reflective of the good manufacturing practice. It is a useful indicator of the process steps. The loads beyond the proposed limits are indicators of microbial overgrowth and or build-up in the parts of the manufacturing unit. It is mandatory to control the source of contamination in PIF milk products since there a varied range of mesophilic microorganisms especially when the children who consume the products are infants less than six months by age, sick or nutritionally and immunologically debilitated (46). It is important to regularly investigate the amount of contamination of PIF products and control their hygienic status.

As described by FAO and WHO the association of microorganisms with PIF milk products was classified in different categories basing on the evidence of their presence and casualties of illness and members of Enterobacteriaceae are thus classified as category 2 (causality plausible, but not yet demonstrated). In this study, Enterobacteriaceae were isolated in 9.4% (n=10/106) of the study samples and were detected in 33.3% (n=10/24) of brand C and there is a statistically significant association between counts and brands (P=0.01) and producer and counts (p=0.02). A similar study done by palchi *et al*., 2009 found lower results of 1%, and a survey was done in Australia by New South-Wales (NSW), on 94 samples found at 2% (46, 47, and 48).

High values of 47% Enterobacteriaceae were found in a study done by Estningsih *et al*. and Yao *et al*. were reported a high value of 22% as compared to this work (49, 50). Another highest value of 100% Enterobacteriaceae was reported in Chile (parra-Flores *et al*.) in 2015 (51). The results of all of the samples in this study found with Enterobacteriaceae were much higher than the numbers permitted by CAC (2008), which recommends the absence of these groups of bacteria in PIF milk products (52). Therefore, these results should be evaluated concerning hazards associated during consumption of PIF by lower age infants especially less than the first six months, the lack of regulation by producers, and responsible organ for inspection at the health authority of Ethiopia.

As described in the case of Enterobacteriaceae, total coliforms are indicators of conditions of the hygienic status of the course of the manufacturing of the PIF milk products. These organisms can be easily inactivated using sanitizers and cleaners. After cleaning and/or sanitizing the process equipment are not appropriate or inadequate these groups of organisms can easily be detected in the end products which pass through the process (2, 15, and 53). Out of the PIF milk samples tested total coliforms were detected in 8.4% and all of the positive samples belong to the same brand (brand C) and company. Similar results of total coliforms were detected in a study done by Kim *et al*. (2011) in Korea in PIF milk products (54). In most studies done to assess the quality of PIF total coliforms were not detected (49, 50, and 51).

The findings in the present study about the presence of total coliforms is thus an indication of the failer of the production process of the PIF products (45), but the fecal contaminations are not an expected hazard of apprehension in PIF, the usage of these bacteria would be mostly restricted to the group of indicators of re-contamination in the manufacturing process. However, the coliform and fecal coliform examinations are less effective than Enterobacteriaceae counts. Replacing and utilization of coliform organisms as testing with an Enterobacteriaceae group for testing as a process of production controller or indicator was advised and recommended by international organizations (FAO-WHO) consultation groups because these Enterobacteriaceae families include the more hazardous pathogens which alarm concern and are found at alarm more frequently and consistently more than coliforms(2, 15).

A higher ACC and Enterobacteriaceae and total coliform values indicate non-firm adherence to the hygienic code of practices that are recommended by international groups for a

preparation for PIF, which have been stated by other scholars (53, 54) and this can impose a hazard for populations that regularly utilize these milk products (52).

Spearman Correlation test signified statistically significant positive correlations between aerobic colony count and thermotolerant coliforms; total and thermotolerant coliforms were also correlated to each other (Table 3). The correlation between aerobic colony counts, total coliforms and faecal coliforms in this study was consistent with the study done in Chile and could indicate the same source of contamination of PIF (68).

Cronobacter species are well known, studied and serious emphasize been given in the developed world but still, there is very little information in developing countries and no such report in Ethiopia. The incidence or occurrence of *Cronobacter* species was 5.6 % (6/106) among the total of evaluated 106 PIF milk products, in particular, the six samples are from the same brand (Brand C), the same company, and all are imported from the same country. In Korea, slightly similar results of *Cronobacter* species were isolated from 4.4% (n=10) of PIF milk products (55). Another study done in Nigeria retail market on the same products of PIF milk by Enem *et al*., 2020, shows that a contamination rate of *Cronobacter* species was 4.4 %(56), and also similar results are found in the same publication in different parts of the world (57,58,59). In another research done on the Chinese retail market, Cronobacter species was found at a prevalence of 2.8 %(60, 61) and it is slightly lower than the results found in the current study and it may be due to manufacturers strict adherence to GMP due to better awareness of Chinese health authority.

