HADIZA KABIR BAKO
MICROBIAL ASSESSMENT OF READY TO USE FOOD USED FOR THE REHABILITATIONOF MALNOURISHED CHILDREN
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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF NEAR EAST UNIVERSITY

By

HADIZA KABIR BAKO

In Partial Fulfillment of the Requirements for the Degree of Master of Science in Food Engineering

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name:
Signature:
Date:

To Ameena with love…

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ABSTRACT

Microbial growth and contamination are linked to several factors and there are superiority claims of Ready to Use Therapeutic Food (RUTF) and Ready to Use Supplementary Food (RUSF) over other complimentary foods in terms of susceptibility to microbial contamination and therefore use for the rehabilitation of malnourished individuals. This study focuses on microbial assessment of RUTF and RUSF used for the treatment of malnourished children. Samples were collected from three places in the northern part of Nigeria and analyzed by conventional culture methods. *Lysinibacillus sphaericus, Citrobacter youngae and Bacillus licheniformis* were detected and further identified using BD phoenix^{TM}, while *Candida albicans* was identified with Vitek 2 Bio-Merieux. Among the investigated samples, RUTF are the most contaminated with *Citrobacter youngae* as the most prevalent.

Although these bacteria are not thought to be a causal agent of food poisoning, their presence may be hazardous since the malnourished children are immune-deficient. Furthermore, contamination of RUTF can be related lack of stringent food chain control. It is highly recommended that RUTF manufacturers both foreign and indigenous should increase use of Good Hygiene Practices, Good Manufacturing Practices and HACCP systems by food industry, authorities should enhance food monitoring programmes and set the limits in accordance with international recommendations to monitor outbreaks.

Keywords: Malnutrition; *Ready to Use Therapeutic Food; Ready to Use Supplementary Food; Low Moisture Food; Microbial Assessment*

ÖZET

Mikrobiyal kontaminasyon ve bulaştıkları gıdada gelişmeleri bir çok faktöre bağlıdır.Tamamlayıcı gıdalar arasında hazır terapötik besinler (RUTF) ve hazır takviye edici besinler (RUSF) mikrobiyal kontaminasyon bakımından daha hassastırlar ve kötü beslenmiş bireylerin beslenmesinde kullanıldıkları için de bu bakımdan daha dikkatle işlenmeleri gerekmektedir.

Bu çalışma kötü beslenmiş çocukların tedavisinde kullanılan RUTF ve RUSF gibi hazır besinlerin mikrobiyal incelenmelerini amaçlamaktadır. Hazır besin örnekleri Kuzey Nijeryada'ki 3 ayrı bölgeden toplanmış ve konvensiyonel kültürel yöntem ile analiz edilmişlerdir.

Örneklerde tespit edilen *Lysinibacillus sphaericus, Citrobacter youngae* ve *Bacillus licheniformis* varlığı BD Phoenix cihazı ve *Candida albicans* Vitek 2 Bio-Merieux cihazı kullanılarak doğrulanmışlardır. İncelenen örnekler arasında RUTF'un *Citrobacter youngae* ile en fazla kontamine olan örnek olduğu bulunmuştur.

Bulunan bu bakteriler gıda zehirlenmesine yol açan en önemli etmenlerden olmasalar da, kötü beslenmiş çocukların bağışıklık sistemleri zayıf olduğundan kullanılan bu besinlerde bulunmaları tehlike oluşturabilir. Bundan başka RUTF'un kontaminasyonu gıda zincirinde kontrolün eksikliğini akla getirmektedir. Yerli, yabancı RUTF üreticilerinin oluşabilecek salgınları önlemek için, iyi hijyen teknikleri, iyi üretim teknikleri ve HACCP sistemini üretimde uygulamaya ve ülke standartlarını uluslararası standartlarla uyumlu hale getirmeye özen göstermeleri gerekmektedir.

Keywords: Kötü Beslenme, hazır terapatik besinler (RUTF), hazır takviye edici besinler (RUSF), Düşük Su İçeren Gıdalar, Mikrobiyel Kalite

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LIST OF ABBREVIATIONS

ACFDP: Advisory Committee for Food and Dairy Products

a_w : Water Activity

BA: Blood Agar

CFU: Colony Forming Unit

CIFOCOSS: Consumption Database Summary Statistics

CNS: Central Nervous System

CV: Crystal Violet

DALY: Daily Adjustment Life Year

EFSA: European Food Safety Agency

EMB: Eosin Methylene Blue

FAO: Food and Agricultural Organization

FBO: Food Business Operator

G: Gram

GMP: Good Manufacturing Practice

HACCP: Hazard Analysis Critical Control Points

HIV: Human Immunodeficiency Virus

ID: Identification

IDP: Internally Displaced Person

Kcal : Kilo calories

KG: Kilogram

KJ: Kilojoules

LMF: Low Moisture Food

MAM: Minimum Acute Malnutrition

MAX: Maximum

MSF: *Médecins Sans Frontières*

MG: Milligram

MIN: Minimum

MUFA: Monounsaturated Fatty Acids

NHS: National Hospital System

pH: Hydrogen Ion Concentration

ppb: Parts per billion

PUFA: Polyunsaturated Fatty Acids

RTE: Ready To eat Food

RUF: Ready to Use Food

RUTF: Ready to Use Therapeutic Food

RUSF: Ready to Use Supplementary Food

SAM: Severe Acute Malnutrition

SDA: Sabouraud Dextrose Agar

SCN: Standing Committee on Nutrition

UN: United Nations

UK: United Kingdom

UNICEF: United Nations Children's Fund

US: United State

USAID: United States Agency For International Development

WFP: World Food Program

WHO: World Health Organization

***:** Asterisk

^{O}C: Degrees Centigrade

>:	Greater Than
<:	Less Than
µg:	Microgram
-VE:	Negative
ω:	Omega
%:	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background

According to World Health Organization (WHO), Malnutrition is defined as "insufficiencies, surpluses or imbalances in an individual's consumption of energy and/or nutrients. It comprises of two conditions 'Under-nourishment' - which covers low height for age, low weight for height, low weight for age and micronutrient deficiencies. And 'Excesses' - which include overweight and obesity causing food-related non-communicable illness like diabetes" (World Health Organization, 2017).

About 180 million kids suffer from under-nutrition Worldwide. In 2016 alone, 22.9 % out of children below 5 are stunted globally although there have been a significant decrease from former estimation of 32.2%; with Eastern Asia and the Pacific from 24.4% to 9 %, South Asia 51.3% down to 35.8%, Middle East and North Africa 22.8 to 15.3%, Eastern Europe and Central Asia 13.3% to 6.2%, Eastern and Southern Africa 45.4% to 34.4%, Latin America and Caribbean 18.4 to 11% and North America 3% to 2.3% (Figure 1.1).With the decline of malnutrition globally between 2000-2016 from 198 million to 155 million among children below 5 years there have been a drastic increase of stunted children in West and Central Africa from 22.9 million to 28.1 million with a significant rise of 23% (Figure 1.2). On the other hand, 6 % were overweight with East Europe and Central Asia having the leading overweight predominance of 12.8 %, then the Middle East and North Africa with 10.7 percent and North America with 7.8 percent affected. The lowest overweight occurrence in 2016 was recorded in Central and West Africa, at about 3.7 percent, then East and South of Africa at 4.2 percent. Similarly, East Asia and the Pacific had the highest predominance of obese young ones in 2016 of 8.6 million, followed by South Asia with an approximate 7.4 million overweight. With both accounting for nearly two in every five overweight kids globally. 52 million young ones below 5 were

undernourished of which 17 million were seriously wasted. This results in a predominance of 7.7 % and 2.5 % respectively. Over half of all undernourished kids reside in South Asia and around one-quarter in sub-Saharan Africa, with the same estimate for seriously undernourished kids. The 16.0 % South Asia's, under-nourishment predominance indicate a 'serious' public health concern; that of the Middle East and North Africa is reaching a 'urgent' need for intervention with proper rehabilitation programmes (UNICEF, World Health Organization, & World Bank Group, 2016).

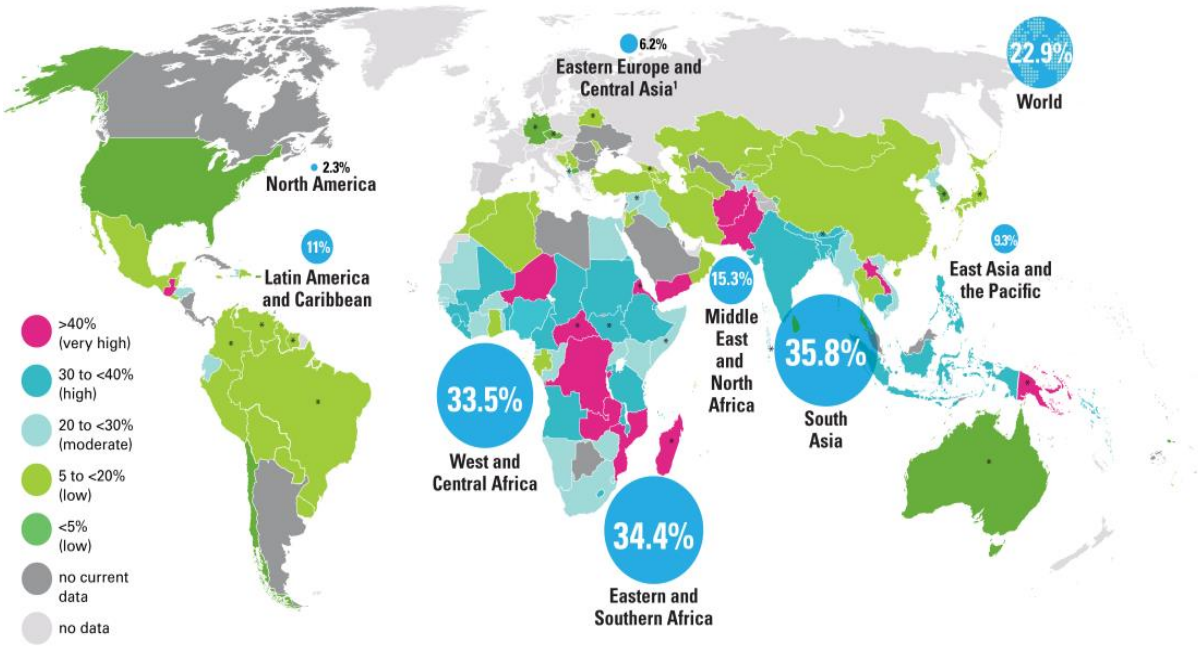

Figure 3.1: Current Status of Global Malnutrition (UNICEF, 2017)

The most recent overall data of the world malnutrition status between 2011 to 2017 with the exception were older estimation are used (2005-2010) are indicated with asterisk (*) and where information up-until 2005 are available the dark grey shows no updated estimation used.

Almost half of all deaths in kids below the age 5 are linked to this menace, resulting in the loss of about 3 million children yearly. Children suffering from malnutrition are at higher risk of dying from a simple infection, elevate the frequency and harshness of such infections, and add to slow healing. Malnutrition and infection can cause a deadly cycle of serious sickness and decaying nutritional state. Inadequate nourishment in the early 1,000 days of an infant's life can additionally affect growth, which is linked to impaired learning

ability and children not reaching their full potential (FAO/ WHO, 2016; Latham et al., 2011; Unicef, 2017).

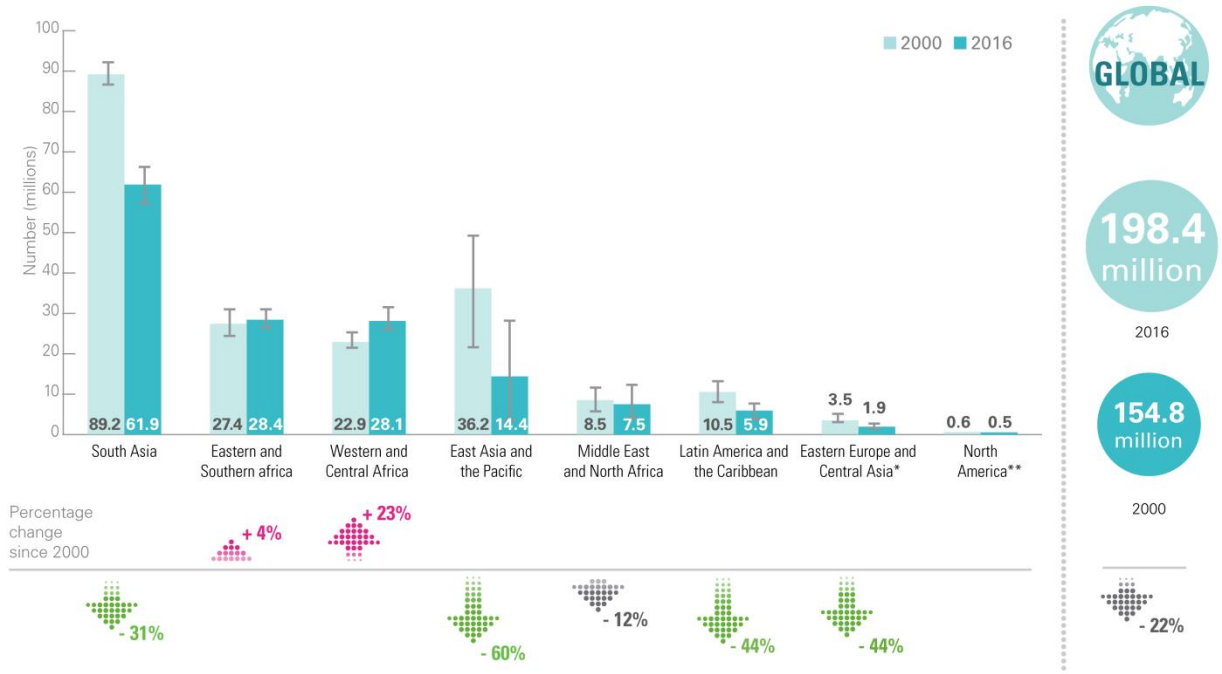

Figure 1.2: Stunned children below the age of 5 by region between 2000 to 2016 (UNICEF,2017)

As an intervention on under-nutrition and Constant commitment to produce safer food for the younger ones, especially at the period of complementary feeding within 6 months to 1 year and the time of fast growth up to 5 years, the Food and Agriculture Organization (FAO) of the United Nations (UN) and the World Health Organization (WHO) organized a technical meeting in FAO headquarters Rome that addressed the microbial safety of Ready-to-Use Foods (RUF) for the rehabilitation of acute malnourishment. Additionally, In April 2016, the United Nations General Assembly adopted a commitment declaring the UN Decade of Action on Nutrition from 2016 to 2025. The Decade intends to prepare policy commitments that result in significant action to address all types of under-nutrition. The purpose is to ensure people have access to healthier and safer food to eradicate all kinds of under-nourishment globally. Ready to Use Food may be lipid-based produce packaged in potpourris or containers or non-lipid-based food the likes of biscuits and bars. The deliberation was centered on the microbial safety of lipid-based RUF used for the

therapy of moderate acute malnutrition (MAM) and severe acute malnutrition (SAM) in infants aged 90 days - 5 years. When specificity is needed, lipid-based Ready to Use Food used for management of Moderate Acute Malnutrition in children is known as Ready to Use Supplementary Food and the lipid-based RUF used for rehabilitation of SAM are called Ready to Use Therapeutic Food (FAO/ WHO, 2016; World Health Organization, 2017).

For kids afflicted with malnutrition, instant hospital admission is not needed only and unless when there are complications (Kapil, 2009). RUTF in the last few years has made an immense contribution towards nutrition which is used in the therapy of SAM, as a product of broader outreach. It is said to be a nutrient of thick consistency and high energy, mostly peanut paste originally designed specifically for the therapy of the severe acute dietary deficiency in infants. It may be eaten from the package by the kid and dilution with water is not needed. Any infant eating RUTF will, however, require water in addition. It can be stored for 3-4 months without cooling, even at temperature regions. There are varieties of RUTF virtually all are merchandizing products. Plumpy'nut is commonly used, which are trademarked goods, a prototype developed at the late 1990s; it is produced by Nutriset, a French company. It is packaged in 92-gram film sachet, contributing 500 kilocalories. In 2009 Nutriset produced 14,000 tones, predominantly shared and donated by the UN Children's Fund (UNICEF), to be given to over five hundred thousand infants resulting to $US 66 million worth. UNICEF has asserted that this 'nutritional paste (peanuts, powdered milk, refined plant oil, nutritive sweeteners, vitamin, and mineral mix) comprise of the appropriate formulation of nutrients as therapy for kids with SAM, and in a way that is simple to be eaten and safe'. It is used in healthcare centers and in the neighborhoods (Latham et al., 2011). This initiative was backed by WHO, which also support short, aggressive therapy periods in a kid with a dietary deficiency that permits recovery within 14-40 days. Although there are great proof for various nutritional interventions internationally, notable impact of such interventions is still yet to be achieved (Steenkamp et al., 2015). The FAO/WHO Codex Alimentarius Commission has not long ago set guidelines for food supplement for toddlers and infants, which will include RUTF. No United Nations agency, or any cooperation, has so far standardized or in a way defined

the composition or quality standards of what may be referred or ingredients used as or for RUTF (Latham et al., 2011).

Although both Therapeutic and Supplementary Food have been produced to give high energy options than more regular supplements (Steenkamp et al., 2015). It was strongly acknowledged that the beneficiaries of Ready to Use Therapeutic Foods (RUTF) are not same as of Ready to Use Supplementary Foods (RUSF), with RUSF being different. At the 37th assembly of the Codex Committee on Nutrition and Foods for Special Dietary Uses held November 2015, The UNICEF recommend at Codex level a conference paper for a Codex guideline that particularly point out RUTF for the therapy of SAM; this new recommendation redress an earlier recommendation that include all Ready-to-Use-Foods (RUFs). This considerate amendment admits that as the numbers of beneficiaries of RUSF and RUTF increases so will the need for different standards for these products. For an instant, RUSF production is based on the taste and requirement of pregnant mothers or individual with HIV and those who are stunned or for the elderly. A ‘‘one-size-fits-all’’ standard RUF product that serves the desired health result becomes unrealistic. The recommendation of UNICEF guidelines whose scope is specific to the desired outcome for a targeted state of health and precise individuals is circumspective, science-based approach (Schweitzer, 2016).

WHO proposed that RUTF should be produced locally by each country, while maintaining its International Standards (Kapil, 2009) however, errors may occur in production due to choice of ingredient, Aflatoxin formation, oxidation of fatty acid, mistake in formulation or bacterial contamination and also post-process contamination can occur in both indigenously manufactured and imported RUTF.

1.2 Statement of Problem and Justification of the Study

Nigeria was counted in the topmost in acute malnutrition globally and was grouped 13th by UNICEF in its world ranking of high rates of Global Acute Malnutrition in 2013. It is particularly alarming to observe Nigeria’s Global Acute Malnutrition rate at 14% higher than that of West and Central African mean. It is higher than 5 percent of Sub-Saharan average of 9%. Statistically, it was observed that infants under 5 years in Nigeria; 37% are

stunted, 21-29% are underweight, an indication that about 34-42% are wasted. Nevertheless, reoccurring incidence of insurgency in North-East geopolitical zone, with the aftermath of IDP's in different camps, has created an immediate need to boost the programme out-reach for the therapy of malnutrition, thus generating an even greater need for relief food (FIIRO, 2016).

Microbial growth and contamination are linked to several risk factors; survival of pathogen and cross contamination may cause infection if the food is not properly store, handled or used particularly among most vulnerable group (children), in regards to Nigeria having no indigenous plant for the manufacture of RUTF and RUSF, and relies on import where the product is subjected to different handling, storage condition and shipping delays, and an enabling condition favorable for microbial growth.

Therefore it is of utmost important for such study to assess the microbial safety to give food scientist, food producers, and related authorities more ideas on the manufacture of safer products.

1.3 Aim of the Study

The purpose of the research is to conduct microbial Assessment on Ready to Use Food: Ready to Use Therapeutic Food and Ready to Use Supplementary Food used for the rehabilitation of infants from malnutrition and to determine the product complies with an established microbiological specification**.** The objectives are:

To determine the microbial loads of three types of Ready to Use Food using aerobic mesophilic bacterial, yeast and fungi count (*Escherichia coli, Coliforms, Cronobacter*, pathogenic *Staphylococci, Salmonella, Listeria*, *Aflatoxin*, total aerobic and spoilage bacteria, yeast and fungi)

1. To identify discrete colonies using standard biochemical method
2. To screen the identified isolate and confirm their occurrence

This study will be limited to RUTF and RUSF with regards to their notable contribution to undernourished child in Nigeria.

1.4 Overview on Lipid –Based Paste

RUTF and/or RUSF are viscous mix of powdered formulation combined to form a spread, resulting in great energy food (FIIRO, 2016). The spread composes of powdered milk, minerals refined oil, sugar, granulated vitamins, and peanut-butter. As the name entails, Ready to Use Therapeutic Food requires no cooking before consumption, making it handy where cooking opportunities are unavailable. RUTF have reduced Water activity (a_w), therefore almost unlikely for bacteria to grow in these foods and highly nutritious, approximately 23 kJ/gm (5.5 kcal/g). A serious under-nourished kid can devour only a couple of spoonful of RUTF between 5 to7 times in a day, to attain adequate energy required for total recovery. While water requires to be taken after RUTF, additional foods are not fundamental for the recovery of the malnourished child (Manary, 2005). RUSF is akin to RUTF, but is specifically developed for infants, expecting mothers or people with a weak immune system like HIV, or to prevent malnutrition. After series of investigation peanut-based RUTF proof to be equally or more effective than F100 (a therapeutic milk powder fortified with vitamins and minerals) in the rehabilitation of malnourished youngsters, in 2007 the WHO and WFP together propagated outpatient, community-based treatment of seriously malnourished children with RUTF (Beckett et al., 2016).

Nevertheless, as these supplements have a low water activity, making them safer and convenient for use in third world nations where clean drinking water and most favorable storage are often problematic issues (Steenkamp et al., 2015), microbiological investigation has been and keep on being an important means of ascertaining the capability of a food to enable the growth of spoilage bacteria or pathogens and also play a vital role in the validation of methods that are intended to deliver (Comprehensive Review In Food Science And Food Safety, 2003). Investigating the ability of food products to support the of growth microorganism is not easy since several Therapeutic Food and Supplementary Food are locally produced with various constituents that have influence on the fate of pathogen of concern and many new companies are emerging producing variety of imported RUT foods seeking to buy out competitors from the market.

1.5 Significance of Food Safety

Foodborne sicknesses are preventable. These diseases when underreported are a concern to public health and increase healthcare expense. They act as a serious risk to some individuals. Although anyone can get a foodborne sickness, some are at higher risk. For instance:

- Infants below 48 months have the greatest cases of laboratory-diagnostic diseases from certain foodborne pathogens, the likes of *Campylobacter, Cryptosporidium, and Salmonella, Shiga* toxin producing *Escherichia coli* O157, *Shigella*, and *Yersinia*.
- Adults above 50 years and immune-deficient individuals are at higher danger of hospital admission and even death from intestinal pathogens usually eaten in foods.

Safer food assures healthier, better lives, in-expensive health care, and a more vibrant food industry.

Microbial assessment is used to explain areas of vagueness and establish growth characteristic for products with chemical properties close to the growth – no growth border (NSW Food Authority, n.d.). However, reports from food manufacturers and testing-laboratories showed a necessity for the evaluation of the technical guidance report to expedite such analyses, the European guidance report, with major emphasis on the idea of investigative studies to assess the growth characteristic of pathogens in food products. It was stressed to define microbial test, when one is desirable, the circumstances to be reflected on and the laboratory method to be employed when carrying out one to investigate the growth ability or presence in a given sample. The Food Standards Agency of New Zealand has newly issued standards for carrying out investigative studies (Álvarez-ordóñez et al., 2015) Thus Microbiological process maybe be listed as quantitative or qualitative. While the former (enumeration) is used in determining the number of bacteria directly or indirectly, in a food matrix the latter describes presences of the target bacterium or not in the sample (Välimaa, 2015).

Ready-to-use foods bearing harmful microorganism may not certainly make one sick but it has been proven microbiological and epidemiological that small numbers of pathogens in

foods have caused sickness. The ACFDP is of the opinion that there is no justification for processed RUF foods being contaminated with these pathogens and that their presence, even in minute amounts, makes such kind foods being of unacceptable quality/potentially dangerous (Gilbert et al., 2000). Foodborne pathogens in RUF foods endanger consumer's health and their absence is of significant importance.

Except for anaerobic or aerobic bacterial spores, a discovery of pathogenic organisms at any level is of great concern and should be investigated with a sense of urgency and response should be proportionate to the extent of exposure and danger to consumers. Although few pathogens, like coagulase-positive staphylococci in Ready-to-Use foods, may pose a little danger to immunocompetent consumers, they are more threatful to the immunocompromised and vulnerable individuals.

Low levels may as a result of contaminated raw materials employed in the manufacture of those foods, their ubiquity signify lapses in the making or following handling of food which could lead to an unacceptable rise in the hazard. A call for action may be required when few of these organisms are detected in RUF as a result of the difference in host susceptibility and interstrain change in the pathogenicity (Health Protection Agency, 2009). Nevertheless, there are many rare and uncommon pathogens which are also life-threatening.

CHAPTER 2

THEORETICAL FRAMEWORK

This chapter gives an insight on Ready to Use Food, method of production and storage, the significance of microbiological quality assessment, sampling method and method of analysis

2.1 Ready to Use Food (RUF)

Ready to Use Therapeutic Food (RUTF) and Ready to Use Supplementary Food (RUSF) are types of RUF which are nutritious, water-soluble, lipid- based paste. The thick liquid are composed of tiny particles of protein, carbohydrate, minerals, and vitamins all combined in this edible paste.

Table 2.3: A typical composition of Ready to Use Therapeutic Food (Manary, 2006; Wagh & Deore, 2015)

Nutrients	% weight basis
Full fat milk	30
Sugar	28
Vegetable oil	15
Peanut butter	25
Mineral/Vitamin Mix	1.6

This Lipid-based Ready to Use Food gives protein, energy, fatty acids and micronutrients custom-fit for the requirements and organoleptic tastes of the kids given during emergency feeding plans relies on a standard combination of constituents (Table 2.1 and Table 2.2).