Some studies were done in the Chinese retail market and Egypt showed that high results of Cronobacter species of 12.3% and 17.5% respectively (62, 63). The results observed in this study are as high as results presented by previous scholars and this high positivity rate should aware the individuals and authority at the health office as this organism is very lethal and has very high neurological sequelae in high risky individuals especially immunocompromised infants and low birth weights(<1500g) (12,15,64,65). Also, many studies have been emphasized the isolation and identifications of Cronobacter species for evaluation of the level

of contamination of PIF (2, 15, 51, 52, 66, and 67). Even though the finding of *salmonella* species was null in these work there are situations which warn the utilization of microbial quality control in the production. The microbial quality hazard during utilization of PIF milk products can't be relaxed as these products of PIF are used by very sensitive populations. In 2015 all European countries were affected by *Salmonella* infections from contaminated PIF milk products and there was a recall of these products from the markets of Argentina due to contamination of the same organism (68) thus, unsatisfactory microbial quality of PIF milk products to be consumed by infants lower than six months was found in the present study. The finding of Enterobacteriaceae, total coliforms, and *Cronobacter* species were identified, and this will raise the producers and health regulatory body of Ethiopia since the consequence of these products could pose very great public damage as it is imported and consumed in mass to the public in Ethiopia.

7 Strength and limitation

7.1 Strength

This study has greater attention from public consumers, health authorities, importers, and stakeholders since food safety, especially in infants, is currently up-to-date health agenda. In this study, internationally recommended methods were employed with appropriate steps by utilizing control strain organisms.

7.2 Limitation

Due to the shortness of duration of study and budget we collected and investigated a few sample size which doesn't represent the whole city and country. Due to this, we couldn't conclude the exact situation of the microbial safety status of PIF in the country. It is recommended to undertake further study to generalize the microbial quality status of the PIF milk products.

8 Conclusion and Recommendation

8.1 Conclusion:

The finding of this study shows that the PIF was contaminated by ACC, Enterobacteriaceae groups and coliforms. The incidence or occurrence of *cronobacter* species was found to be 8.6% (6/106). These all findings are above the recommended international specifications of Codex Alimentarius Commission.

8.2 Recommendation:

The result of this study contributes to the knowledge used in monitoring the health problems of infants that come from PIF milk contamination. It provides basic information used to reduce infants and neonatal exposure to infections from *cronobacter* species and up-to-date information for the work to be done on infant formula products to prevent further health risks of infants health stack holders and finally there are high risks for consumers health and requires an immediate call for action.

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10 Annex Protocols

10.1 Appendix A

Request form:

Microbiological Safety of powdered Infant Formulae and Dried Dietary Foods for Special Medical Purposes Intended for Infants below 6 months of age

General Information:

- Sample collector Name: _____
- Sample Reference Number *(i.e. collector own personal reference number for the sample)*_____
- Laboratory Reference Number *(upon receipt of laboratory)*_____

Source information

Supermarket ⬭

Pharmacy ⬭

Hospital ⬭

Distributor/ Importer ⬭

Other

Sample information

Product name:_____

Batch no:_____

Expire date:_____

Other info:

Microbiology results

Salmonella Species: present/absent in 25g
Enterobacter sakazakii: present/absent in 25g
Other Enterobacteriaceae in 25g

10.2 Appendix B: SOP for isolation of *C.sakazakii* from milk and milk products.

(Adopted from ISO 22964:2006)

This procedure provides instructions for the detection of C.sakazakii from milk and milk products (powdered infant formula).

Materials and supplies

1. Buffered peptone water (BPW)
2. Modified lauryl sulfate tryptose broth (mLST)/vancomycin medium
3. *Enterobacter sakazakii* isolation agar (ESIATM)
4. Tryptone soya agar (TSA)
5. Biochemical tests, general laboratory equipment, and others.

Procedures

1. **Pre –enrichment**. Add 25 gm of PIF to 225ml of BPW (pre-enrichment medium) and incubate at 37^0c±1^0c for 18±2 hrs.
2. **Selective enrichment**. After incubation of the inoculated pre-enrichment medium, transfer 0.1 ml of the obtained culture into 10 ml of mLST/vancomycin medium. Incubate at 44 °C ± 0.5 °C for 24 h ± 2 h. (It is recommended to use either a water bath or a forced-air incubator to ensure that the maximum temperature (44.5 °C) is not exceeded).
3. **Presumptive isolation**. After incubation of the inoculated mLST/vancomycin medium, streak a loop full (*ca*. 10 µl) onto the surface of the *Enterobacter sakazakii* isolation agar plate. Incubate the plate at 44 °C ± 1 °C for24 h ± 2 h.
4. After incubation, examine the Chromogenic plate for the presence of typical colonies (small to medium-sized (1 mm to 3 mm) green to blue-green colonies, non-typical colonies are often slightly transparent and violet-colored) of presumptive *Enterobacter sakazakii*.
5. Streak the selected colonies (typical colonies) onto the surface of the TSA plate so that after incubation separate colonies can be observed. Incubate the plate at 25 °C ± 1 °C for 44 h to 48 h. After incubation, examine the TSA plates for the presence of yellow-pigmented colonies.