These incorporate groundnut, chickpeas, soy flour, milk protein (powered skimmed milk and/or whey), sugar, vegetable oil, vitamins, minerals premix (Table 2.3), emulsifiers and

thickening agent. The components are relatively cheap goods that are widely available. Although manufacturing of these RUFs has advanced notably from its former times of using simple kitchen tools, lipid-based Ready to Use Food are comparably easy to make without sophisticated or specialized machine, which simplifies their manufacture in the locality of use. Presently, there are about 20 producers of lipid-based RUFs in Africa, Asia, Europe and North America (FAO/ WHO, 2016; WFP, Unicef, 2016).

Table 2.4: A Typical Nutritional values and composition of RUSF (VALID NUTRITION, n.d.)

Nutritional values of 100g RUSF	Composition
530kcal energy	Peanut-Paste
10.4% of total calories as protein	Skimmed Powdered Milk
58% of total calories as lipids	Vegetable Oils & Fat
5.5g ω-6 fatty acids	Sugar
0.4g ω-3 fatty acids	Vitamins & Minerals
	Stabilizer: Vegetable-Monoglycerides

2.2 Principles for Production of Ready to Use Food

To gain desired result, a specific blending method must be compiled. The lipid ingredients of RUTF and or RUSF are combined and often cooked; the dry ingredients are gently combined to the lipids during rapid blending. On inclusion of the dry ingredients, the blend is combined at a high pace for sometimes (Figure 2.1). It will be worthy of mention that powdered ingredient not more-than 200 microns, the blend does not easily crumble. However, when the paste is produced with bigger particles, it must be massage slightly by hand just before consumption, to combine the larger particles in the paste. Addition of oils at room temperature eases the blending stage. RUFT are packaged in containers or tubes, manually (simply pouring it) or using an automated instruments. The production of RUTF have was realized in Malawi, Niger, and Congo utilizing similar standards (Manary, 2005). Moreover, RUTF is developed from collective reports of WFP, UNICEF, SCN, and WHO for SAM, and RUSF are based on WHO technical statement of foods for treating MAM. The nutrient compositions (min-max) that are explained in these guidelines represent the

required nutrient intake as a yardstick for formulating product specifications. Due to various nutrient benefits from ingredients and vitamin/mineral premix with 10% dosing variation (Table 2.4), different processing methods among manufacturers, in addition to loss of nutritional value during storage, the minimum and maximum range of product specifications, i.e. the product composition between manufacture and after 2 years of storage (at 30°C for 24 months) is wider than the WHO min-max for intended nutrient intake (WFP, Unicef,USAID 2016).

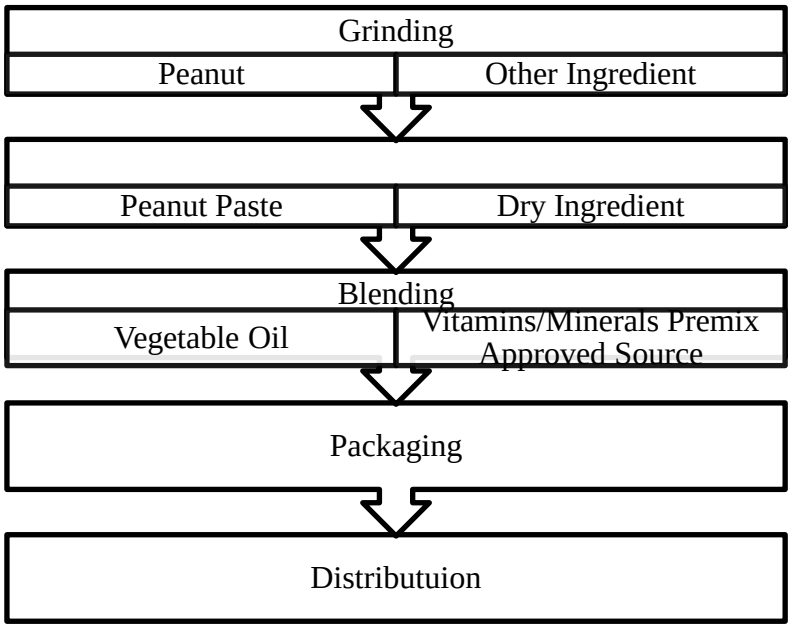

Figure 4.1: General flow chart for the manufacture of lipid-based Ready to Use Food for rehabilitation of Minimum Acute Malnutrition and Severe Acute Malnutrition

RUSF shall be made in a quality and safe environment using the most current version of accepted international standards and best methods and references like as (WFP, 2016) :

- Recommended International Code of Practice. Acceptable standard of Food Hygiene CAC/RCP 1-1969, of the Codex Alimentarius
- General principles for addition of essential nutrients to foods: CAC/GL 09-1987, of the Codex Alimentarius
- ISO 22000:2005: Food safety management systems
- ISO/TS 22004 – Guidance on the application of ISO 22000:2005

Precautionary care should be ensured to avoid water getting into RUTF during the course production since raising the water level of RUTF permit bacteria and mold to grow within the food which increases food spoilage and endangers the undernourished child with potential pathogens. Left over water from cleaned processing utensil are prone to be added to the mix. laboratories (Ciliberto et al., 2005).

2.2.1 Ingredients

Therefore, it is important to narrow the times the processing tools are washed with detergent and water and to preferably dry-clean. Regular used utensils should be washed with cleanser and water on a weekly basis depending on the design of the equipment or need for the clean-up (clean in place or out of place). If the packaging of RUTF to be placed into first requires washing, caution should be ensured that they are properly dry. Enteric bacterial contamination may occur from faecal contamination of stored components or during the blending. Operators shall clean and completely dry their hands prior to handling the food and wear clean gloves, head gears, and protective coats when processing RUF and ingredients should be frequently examine for *Salmonella* contamination by acceptable microbiological method in standard.

Dairy is a great nutritional constituent that is vital in the treatment of severe under-nourishment and for stunning. Dairy is an important ingredient in RUTF (WFP, UNICEF and USAID 2016), The recipe of RUTF was developed from F-100 (Manary, 2005). Some RUSF contains dairy whereas others are replaced with soy isolate. WHO recommends that half of the protein in Ready to Use Therapeutic Food should be from dairy.

Protein quality has been described, which can be accomplished with 1/3 of protein source from dairy or replacing with qualitative soy products. The requirement of RUSF is same quantity as RUTF but protein is derived from dried skimmed milk, equivalent to 10% powdered milk in the product. Approximately 20% powdered milk, or whey protein concentrate are used alternately (WFP & UNICEF, 2016). The inclusion of groundnut butter changes the characteristics of the product to a thick paste food instead of a powdered (Manary, 2005).

As the original lipid paste blend include peanuts, powdered skimmed milk and/or whey protein concentrate and/or soy protein isolate, refined oil, sugar, maltodextrin thus this

premix are major ingredients, peanuts can be replaced with chickpeas, a mix of rice-lentil, sesame, almonds, or cashews. As long as the product nutritional and safety standard are not compromised, and that the product is been accepted by targeted consumers, the utilization of raw ingredient choice is possible. This allows the utilization of local produces, which may reduce cost, take local taste into consideration and lower the hazard of Aflatoxin contamination, specifically affecting peanuts and maize (Wagh & Deore, 2015; WFP & UNICEF, 2016).

Table 2.3: Typical minerals and vitamins composition in 100g of Ready to Use Therapeutic Food (Manary, 2006; Wagh & Deore, 2015)

VITAMINS	MINERALS
Vitamin A (57 mg)	magnesium (587 mg)
Vitamin B12 (110 mg)	copper (92 mg)
Vitamin D (1 mg)	Potassium (36 g)
Vitamin K (1.30 mg)	zinc (717 mg)
Vitamin B2 (116 mg)	Iodine (5 mg)
Vitamin B6 (37.5 mg)	selenium (1.54 mg)
Vitamin B1 (37.5 mg)	Iron (704 mg)
Vitamin E (1.25 g	
Vitamin C (3.3 g)	
biotin (4.1 mg)	
folic acid (13 mg)	
niacin (332 mg),	
Pantothenic acid (194 mg)	

Table 2.4: Composition of Ready to Use Supplementary Food (Peanut Formula of 100g equivalent to 530kcal) (Valid Nutrition, n.d, Wagh & Deore, 2015)

VITAMINS	MINERALS
Biotin (60 µg)	Calcium (535-750 mg),
Niacin (13 mg)	Copper (1.4-1.9 mg),
Folic acid (330 µg)	Iodine (100-140 µg),
Panthothenic acid (4 mg)	Iron (10-14 mg),
Vitamin A (550-1150 µg)	Magnesium (150-225 mg),
Vitamin B1 (1 mg)	Manganese (1.2-2.4 mg),
Vitamin B12 (2.7 µg)	Phosphorus (excluding phytate) (450-750mg)
Vitamin B12 (2.7 µg)	Potassium (900-1400 mg),
Vitamin B2 (2.1 mg)	Selenium (20-40 µg),
Vitamin B6 (1.8 mg)	Sodium (270 mg max),
Vitamin C (60 mg)	Zinc (11-14 mg)
Vitamin E (16 mg)	
Vitamin D (15-20 mg)	
Vitamin K (27 mg)	
(N.B: The ranges for vitamins are in minimum)	

2.2.2 Packaging and Storage Condition

RUTF pouches are packaged in tablets of 92g-500 kcal. While RUSF contains 550 kcal-100g lipid paste in grease-proof paper.

These finished products are packed in a carton containing 150 sachets, containing detailed information indicating nutritional composition of RUF including list of all minerals and vitamins.

RUTF have a shelf life of 48 months from the production date to period of storage. Little reduction in the level of some vitamins may occur depending on the duration and

temperature of storage condition. When the aluminium bag is opened, RUTF bag should be used one or two weeks.

2.2.3 Consumption

It was impossible to use a single yardstick for collecting information regarding the facts that the authorities required to be analyzed even when simplified. This was not a simple field for which to gather information and so a combination of reports from various sources and proficient search was employed to grade these sub-criteria(FAO and WHO, 2014). Research conducted in provincial Bangladesh revealed that supplementary foods are very much lacking in essential micronutrients. In a more recent study, the sufficiency of consumption of 11 micronutrients between 24 to 48 months infants in provincial Bangladesh was evaluated and the total mean prevalence of capacity of micronutrient intakes for infants was only 43%. The predominance of adequacy was below 50% for riboflavin, folate, iron, calcium, and vitamin B12. In the same community it was recognized that infants eat small amounts of fat and among most kids, only 1%-4% total percentage of energy is derived from essential fatty acids. These observations indicate food insecurity which affects about 20% to 30% people, with a low dietary difference and lower feeding rate of young kids among a larger section of the society.

Although adequate awareness to better the nature of complementary feeding attainable in food secured neighbourhoods, supplementation with nutritious food may be necessary for infants, particularly those who can- not afford a balanced diet meal (Ahmed et al., 2014a).

All wasted infants ranging from 6 to 60 months of age in the settlements greater than 80 percentages were different in thinness Z score between the RUTF intervention and kids not on RUTF intervention at the start and after 240 days of follow-up were -010Z and 0.12Z each. The shift in outcome of the programme was found to be 0.22. RUTF treatment outcome is 36 percentages (95% CI, 17% to 50%, $p<0.001$) with a decline in the prevalence of malnourishment and 58 percentages (95% CI, 43% to 68%, $p< 0.001$) decrease in critical malnutrition.

Observation made at the brief period of RUTF intervention reduce the decrease in thinning Z score and number of malnourishment within 8 months follow-up (Wagh & Deore, 2015). The 10 largest RUF consuming countries in the span 2010 to 2013 were Niger,

Nigeria, Ethiopia, Somalia, Chad, Yemen, Pakistan, Democratic Republic of Congo, Mali and Burkina Faso (UNICEF, 2014).

Food and Agricultural Organization & World Health Organization Chronic Individual Food Consumption Database Summary Statistics (CIFOCOSS) was adopted as a competent specific food intake resource accessible world-wide. It was observed that it was impossible to produce a credible estimate of median and standard deviation of some LMF (i.e. dried protein products) as a result of few consumers detailed in the study. The mean consumed in grams per day for the average population and amount eaten by those estimated to be the high consumption in the Preliminary communiqué of FAO/WHO expert deliberation on the ranking of reduced moisture foods was therefore employed for ranking purposes (FAO and WHO, 2014)

(i) **Ready-to-Use Therapeutic Food (RUTF**) is energy and nutrition-dense, comprising of 520-550 kcal/100g.Therapy suggestions for SAM is to provide 100-135kcal/kg/day of a RUTF, for a span of 6- 10 weeks, continuously for the child to achieve satisfactory weight. An average critically malnourished child can consume about two sachets per day (1000kcal) and can obtain enough nutrient intakes for total recovery. While clean drinking water requires to be taken after consumption of RUTF , no other foods beside breast milk are needed (UNICEF, 2014).

(ii) **Ready-to-Use Supplementary Food (RUSF)** is a kind of RUF that is specially produced for children between 6 to 59 months for the therapy of MAM. 92-100 g RUSF, with an energy density of 513-550 kcal/100g, as a daily portion is recommended. It is consumed by the child in along with breast milk and other foods for about 3 months (UNICEF, 2014).

Babies and toddlers (0 – 35 months) and the aging (>65 years) with Moderate Acute Malnutrition and Severe Acute Malnutrition are regarded to be the most vulnerable group, with the data obtained from these figures it was not feasible to relate such information to the LMF products and therefore this would not differentiate those group which may be commonly eaten by the under-nourished individuals (FAO and WHO, 2014).

2.3 Safety Concern of Lipid-Base Ready to Use Food

There are no specific guidelines in the manufacture of Ready to Use Food, although flexible guidelines exist to encourage innovations and modifications of the products. Reports issued by UNICEF and some non- government organizations do not cover all areas. The guidelines on the nutrient composition of any RUF are important, particularly as it is being considered as the only food significant for the treatment of SAM which includes the amount of calories and protein, and also all important minerals and Vitamins. Guidelines on safety is needed, and on acceptable levels of toxins and other possible toxic ingredients present in any RUF. (According to reports, Aflatoxin was discovered in RUTF being fed to children in Haiti. The Incident was from contaminated peanuts) (Clark, 2018; Latham et al., 2011)

Food safety is an issue of world interest that urged the WHO to begin an action Global Burden of Foodborne Diseases intend to globally outline and measure illness linked to hazardous food. Numerous points might now be recognized in food microbial safety that comprises of differences in consumption behavior, variations in applicable production techniques (increasing large size industrial production or free, outdoor and organic production), food production method (inadequate knowledge of the preservation processes employed in conventional local foods, mildly preserved foods), microbial resistance and climate changes. These are vulnerable areas in manufacture of food and circulation channels where food pathogens are somewhat destroyed, re-enter and adapt to food, manufacture and handling conditions, and therefore grow and produce toxic microorganisms resulting in numerous foodborne disease (Clark, 2018; Martinovi et al., 2016).

These illnesses are result by consuming food spoiled by pathogens or their toxins which are quickly spread and as a result becomes a global public health concern. In 2013, 5196 foodborne related outbreaks were published in the EU, leading to 43,183 people infected, an approximate of 5946 hospitalizations and 11 deaths. In the US, an approximate of 9.4 million incidents of foodborne sickness occurs yearly 55,961 have been hospitalized and 1351 reported deaths. There are notable increase in the number of foodborne diseases associated with new nutritional trends that encourage eating unprocessed and fresh food,

dry products, and exotic ingredients. Subsequent to these options, globalization of the food business added up to foodborne illness outbreaks, making food security and safety a universal subject. This is proofed incidence of outbreaks of food poison caused by a foodborne Shiga toxin producing Escherichia coli O104:H4 in recently in France and Germany. About 3816 incidents with 54 deaths were reported in 2011. Hence, identifying pathogens in food and safeguarding the food against spoilage is a responsibility of great public health, social, and economic significance. Bacteria, viruses, parasites, and fungi that infect food in various steps of processing and handling cause foodborne illness referred to as foodborne pathogens. Additionally, some fungi and bacteria can as well produce toxins, and at this stage identification of the pathogen alone is not an adequate preventive step for food safety. Several pathogens and their toxins are heat resistant, and cannot be killed by simple food preparation techniques such as frying, cooking freezing, , food safety control becomes a more difficult issue (Aruwa & Akinyosoye, 2015; Martinovi et al., 2016).

2.4 Importance of Microbiological Assessment

Although the precise prevalence of food-borne diseases is unknown, specialists admit that prevailing levels are alarming. Information from population-based investigations and national monitoring gathered from 1996 to 2000 in the UK each year, almost 2 million incidences of indigenous foodborne illness happen. While many cases are self-limiting, some, particularly in children, pregnant women or immune-incompetent persons can be dangerous and even life-threatening. The strain on health-care systems is substantial and the price to the National Hospital System (NHS) and business was valued as £350 million yearly by the Food Standards Agency in 2000. Although control of foodborne sickness is a top-most of the European Food Safety Agency (EFSA) agenda, data indicates that majority of incidences go unreported (Clark, 2018; Tebbutt, 2007). In the field of food Microbiological assessment, According to EC Regulation No. 2073/2005, "microbiological criterion is a basis of assessing the acceptance of a goods, a lot of food-produce or a method, based on the nonexistence, occurrence or quantity of microorganisms, and or on the amount of their metabolites, per unit of the volume, area, mass or batch" and "food safety criterion defined a standard describing the acceptance of a produce or a lot of food

products suited to place a commodity on the market" (Mashak et al., 2015) therefore it is a vital quality preventive measure in determining the safety of food.

The most frequently utilized methods of bacteriological study in food microbiology are detection and enumeration. The presences of a particular kind of pathogen and their concentration must be known, to evaluate and manage safety risk, the ability to cause deterioration or to determine their specific properties. The bacteria of concern to food maybe categorized as infectious agents, causative agents of foodborne illness, spoilage agents, and manufacturing aids so as the Metabolic activity of a bacterium in food can be regarded as trigger of deterioration or as a production aid based upon the usefulness of the effect that occur. Detection of particular kinds of bacteria can be accomplished by cultural isolation, or by indicators such as biomolecules specific to the organism (example nucleic acid sequences, antigens, metabolite, toxins) or products of metabolism (e.g. gas, acid, substrates with chromogenic products). For enumeration, cell-concentration can be assessed by dividing the specimen on a solid exterior (such as membrane or agar media), in liquid portion (example MPN) or by direct or indirect amount of biomass (like; optical density) (Gill, 2017).

However, the possible extent of contaminant organisms are numerous and comprises of one large major group, the Enterobacteriaceae which include Gram-negative, facultative anaerobic, bacillus of worldwide population that are discovered in soil, water, plants, animals and transmitted from insects to humans. Although, not all genera and species in the family infect and result to sickness in humans, some do either as opportunistic pathogens or as pathogenic bacteria with characteristics that ranked them as very or not so dangerous to living thing (FAO/ WHO, 2016).

Microbial examinations are conducted to investigate the growth level of microorganisms whose presence in a product at certain levels is used to evaluate the quality and\or safety.

2.4.1 Microbial Specifications

The 37th Concourse of the Committee on Nutrition and Foods for Special Dietary Uses agreed to commence a new task on the guideline for: "Ready to Use Therapeutic Food" (RUTF) employed in the therapy of Severe Acute Malnutrition (SAM). Approved

by CAC39 that relevant standards and limit for associated microbiological hazards and chemical contaminants (Such as; pesticides and heavy metals) with reference to CODEX STAN 193-1995 and During the deliberation, the Chairs suggested that the current Codex texts with the Joint WHO/FAO technical consultation meeting reports for 2012 and 2014 and their suggestions would be adopted as a foundation for the creation of microbiological safety standards for RUTF in the Guideline.

At CAC37 the Code of Hygienic Practice for LMF was approved as a final Codex Code of Practice19. RUTF is mentioned in this Codex Code as an Annex to the Code, which includes microbiological criteria for Salmonella in low moisture foods, was adopted by CAC in June 2016.

The WHO/FAO 2012 expert convention also conducted a risk evaluation of the microorganisms reported in the 2007 Joint report and reexamined a number of pathogens in food that cause illnesses of different severity in infancy diseases and assessed their possibility of being carried by low moisture foods. Of the seven microorganisms formerly listed in the 2007 Joint statement, the greatest risk believed to be found in RUTF was Salmonella spp.

The panel suggested that Salmonella should be the top priority danger and its control as the primary food safety programme goal. At CAC39 (2016) an annexed of standards of microbiological guidelines was approved and the addition will be included in the Code of Hygienic Practice for Low Moisture Foods.

However, an agreement reached among the eWG Members that the 2012 and 2014 Expert Deliberation meetings and other existing Codex texts sufficiently addressed the risk of pathogens in RUTF (FAO/WHO, 2016).

However, the current stipulations of Ready-to-Use Foods for the control of MAM and SAM highlighted the criteria for Enterobacteriaceae, Salmonella species, Listeria species. Cronobacter species, coliforms and mesophilic aerobic bacteria (Table 2.5), although, Microbiological specifications differ somewhat between UNICEF, WFP and MSF, the target values for *Salmonella* species, *Cronobacter* species and mesophilic aerobic bacteria are the same. Equivalent microbiological specifications were utilized in 2005 to RUTF as are employed for therapeutic milk formulas.

There are significant variations between F-100 and RUTF that determine the fitness of microbial targets for specifications and concentrations of concern. For instance, F-100 is a powdered milk that is prepared with water prior to serving, like PIF, if F-100 carries *Cronobacter* species and is kept at warm ambient temperature after it is prepared, the small numbers of *Cronobacter* species that is in the dry powder may increase to higher amounts that are considered to produce dangerous invasive infection. Because of Cronobacter's short reproduction time in the prepared formula at favorably warm temperatures, bacteria can multiply a thousand times in matter of hours. To the contrary, RUTF is administered as a dense paste with essential reduced water activity and is not prepared with water.

It is packed in one-serving package, which are to be eaten at once. The Product characteristics present little chances for pathogenic growth even when consumption extended to more hours; furthermore, while people nourishing on RUTF are without a doubt at higher jeopardy of infections with severe complexities than are well-fed infants of comparable age, they are not part of the age group acknowledged to being at greater danger for serious complication of Cronobacter spp. infection or meningitis (FAO/ WHO, 2016; Piper et al., 2018). Therefore, the microbiological standard for no detected counts of Cronobacter species in 30 samples of 10 g each of product, used for PIF and other formulas of particular medicinal plans made for children, may not be a suitable indicator of safety hazards linked with peanut RUF. Additionally, consideration of Codex PIF standards for guidance on relevant microbiological standards for peanut RUF, the quality assurance and food safety teams from UNICEF, WFP and MSF re-evaluated and made other Codex and EU food standards for more microbiological specifications that seemed appropriate to peanut paste RUF.

UNICEF, WFP and MSF purchase nearly 95% of RUF, and their safety and quality teams collaborate on inspection of producers, based on Codex and ISO 22000 standards, to confirm regularity around all emergency intervention feeding programmes that fight malnutrition. Nevertheless, each relief agencies keeps its own independent quality system and decision tree for the validation of products and producers.

Table 2.5: Maximum microbial levels as detailed in Joint statement of FAO/WHO expert panel

MAXIMUM MICROOGANISM	LEVEL
Coliform test	(-ve) in 1 g
Clostridium perfringens	(-ve)in 1 g
Pathogenic Staphylococci	(-ve) in 1 g
Salmonella	(-ve) in 125 g
Listeria	(-ve) in 25 g
Aflatoxin level	5 ppb (max)
Microorganism content	10,000/g (max)
Yeast	10 in 1 g (max)
Molds	50 in 1 g (max)

Total aerobic count 10,000 colony forming unit (cfu)/g maximum

2.4.2 Microbial Hazards

The superiority claim of RUTF over other commercial complementary foods; it carries no water, is not susceptible to bacterial contamination, and hence is safe to use, no longer stand (Latham et al., 2011) the point remains that RUTF and RUSF are characterized as low moisture products which have reduced water activity, Hinders microbial growth, Does not destroy microbial contaminants, and May store them in a metabolically dormant state. Many Microbiological risks have been associated to reduced moisture foods like Peanut butter – salmonellosis, botulism, Milk powder - salmonellosis and Powdered infant formula (WHO/FAO, n.d.) Although salmonella is considered as the largest vegetative pathogen that serves as a limit for growth in both RUTF and RUSF, there are numerous others that possess severe health implications to people consuming them.

The use of peanuts is vital (and promising) in the manufacture of RUTF and RUSF which are frequently being linked with good nutrition and general health and well-being. They have rich oil content with an exemplary fatty acid form of MUFA and PUFA. They also have rich proteins and varieties of minerals and vitamins. Peanuts are extensively

cultivated in several Africa countries and are usually processed by women using simple conventional technologies.

Aflatoxin contamination is popular in peanuts and products made from it; Aflatoxin and Salmonella contamination were reported in peanut pastes. Epidemiological and environmental probes of those outbreaks have proposed and suggested that cross-contamination plays a significant part in the adulteration of these products as it is the transfer of bacteria from one place, item, or surface to another. Related food safety hazard may occur when there is a transfer of a pathogen when the product is ready to use, with no further Salmonella kill-step in the process. In a 2004 survey conducted by the WHO, which showed a notable proportion of European foodborne outbreaks could be sketched back to cross-contamination. The statement showed the factors adding to the ubiquity of pathogens in product involved inadequate sanitation (1.6%), cross-contamination (3.6%), production or warehousing in an unsuitable location (4.2%), contaminated equipment (5.7%), and adulteration by employees (9.2%).

In a report of outbreaks across UK, the causative factor was known to be cross-contamination accounting for 57% of total occurrences (Podolak & Enache, 2010).

Therefore, microbial pathogens may have access to reduced-water activity foods through ingredients or from the production facility because of lack of proper sanitary practices, such as non-compliance to Good Manufacturing Practice (GMPs).