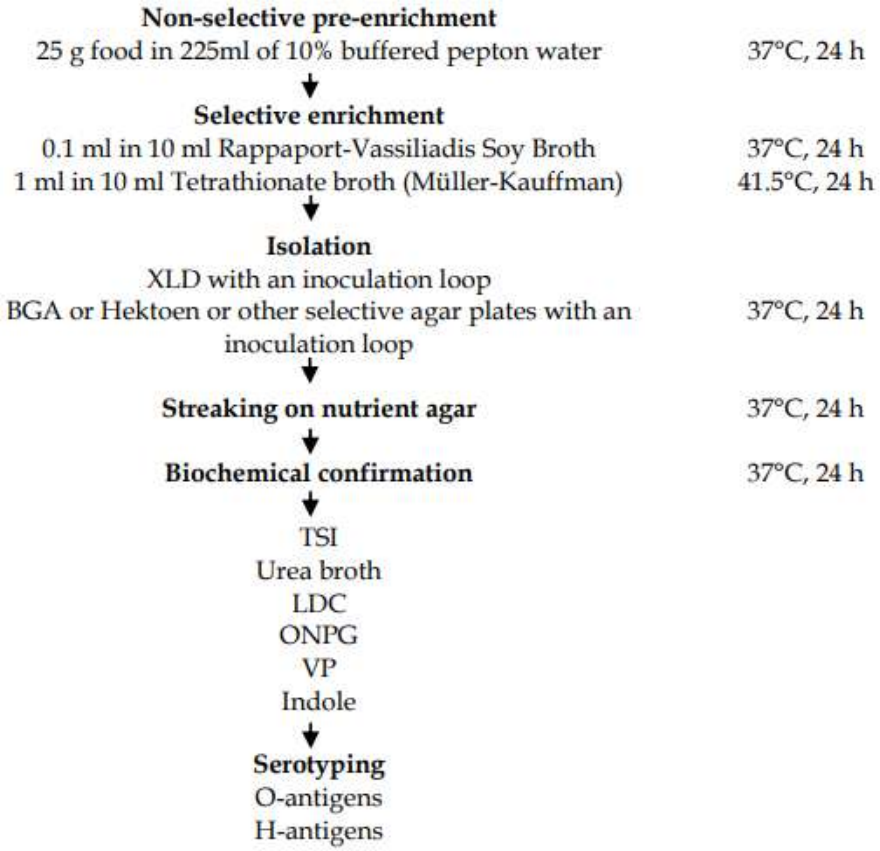
6. Select one yellow pigmented colony from each tryptone soya agar plate for further biochemical characterization and/or miniaturized biochemical identification kits commercially available.
7. Report the result as Present/Absent per 25gm.

Quality Control

To check the ability of the enrichment and isolation media to support the growth of *Enterobacter* species, introduce low-level inoculums of a reference culture of a recently isolated *Enterobacter sakazakii* strain, or of a reference strain from a recognized culture collection center, into control flasks of the pre-enrichment medium. Proceed with this control flask as for the test cultures to demonstrate that the positive control culture is recovered.

10.3 Appendix C: SOP for *Salmonella* from PIF: A Short Description.

Standard - Microbiology of food, Horizontal method for detection of Salmonella spp. (ISO, 2017). Under the above regulation, detection of Salmonella in food should be carried out for such products as raw meat, meat products intended for consumption in the raw state, gelatin, cheese, butter, cream, unpasteurized milk, powdered milk, eggs, and products containing raw eggs, crustaceans, mollusks, fruit and vegetables, unpasteurized juice, **powdered infant formulas**, and dietary food for special medical purposes.



10.4 Appendix D: Detection of Total coliforms, fecal coliforms, and E.coli in PIF (all microbiological safety precautions are extremely valued.)

Isolation of Enterobacteriaceae will be done by using a version of (*NMKL No 144, 2005).*

1. A total of 25g of infant formula powder will be mixed with 225 ml of diluents.
2. Then serial dilution will be carried out using 9ml of normal saline.
3. For primary isolation of Enterobacteriaceae, an overlay method will be carried out where 1ml of each dilution will be pipetted into a Petri dish and then mixed with 10-15ml of Violet Red Bile Lactose Agar(VRBLA) after which about 10ml of VRBLA will be poured on to the solidified medium.
4. The plate will then be incubated at 37^{o}cfor 24h.
5. After incubation five suspected colonies will be subcultured from VRBLA to tryptone soya agar (TSA).TSA will be incubated at 37^{o}c for 24h ±4h (31).

Quality control: Control cultures (use ATCC cultures or equivalent): positive control(s): E. coli that are known to produce gas at 44.5 / 45o C and is capable of fermenting lactose to produce typical reactions on L-EMB agar; if using EC-MUG, a strain that is known to produce ß-glucuronidase EMB / IMViC negative was used.

Declaration

I, the undersigned agree to accept responsibility for the scientific ethical, and technical conduct of the research project and for the provision of required progress reports as per the terms and conditions of the research publications office.

M.Sc. candidate: **Waktole Gobena (B.Sc.)**

Signature: _____

Date of submission: _____

This thesis has been submitted with our approval as advisors.

Advisor: **Kassu Desta (MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.