Groundnut, chickpeas and soybeans which are the major raw materials used in RUF of lipid origin mix containing a variety of naturally occurring bacteria and fungi, some can cause human diseases. Therefore, even reduced moisture foods with adequately reduced a_w can inhibit the growth of bacteria and be a carrier of pathogens in incidence of foodborne disease (FAO/ WHO, 2016).

Lysinibacillus *sphaericus* earlier identified as 'Bacillus *sphaericus*' is strictly aerobic and catalase- positive have been linked to carryover of pesticide residue from farm or exposure of ingredient to insecticide at either production facility or during storage since it is utilized in commercial insecticides for the prevention of crop insect infestation (Geser, Stephan, & Hächler, 2012).

Candida is found in the environment and also in food. Highly-processed foods are not usually contaminated with yeasts of the *Candida* genus. Moreover, fermented food products and products with a reduced level of processing are not yeasts free as well. *Candida* yeast species acknowledged as the most prevalent etiological pathogen of systemic and invasive thrush in humans. Invasions can affect all tissues, organs and systems of human in different growth stages (Maroszyńska et al., 2013).

Clostridium licheniformis is catalase positive organism, One or more incidences of bovine toxemia, peritonist and ophthalmitis were reported and several cases of bacteremia or septicemia and many cases of food poisoning have been clinically reported globally and Food deterioration like as ropy bread, and cases of food-related gastroenteritis. *B. licheniformis* has also been linked with septicemia, peritonitis, ophthalmitis, and food poisoning in humans, bovine toxemia and abortions. *B. licheniformis* is a typical contaminant of dairy products Although, Food-borne *B. licheniformis* cases are mostly connected with cooked meats and leafy vegetables, Reports was made of toxin-producing isolates of *B. licheniformis* gotten from foods related to food poisoning cases; un-pasteurized, and industrially manufactured children's food (Salkinoja-Salonen et al., 1999).

2.4.3 Low Moisture Food (LMF)

The freight of foodborne sickness and many food products recalls linked to microbial contamination in LMF have risen more recently.

LMF's naturally have low water activity or are derived from foods of higher moisture by drying or dehydration method. The reduced water activity (a_w) of these products add to a prolonged shelf life, such Low Moisture Food products may comprise of; confections (e.g. chocolate), powdered-protein products (such as powdered egg and dairy), cereals grains, dried fruits and vegetables, nuts and products of nuts origin (like peanut butter), honey, spices, seeds amidst others.

LMF's are usually regarded as safe which are consumed as ready-to-eat foods that requires no any kill step like cooking before consumption. LMF's are prone wide variety of microbial contamination, although most pathogen cannot grow in LMF because of low water activity, numerous microorganism can live and remain active for quite a while in these foods, posing as a severe threat to consumers.

It is challenging to decrease microbial contamination of reduced moisture food by notable margins (e.g. >5 logs) and to zero detection employing conventional processing interventions with the likes of heat treatments that are used on high moisture foods. However, the mix of low a_w with the more sugar content and or fat level of many Low Moisture Food is gathered to enhance the survival and susceptibility of these pathogens in food products (FAO and WHO, 2014). Since Lipid-based RUF for malnourished populations are termed as LMF's but excluded from ranking which is also considered relevant to general principles of hygienic practice (FAO/WHO, n.d.) in similar regards, nearly all of the constituent of RUF have been ranked and grouped such as dehydrated dairy products (e.g. milk powder or whey), nuts and products of nuts origin tree nuts (e.g. pecans, pine nuts, pistachios, almonds, brazil nuts, cashews, hazelnuts, macadamia nuts, walnuts) groundnuts and peanut produces (e.g. groundnut spreads, peanut butter) mixed or unspecified nuts, sesame seeds or tahini (sesame paste), halva (deserts made from sesame paste) other and unspecified seeds (e.g. pumpkin seeds, sunflower seeds, melon seeds, flax seeds, poppy seeds, mixed or unspecified edible seeds). However, the Total Disability-Adjusted Life Year (DALYs) in outbreaks from 1990 to 2014 for each category was reported to be; Nut Products 118.51, Dried Protein Products Nuts 136.44 and edible Seeds (Ahmed et al., 2014b; FAO/WHO, n.d.). But the actual statistics cannot be known unless reported.

2.5 Quality Control

Quality control is accomplished by utilization of operating procedures that are globally recognized as criteria for food processing, the Codex Alimentarius and the Hazard Analysis and Critical Control Point Program. These standards guides on raw ingredients procurement, storage of acquired product, blending of raw materials and storage of the final goods. Apart from international standards, each nation has a department of Standards which governs the food manufacturing. These agencies also designate manufacturing procedures; carry out inspections of companies and giving permits to produce food. Examinations are used to check the quality of the manufacturing method, and hence should be conducted with all enormous batch of final produce, weekly. In Malawi, the finished food is analyzed each week for contaminating microorganisms (like *salmonella*,

staphylococcus, total flora of aerobic mesophilic bacteria, coliforms, *E. Coli*, yeast, mold), *Aflatoxin* and food content (fat, protein and potassium). The examination is normally conducted internally so as to recognize errors in production quality. However, RUTF shall not be shipped to consumers without confirmation of food quality. Virtually all countries have a laboratory allied with Standards organization that can carry out the independent examination (Manary, 2006; Wakhu-Wamunga & Wamunga, 2017).

2.6 HACCP

HACCP is a control system in which safety of food is approached by investigation and checked of physical risks, biological, chemical, and from raw ingredients processing, purchase and handling, to manufacturing, circulation and consumption of the final goods (FDA, 1997). A strong HACCP design shall take consideration of the way food will be handled and eaten after being sold, as this can determine the method of production or manufacture at the production facility as Indicator organisms can act as part of validating HACCP or other food safety control measures. Their presence indicates processing failure, inadequate hygiene practice and contamination by pathogens. Given that indicator organisms occur more frequently than pathogens, the two main food safety hazards in RUF productions are the presences of Aflatoxin and the hazard of pathogenic microbial growth. The growth of pathogen can be eliminated or reduced by utilization of effective HACCP system (Clark, 2018; Henry, Lim et al., 2014; Tebbutt, 2007).

2.7 Sampling Method

The sufficiency and properties of the food samples collected for investigation are fundamental. If samples are inappropriately collected and mishandled or are not representative of the sampled lot, the end result will be insignificant. Because interpretations of a bulk of food rely on a relatively few samples of the lot, accepted sampling procedures must be used consistently. A representative sample is necessary when organism and their toxins are widely distributed inside the sample or when the distribution of a food lot is established on the manifested bacterial content in line with a legal standard.

The amount of units that constitute a representative sample from a chosen portion of a foodstuff shall be statistically significant. The properties and attributes of each portion

have an influence on the uniformity and regularity of the overall sample quantity. The suitable analytical sampling method, according to the nature of the food: as either liquid, partially-solid, viscous, or solid, need to be decided by the sampler at the period of sampling according to the Investigations Operation Manual (Andrews & Hammack, 2003)

For ready to use food the following Codex guidelines were recommended for manufacturers, especially for microbial and contaminants not addressed in CODEX STAN 234-1999:

• General Standards for Contaminants and Toxins in human Foods and animal Feeds (CODEX STAN 193-1995),

• Code of Hygienic Practice for a_w Foods (CAC/RCP 75-2015),

• General Principles of Food Hygiene (CAC/RCP 75-2015)

• Recommended International Code of Practice – General Principles of Food sanitation (CAC/RCP 1- 1969); Code of sanitary practices for Powdered Infant and toddlers Food (CAC/RCP 66--2008) and its annexes;

• And the standards for the setting and use of Microbiological limit for Foods (CAC/GL 21-1997)

However, in developing a sampling plan, certain aspects should be considered including food properties, production steps, and storage condition of the finished goods, the risk involved, targeted consumers and realistic constraints. Each food produce shall be take into account separately (Centre for Food Safety, 2014).

The sampling plan should include at least the following:

- Number of microorganisms of interest;
- Number of sample units(n);
- The maximum permissible number of defective sample units in a 2-class plan or marginally acceptable sample units in a 3-class plan (c);
- Method of analysis(s);
- microbial limit

- a microbiological limit which in a 2-class plan, set apart good quality from faulty quality or, in a 3-class plan, separates good quality from marginally acceptable quality(m)
- a microbiological limit which, in a 3-class plan, set apart marginally acceptable quality from faulty quality(M)
    i. Acceptable (< m)
    ii. Marginal acceptable (> m and < M)
    iii. Unacceptable (>M)

CHAPTER 3
RELATED RESEARCH

This chapter presents related work to this study that has been conducted on same interest area. It highlights on; low moisture food, consumption, microbial hazard, microbial specifications, previous works and increasing risk of contamination.

Surveillance and disease reporting is inadequate amidst kids with either Minimum Acute Malnutrition (MAM) or Severe Acute Malnutrition (SAM) and how thoroughly the data and the resolutions gotten from them correlate to the overall circumstances of susceptivity to disease and critical illness in malnourished infants has not been thoroughly investigated related to RUTF and RUSF. As it would be presumed that the prevailing rise in sensitivity to infection as a result of under-nutrition would also raise the susceptibility of starved infants to diseases do not seem to have been stated in many research involving malnourished kids. However, related studies exist for reduced moisture food.

The review on the 'challenge of challenge testing to monitor *Listeria monocytogenes* growth in RUTFs in Europe by following the European Commission (2014) Technical Guidance document' highlighted on the 'European Regulation (EC) No. 2073/2005' and laid down microbiological standards for some organisms in foods product and the regulations to be implemented and abide by Food Business Operators (FBO) when performing common and particular sanitary standards. Concerning to *L. monocytogenes*, this regulation includes mainly RUF products, and expects RUF products meant for children and for specific medical goals to be free of *L.monocytogenes*, and in other RUF products apart from those for kids and specific medical plans other microbiological criteria must be implemented base on the food products capacity to enable the growth of this organism. Therefore, for RUF that do not enable the growth of *L.monocytogenes*, the levels should not exceed 100 CFU/ g all-through the shelf-life of the food.

On the other hand, in RUF products that enable the growth of the bacterium, *L.monocytogenes* must not be in products at the point of existing the manufacturing facility; however, if the manufacturer can prove, to the regulatory authority, that the food will not surpass the limit of 100 CFU/g during its shelf-life.

In addition, this guideline proves FBO are obligated to the safety of food; who can carry out investigations to estimate the growth of *L.monocytogenes* that may possibly occur in food through the shelf- life in foreseeable rationally anticipated condition like storage state while distribution, handling and in sequence to compliance with the stated guidelines during the shelf-life of the food. This arouses the question of how the FBO determines if the food is capable or not to enable the growth of *L. monocytogenes*, and how to abide with the 100 CFU/g limit throughout the shelf-life can be proved (Álvarez-ordóñez et al., 2015).

In a related study by (Kotzekidou, 2013) and (Mashak et al., 2015), it was narrated that Switching lifestyles; adding more convenience foods for instant eating had contributed to numerous surfacing microbiological concerns in food safety taking into account *Staphylococcus aureus* as an opportunistic pathogen, having the inclined to grow on products with a_w and linked to contamination of raw ingredients or cross-contamination happening as a result of poor-handling during production or storage, another pathogen *Bacillus cereus* which is a spore-producing bacterium, common in nature and have a resistant endospores which may survive in food of starch origin that have undergone heat treatment, where the growth of the bacterium can occur as a result of inappropriate cooling of the food products after heat treatment.

And *Listeria monocytogenes* is capable of causing severe sickness in vulnerable people (pregnant women, cancer and AIDS patients, adults >65 years old, or organ transplant recipients). Storage at above prescribed period and produce with a shelf life of > 120 hours according to European Commission Regulation, 2073/2005 & 1441/2007) verily raise the danger of *L. monocytogenes*. If present will multiply that may cause disease in human.

Podolak et al (2010) further identified that low water activity (a_w) hinders the growth of several vegetative pathogens, like *Salmonella* species. Processed goods such as

of groundnut butter, powdered milk, infant foods, and cereals are characteristically low water activity foods.

While these foods, do not enable the growth of *Salmonella* but has been linked to outbreaks of salmonellosis. Epidemiological and environmental research of these outbreaks has indicated that cross-contamination is a significant part of the contamination by *Salmonella* of these foods. The authors also stated that Salmonella in sugar might live for long in products such as potato chips chocolate, and peanut butter. The mixture of high fat and low moisture activity can have a synergistic impact on *Salmonella* survival. An insight was given on the causes and danger for contamination by *Salmonella* in reduced-water food and addressed the survival and heat resistance of the microorganisms with precise sources that can be employed to help perfect suitable formulations and methods for these foods; Contamination linked with inadequate sanitation practices.

Contaminated constituents used as raw materials without an extra processing could carry the microorganisms straight into finished foods, Contamination connected with lack of Good Manufacturing Practices: manufacturer, particularly those supplying foods that will require little or no additional preservation technique from consumers, need to be conscious of possible contamination hazards and apply good manufacturing practices (GMPs) to fully protect the well-being of their customers, Pest control and *Salmonella* contamination also Pest control is an essential food safety measure in all production plant.

There are no recorded cases where insect activity was straight associated with *Salmonella* cross-contamination, there are investigations that prove regular rodents and pests can be carriers for *Salmonella* transmission. Nevertheless, *Salmonella* can adjust to severe circumstances less or greater than optimal temperatures, pH values, or desiccation. Despite the optimal growth temperature is 35 - 37 ^{o}C, *Salmonella* can reproduce from 2 ^{o}C up-to 54 ^{o}C. Although the optimal pH for growth of Salmonella is between 6.5 - 7.5, it has also been recognized at pH of 3.8 - 9.5. Usually, it is studied that no growth of pathogenic bacteria occurs below around water activity of 0.85, but with increase to 0.93 is enough to stimulate the growth of *Salmonella*. When these conditions are lower outside growth conditions, *Salmonella* may live for very long in some low- moisture foods

In another literature (Henry et al., 2014), stated that 'Aflatoxin contamination have a significant safety concern in the production of Ready to Use Therapeutic Food that comprises of peanuts as main constituents'. Aflatoxins are toxic mycotoxins produced by the fungus *Aspergillus flavus*, which reproduces on groundnuts and other products in situations like heat or drought intensity. Eighteen kinds of Aflatoxin have been classified. The more dominants are B1, B2, G1, and G2. B1 is the most well-known and poisonous, and its consumption may result to gastrointestinal complications, cancer, or even fatal liver failure.

Aflatoxins grow in peanuts that are not stocked well or are bared to a rainy weather condition, extreme temperatures or high humidity. It is remarkably so for shelled groundnut, where the natural guarding barrier of the kernel has been unshelled. Peanuts may become infected if they are damp or damaged unintentionally. Human Foods may contain up-to 4 - 30 µg/kg of Aflatoxin, differs among country. In 1998, the EU set a stricter directive, with a limit of 2µg/kg for B1 Aflatoxin and 4µ/kg for all Aflatoxins. The Codex Alimentarius Commission recommended a limit of 15µg/kg for all Aflatoxins. International standard proposes that food comprising concentrations as high as 10 µg/kg of Aflatoxin B1 would be admissible for all kinds of food products if the overall level of Aflatoxins shall not pass 15µg/kg.

The processing of RUF should be frequently observed against Aflatoxin contamination to decrease the danger of food-borne diseases. The optimal states for growth of *A. flavus* are 12% - 35% moisture at 27° - 38°C. Shelled goober-peas, shall be kept at reduced relative moisture to sustain low moisture and kept in a serene environment, can be stored for up-to18 months, whereas unshelled peanuts can stay as long as 528 days.

High temperature treatment prior to production has demonstrated to inhibit Aflatoxin contamination while in storage. Roasting decreases the amount of water in peanuts to a much-reduced level, making it hard for most bacteria and molds the likes of *Aflatoxin flavus* to grow. The two roasting methods are: dry and oil roasting; dry roasting is carried out at 160°C for 25 - 60 minutes to roast the groundnuts. During oil roast, the peanuts are fried at about 140°C for 3 -10 minutes. It was suggested that peanut-based products need to be protected after processing against cross-contamination considering the expanding interest in the home base produced RUTF since

the objective is to make RUTF using provincially available, seeds and nuts cereals, legumes, and tubers.

Furthermore, it was added that RUTF may also serves as means to deliver extra micronutrients, such as antioxidants, and probiotics. Nevertheless, the challenge ahead is to manufacture inexpensive RUTFs employing simple equipment and the utilization of HACCP principles. The applying GMP will promote the manufacture of RUTFs that are nutritious and microbiologically safe in numerous developing nations.

Manary (2005) describes the processing of RUFT relating to quality control issues; suggested that groundnut should be bought from a supplier that can guarantee all measures to avoid contamination have been achieved during harvest and in storage. It was also stated that Aflatoxin contamination manifest in groundnuts with black color or looking shriveled and irregular consumption may lead to hepatic oxidative stress and elevate to hepatic cancers.

It was further suggested that RUTF should comply with international standards for maximum Aflatoxin level of 10ppb - 20ppb. Elevated level of Aflatoxin may lead to acute intoxication. Moderate content may hinder a child growth. The essential microbiologic safety of RUTF permits it to be packaged in clean and dry environment, but not sterile conditions.

The growing number of infants suffering from malnutrition related to war across the globe in Countries like Syria, Yemen, South Sudan and Nigeria to name but a few have sky rocketed similarly, the spring of locally made RUF, possess a great challenge on safety. With the ongoing public health inspection, systematic collection, discussion and distribution of information for public health action (Porta, 2008), It is necessary to recognize outbreaks and other public health hazards, and to understand the extent of such incidence, observe changes in causes of disease, and examine interventions (Thacker et al., 2012) due to inadequate official surveillance, it is remarkably hard to discover outbreaks until the incidences become considerable, except the symptoms caused by the disease are unusual and critical (e.g. liver failure with jaundice quickly advancing to death in severe aflatoxicosis). As salmonellosis in infants under the therapy of lipid-based RUF is common compared to the unavailability of reported *Cronobacter* species

infections as it is as a consequence of daily risk to Salmonella species from food, water and the environment, and a low incidence of exposures to *Cronobacter* species within these localities, these incidence have not been documented. Bridging information gaps will be significant to understanding the health consequence of *Cronobacter* species in RUF of Lipid origin.

Since *Cronobacter* species infection in undernourished infants are not frequent and *Cronobacter* meningitis seem not to have been recorded among the vulnerable individuals, septicaemia and death are not unusual among kids with SAM and MAM, and they barely undergo culture-based diagnostics when ill (FAO/ WHO, 2016).

CHAPTER 4

MATERIALS AND METHODS

This chapter states the study area and design, acceptance and rejection criteria, collection of sample, study design, sample analysis, data analysis, quality control and investigation.

4.1 Materials

4.1.1 Ready to Use Food

RUTF and RUSF (Three different samples sourced from 3 states in Northern part of Nigeria; North West Nigeria (Katsina State), North East Nigeria (Borno State), and North West Nigeria (Kano State) were analyzed).

4.1.2 Study Design/Area

This research uses a systematic and scientific approach to investigate the presence of foodborne pathogens in RTU foods; RUTF and RUSF distributed by relief aid in northern part of Nigeria. Samples were sourced from three different regions in northern part of Nigeria (Figure 4.1), RUSF (USAID) was collected from IDP camp north-east Maiduguri, RUTF (USAID) was collected from a relief center in Kastina and RUTF (MANA) was collected form Murtala Mohammed specialist hospital Kano Nigeria.

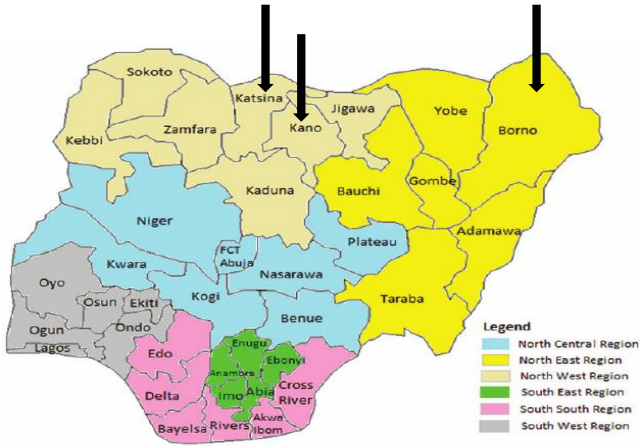

Figure 4.1: Map of Nigeria indicating study Area

4.1.3 Sampling Procedure

Samples were collected using random sampling approach. Six primary samples were collected from the package of 150 each (Figure 4.2), placed in a sampling container, stored at room temperature and transported from Nigeria to Near East University microbiology laboratory Cyprus for analysis. At Near East University Microbiology laboratory, Faculty of Medicine, samples were store in a deep freezer at minus 18^{o}c before analysis was conducted.

Figure 4.2: RUSF at point of sampling

4.1.4 Sample Preparation

Sample preparation was conducted in accordance to the method described by Food and Agricultural Organization of the United Nations FAO, guide on food quality control for microbes (1979). Six primary samples collected from the package of 150 sachets each were

carefully cleaned with a choline swap, cut each with a sterile scissors and homogenized thoroughly in a stomacher making a composite sample. (Figure 4.3) 9 ml normal saline solution was placed in a five series of sterile tube labeled 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Same was repeated for 8ml and 7ml sterile tubes series respectively. 1ml of the RUSF (USAID) was added to 9ml normal saline solution, homogenized for one minute to make a stock solution labeled 10^{-1} dilutions, the syringe used to place the composite sample was discarded. Another syringe was used to collect 1ml from the stock solution and added to the sterile tube labeled 10^{-2} each step the syringe was discarded and 10^{-2} was homogenized thoroughly. The same was repeated for 10^{-3}, 10^{-4}, and $10^{-5.}$ The 1ml solution taken from 10^{-5} was discarded. Caps were replaced each time dilution was made to avoid contamination. 2ml of the composite sample was added to 8ml normal saline solution and 3ml composite sample was added to 7ml normal saline solution repeating serial dilution as that of 9ml normal saline solution. Same sample preparation method was employed for RUTF (USAID) and RUTF (MANA) respectively.

Figure 4.3: Sample Preparation

4.2 Culture Media

Media were prepared according to manufacturer's specification. (Merck 1.01347.0500 EMB agar, 1.05438.0500 Sabouraud Dextrose agar and 1.10886.0500 Blood agar were used) (Figure 4.4)

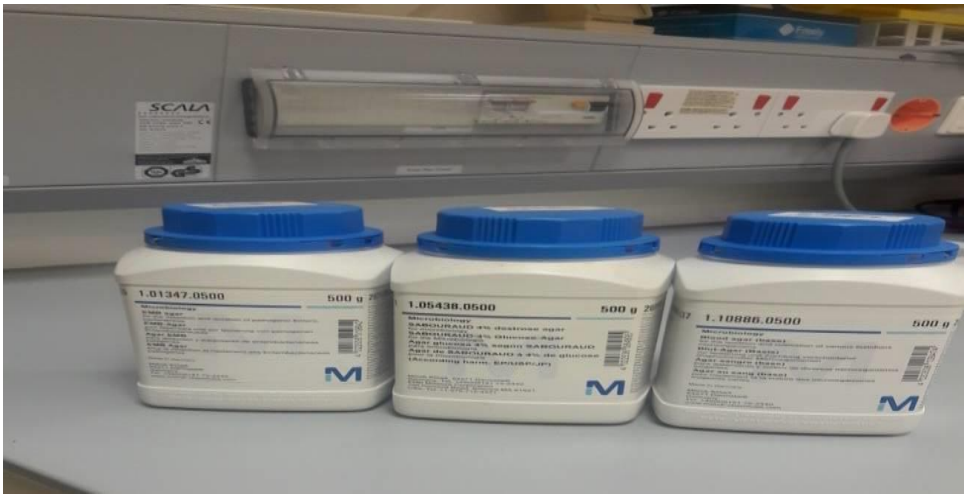

Figure 4.4: Media

Blood Agar

Principles

Blood Agar (BA) is an enriched medium utilized in culturing bacteria or microbes that do not grow readily, these bacteria are referred to as "fastidious" as they require enrichment, enriched nutritious condition as to the regular bacteria. It is employed in growing different types of pathogens specifically those that are hard to grow like Haemophilus *influenzae, Streptococcus pneumoniae* and *Neisseria* species. It is also needed in detecting differentiate haemolytic bacteria, especially *Streptococcus* species which destroys the Red Blood Cell by cytolytic toxins produced by these bacteria, like some strains of *Bacillus, Streptococcus, Enterococcus, Staphylococcus, and Aerococcus*.

Preparation

40g of nutrient agar powder was Suspend in 1 liter of distilled water and brought to a boil while mixing to dissolve completely. The dissolved mix was Autoclaved at 121 ^{o}C for 15 minute and allowed to cool to 40 ^{o}C. 5% (volume/volume) sterile defibrinated blood that has been warmed to room temperature and mixed gently but well was added and Air bubbles was avoided. At a final pH 6.8 ± 0.2 at 25 oc it was Dispensed into sterile plates while liquid foam to set.

Eosin methylene blue agar

Principle

EMB is both a selective and differential medium utilized to identify fecal coliforms. Eosin Yellow and methylene blue are pH indicator dyes which mix to produce a dark-shade of purple precipitate at low pH; they also act as growth inhibitor of many Gram positive organisms. Sucrose and lactose act as fermentable carbohydrate sources which boast the growth of fecal coliforms and give a way of isolating them. Fast fermenters of lactose or sucrose will yield enough amount of acid adequate to produce the dark purple dye complex. The growth of these microorganisms will emerge dark-shade purple to black. A strong fermenter like Escherichia coli outcome is a green metallic sheen and slower fermenters will yield mucoid pink colonies. On a typical circumstance, a colored or colorless colony implies that the organism ferments neither lactose nor sucrose and is neither a fecal coliform.

Preparation

36g of agar powder was dissolved in 1 litre of purified water and heated in boiling while agitated to completely liquefy all constituent. The dissolved mixture was Autoclaved at 121 ^{o}C for 15 minute and allowed to chill to 40 ^{o}c. At pH 7.1±0.2 at 25^{o}c it was poured onto sterile plates while liquid to set.

Sabouraud Dextrose Agar

Principle

Contribution of nitrogen and vitamins source from Peptone (Enzymatic Digest of Casein and Enzymatic Digest of Animal Tissue) is needed by microorganism to grow in Sabouraud Dextrose Agar. The Dextrose serves as the source of energy and carbon. Agar acts as the hardening agent. Broad-spectrum antimicrobials like Chloramphenicol and or tetracycline represses the growth of a variety of gram-positive and gram-negative bacteria. Gentamicin further frustrates the growth of gram-negative bacteria. The neutral pH of the Emmons modification appears to improve the growth of some pathogenic fungi, the likes of dermatophytes.

Preparation

65g of agar powder was Suspend in 1 liter of purified water, and Heated while whirling to completely dissolve all components. The dissolved solution was Autoclaved at 121 ^{o}C for 15 minute. It was ensured that overheating was avoided. At a pH 5.6±0.2 at 25^{o}c it was plated onto sterile plates while in liquid state and let to form.

4.3 Equipment

Figure 4.5: Vitek 2 Biomerieux and BD PhoenixTM

The BD Phoenix- Automated microbiology system is employed for the speedy identification (ID) and antimicrobial sensitivity test of clinically significant bacteria. The Phoenix system produces fast identification for numerous aerobic and anaerobic Gram-positive bacteria and other various aerobic and facultative anaerobic Gram-negative bacteria of human origin. The Vitek 2 offers thorough identification and susceptibility options (Figure 4.5).

4.4 Other Material

Pipette/dispenser, agar plates, cling film, metal spreader, test tubes/stand, Bunsen burner.

4.5 Microbiological Analysis

Microbiological analysis was conducted using the Spread Plate Technique and pour plate method. The principle behind these techniques of CFU establishes that a single microbe can grow and become a colony via division. These colonies differ from one another,

microscopically and macroscopically. This technique gives an insight on how many CFU's are present per mL in the sample the degree of contamination.

Principle

The spread plate method involves using a sterilized spreader with a flat surface made of metal to plate a liquid specimen with the aim of isolating or counting the bacteria present in that sample. The plate shall be dry and at room temperature as is necessary for the agar to absorb the bacteria more easily. A well spread plate will have a calculable number of isolated bacterial colonies smoothly dispersed on the agar (Aryal, 2016). Pour plate technique is same as spread plate method but differ during plating as a fixed amount of diluted sample is added to molten agar then allowed to solidifier. A total of 45 plates were made for each media per technique.

4.5.1 Inoculation and Incubation

Blood agar was labeled direct, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Same was repeated for Eosin methylene blue and Sabouraud dextrose agar respectively (Figure 4.6). Plates were left for 24 hour with the lid on at 37^{o} C, Plates were ensured to be dried so as to foam good spread. 0.1ml of samples was applied at the center of a dry plate, spread steadily on the surface using a metal (flamed and cooled) at about 110^{o} angle and flamed smooth. The rod was moved to and fro over the agar surface, lightly but rapidly, while rotating the petri dish to achieve a uniform application of the sample per dilution per media. Each sample was cultured directly on the three media agar labeled direct. The plates were Retain in upward position until inoculum was absorbed by agar medium (about 10 min on perfectly dried plates). The plates were Inverted and incubate 48 hours at 37°C (blood agar and EMB) (Figure 4.7) while SDA agar plates were store at controlled room temperature of 27^{o}C (All plates were cultured in the biosafety cabin).

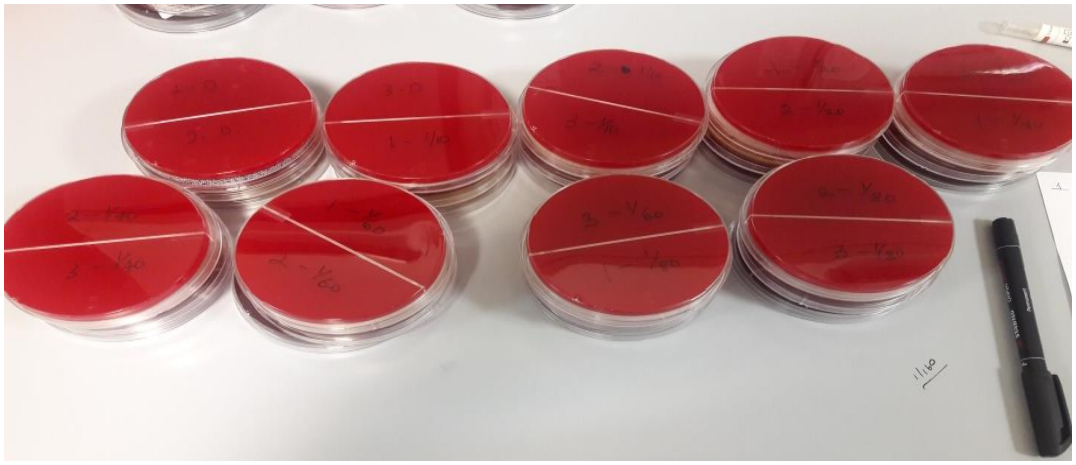

Figure 4.6: A pictorial representation of Inoculated Agar plates

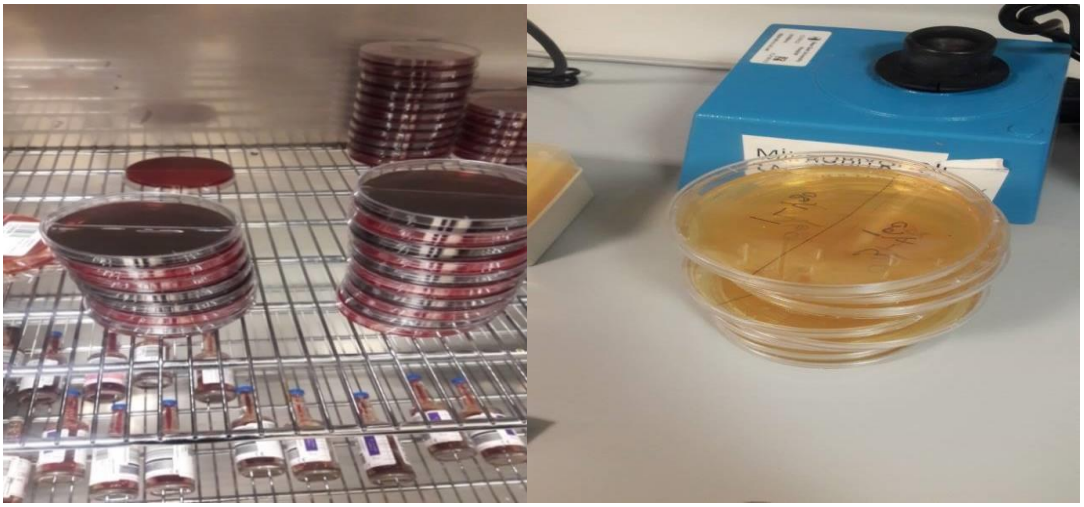

Figure 4.7: A pictorial representation of incubated plates

4.5.2 Observation

Spread plates growth on blood agar was observed as non-spore bearing Gram positive bacilli and spore forming. While growth on EMB agar was recognized as colorless, off-white gram negative non-coliform bacteria. And On SDA agar, white to cream colored, smooth, and glabrous were observer to be Gram negative bacilli and pink colonies were tentatively recognized as yeast cell (Figure 4.7). Counts were made and averages of colonies were taken.

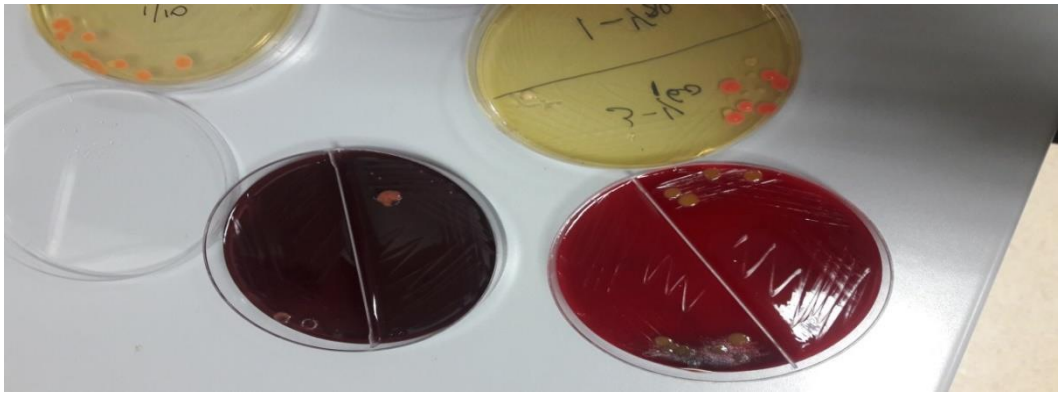

Figure 4.8: A pictorial representation of growth observed in some spread plate agars

4.5.3 Gram Staining

Principle

Staining is an auxiliary process employed in microscopic techniques utilized to improve the visibility of a microscopic image.

Heat Fixing: Ultra distilled water was used to form a smear on a glass slide and the smear was heat fixed in the blue flame of a Bunsen burner.

Stain Reaction: (staining was carried-out using BIOMERIEUX COLOR GRAM 2-F) (Figure 4.9)

- **Crystal Violet (CV)** was flooded onto the heat-fixed smear of bacterial culture. The CV separate in aqueous solutions into CV+ and Cl – ions and absorbed by the Gram-positive and Gram-negative cells wall and cell membrane. The CV+ ions later combine with negatively charged bacterial parts and stain the cells purple.
- **Gram's Iodine was added,** Iodine (I – or I3 –) serves as a fixative and a retaining agent that improves the affinity of the cell wall for a stain trapping the primary stain, consequently producing the crystal violet and iodine create an insoluble complex (CV-I) which gets confined to the cell wall and change the cells to purple.
- **Decolorization was done using** Alcohol which bares the peptidoglycan layer by dissolving the lipid membrane outside of Gram-negative bacteria and enhances the pores of the cell wall. The CV-I complex is then rinsed from the peptidoglycan layer, the Gram-negative bacteria remains colorless while the dehydrating influence of alcohol on the cell walls of Gram-positive bacteria which makes the openings of

the cell wall to shrink. The CV-I complex grips unto several very crossed-linked layers of the Gram-positive cell wall thus retaining the cells purple.

The decolorization stage was carried out with care, to avoid over-decolorization. This step is crucial and it was timed for 10 seconds to avoid the crystal violet stain been washed off from the Gram-positive cells.

- **Counterstain was made using Safranin** positively charged safranin was used to stain the decolorized Gram-negative cells to pink. The pink color which sticks to the Gram-positive bacteria is concealed by the purple color of the crystal violet.

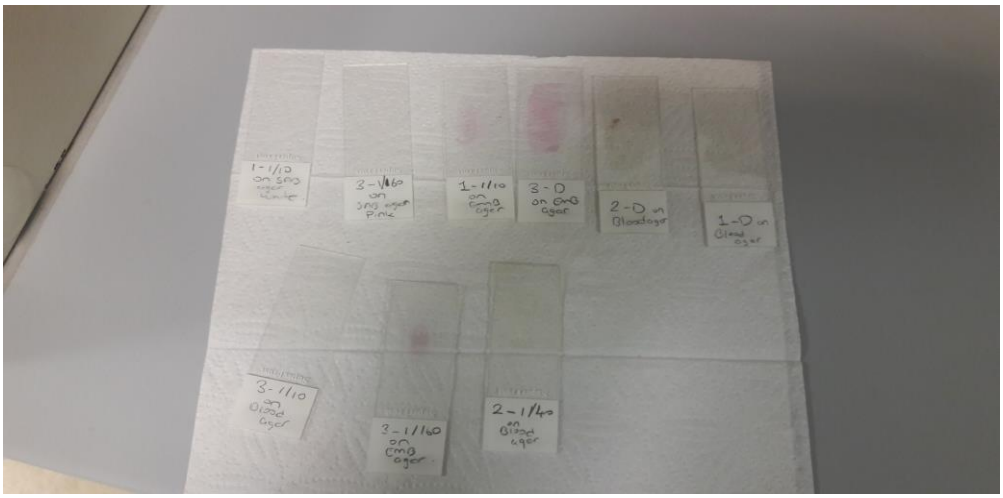

Figure 4.9: A pictorial representation of some Gram stained slides

4.5.4 Microscopic Observation

The spread plate stained glasses were observed under a microscope. Microscopically, they exhibit as non-spore bearing Gram positive bacilli (Figure 4.10) and spore forming (Figure 4.11), gram negative non-coliform bacteria (Figure 4.12) and spherical to sub-spherical large yeast-like cells with budding, blastoconidia, and pseudohyphae, and both (Figure 4.13).

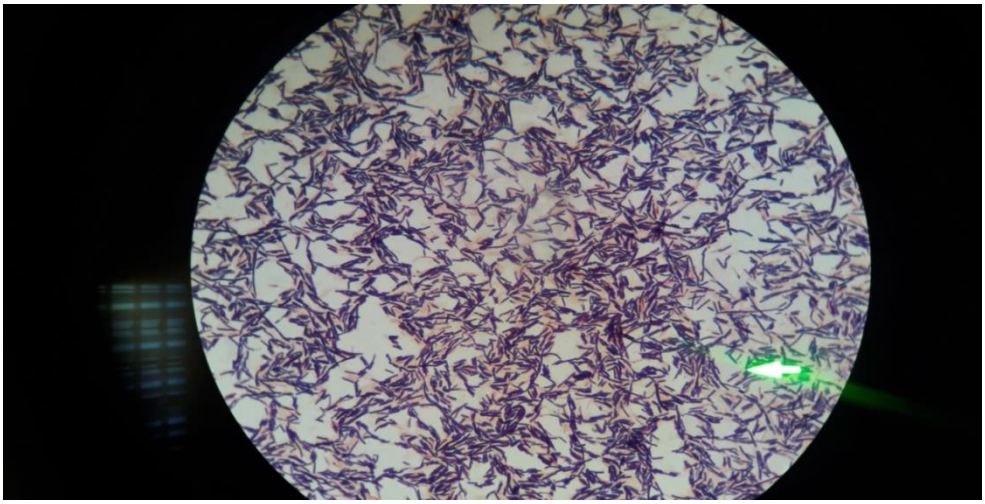

Figure 4.10: A microscopic view Gram positive Bacilli in RUSF USAID direct culture

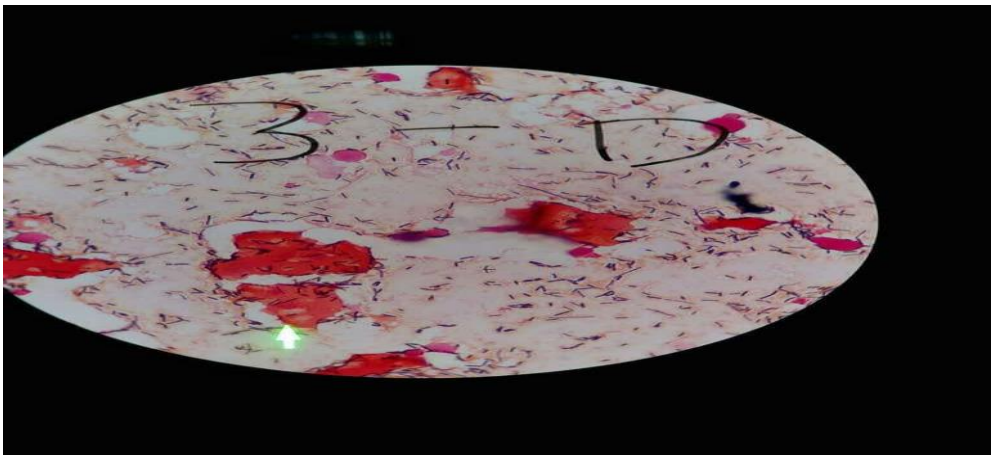

Figure 4.11: A microscopic view of Gram positive Bacilli with spore formation in RUTF USAID direct culture

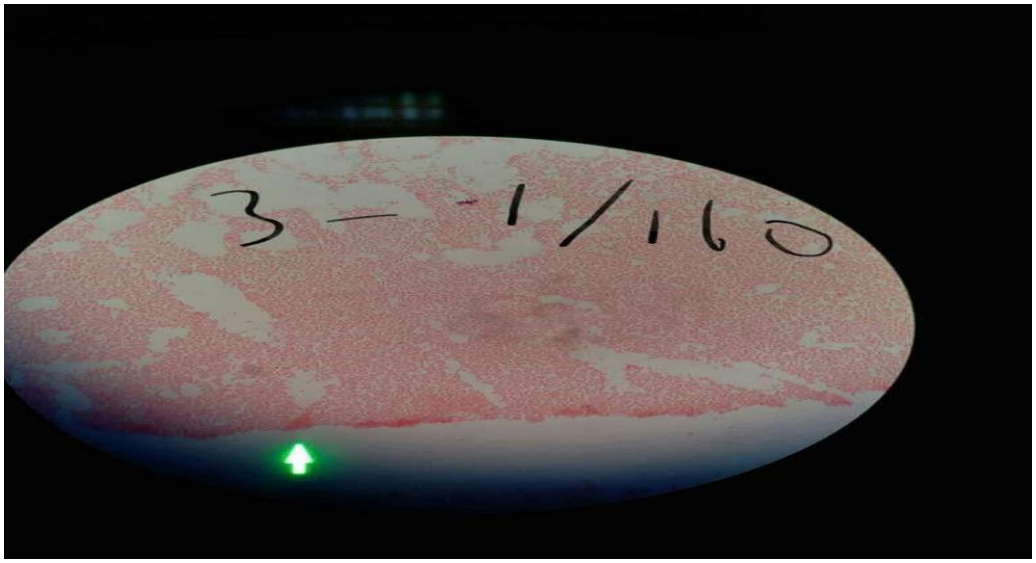

Figure 4.12: A microscopic view of Gram negative Bacilli in RUTF MANA in 10-5 dilution

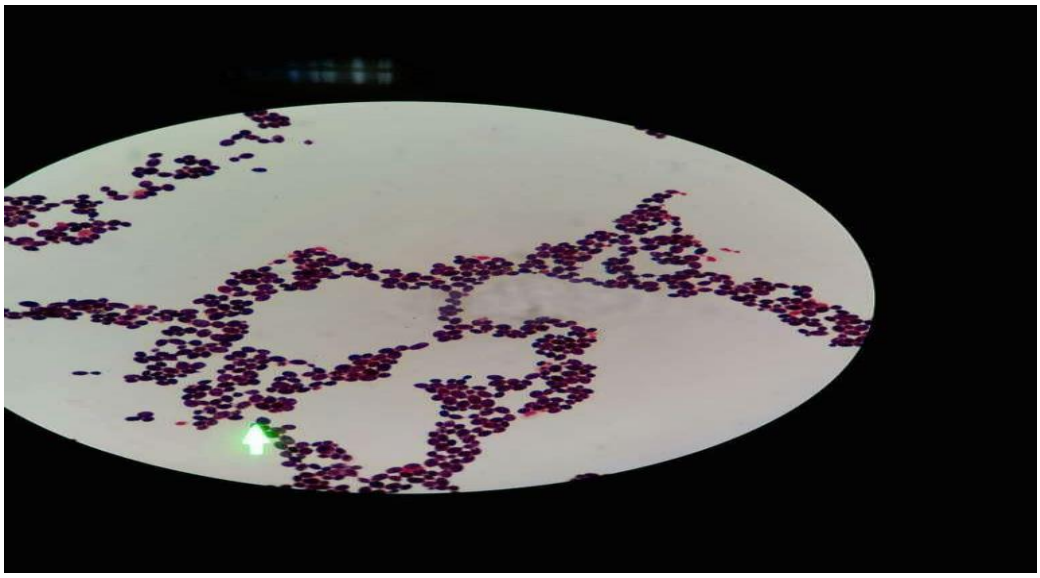

Figure 4.13: A microscopic view of yeast cell in RUSF USAID in 10-1 dilution

4.5.5 Identification Procedure

Microorganisms were identified using BD phoenix^{TM} 100 Bacteria Identification mechanism and yeast was identified using Vitek 2. Identification was conducted according to system user manual.

Principle

Up-to 100 microbial identification and antimicrobial sensitivity test may be conducted in the Phoenix machine at once with the Phoenix combination panels. It has a seal and auto-inoculating polystyrene plate of 136 micro-well with a powdered reagent which acts as disposal. The combination panel comprises of an identification section with a dried substrate for bacterial identification, AST part with different concentrations of antimicrobial agents and growth and fluorescent controls at the fitting wells location. The Phoenix system uses an enhanced colorimetric redox indicator for AST, and a various types of colorimetric and fluorometric indicator for identification. The AST broth is careful regulate to improve sensitivity testing efficiency.

The Phoenix panel is constituted of a 51 identification wells section and 85 wells AST places. The ID parts comprises of 45 wells with a dried biochemical substrate and 2 fluorescent control wells. The AST section potentially carries up to 84 dried antimicrobial agent wells and a growth control well. Panels are available as ID only, AST only, or ID/AST combine.

4.5.6 Bacterial Identification (verification)

The identification part of the Phoenix panel uses a range of classical, chromogenic, and fluorogenic biochemical test to evaluate the identification of the microorganism. Both growth-based and enzymatic substrate is used for the various kinds of reactivity within the range of taxa. The tests are based on microbial use and breaking-down of particular substrates sensor by multiple indicator systems. When an isolate is able to use a carbohydrate substrate, acid production is indicated by a change in phenol red indicator. During enzymatic hydrolysis of p-nitrophenyl or p-nitroanlide compound chromogenic substrates yield a yellow color. Enzymatic hydrolysis of fluorogenic substrates results in the release of a fluorescent coumarin derivative. Microorganisms that use a particular carbon source lower the resazurin-based indicator.

Procedure

Phoenix panels were inoculated with a standardized inoculum. Organism suspensions were prepared with BD phoenix Spec., after inoculation the panels were put in the equipment

and incubated at 35^{o}C in the machine test panels every 20 minutes: on the hour, at 20 minutes after the hour, and again at 40 minutes after the hour for up to 10 hours. Panels were interpreted by the use of Phoenix instrument. *Lysinibacillus sphaericus, Citrobacter youngae* and *Bacillus licheniformis* were identified (See Appendix 1, 2, 3)

4.5.7 Yeast Identification

The Biomérieux VITEK 2 machine is totally automated equipment utilized for the identification and sensitivity examination of organisms. The combination of VITEK Identification -Yeast card with the VITEK 2 system enables the identification of yeasts and yeast-like microorganisms within 15 hours with the aid of a highly sensitive fluorescence-based technology. The ID-YST card comprises of 47 biochemical reactions. The database contains 51 taxa, including newly described species.

Procedure

The combined VITEK 2 machine auto-fills, closes, carry the cards and incubate at 35°C. Each 15 minutes the cards automatically undergo fluorescence measurement. The profile was interpreted according to a specific algorithm. It was incubated for 15 hours; the profile outcome was compared to the ID-YST database, which results to the identification of *Candida albicans* (See Appendix 4).

CHAPTER 5
RESULTS AND DISCUSSION

5.1 Results

Globally, the number of gastroenteritis outbreaks caused by foodborne pathogens associated with RUF is on the rise. *Escherichia coli*, *Coliforms, Cronobacter*, pathogenic *Staphylococci, Salmonella, Listeria*, *Aflatoxin*, total aerobic and spoilage bacteria, yeast and fungi were investigated for in three types of Ready to-Use Therapeutics Food and Ready to-Use Supplementary Food.

Table 5.1: Observation made in RUSF (USAID)

RUSF (USAID)		DILUTION Cfu (ml)					
		10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**DIRECT**
BLOOD AGAR		I7	5	4	1	1	Positive
EMB		2	1	Negative	Negative	Negative	Negative
SDA	**Pink**	17	8	2	Negative	2	Negative
	White	4	Negative	Negative		Negative	

Note: numbers represents Cfu (ml), pink and white growth characteristics

Table 5.2: Observation made in RUTF (USAID)

RUTF (USAID)		DILUTION Cfu (ml)					
		10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**DIRECT**
BLOOD AGAR		21	12	8	4	1	Positive
EMB		2	Negative	2	1	Negative	Positive
SDA	**Pink**	10	7	3	1	Negative	Negative
	White	2	1	1	Negative		

Note: numbers represents Cfu (ml), pink and white growth characteristics

Table 5.3: Observation made in RUTF (MANA)

RUTF (MANA)		DILUTION Cfu (ml)					
		10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**DIRECT**
BLOOD AGAR		12	9	1	10	62	Negative
EMB		4	2	1	1	40	Positive
SDA	**Pink**	11	1	2	3	8	Negative
	White	3	1	1	Negative	10	

Note: numbers represents Cfu (ml), pink and white growth characteristics

Results obtained from spread plate technique by direct culture, *Lysinibacillus sphaericus* was found in RUSF (USAID) from North East (Nigeria) and RUTF (MANA) from North West (Kano) Nigeria. While Bacillus *licheniformis* was detected in RUTF (USAID) from North West (Kastina) Nigeria (Table 5.4).

Table 5.4: Microorganism Identified in Direct culture

Region	**Product**	**Media**	**Pathogens**
North East (Maiduguri)	**RUSF (USAID)**	Blood Agar	*Lysinibacillus sphaericus*
		EMB	-
		SDA	-
North West **(**Kastina)	**RUTF (USAID)**	Blood Agar	Bacillus licheniformis
		EMB	Bacillus licheniformis
		SDA	-
North West (Kano)	**RUTF (MANA)**	Blood Agar	-
		EMB	Lysinibacillus sphaericus
		SDA	-

In RUSF (USAID) from North East (Maiduguri) Nigeria *Citrobacter youngae* was present in all dilutions of Blood agar although with varying concentration, *Citrobacter youngae* also appeared in EMB in two dilutions where-as *Citrobacter youngae* and *Candida albicans* were identified in SDA in a single dilution. Also, in RUTF (USAID) from North West (Kastina) Nigeria *Citrobacter youngae* was identified in all dilutions of Blood agar, in two dilutions in EMB, three dilutions in SDA and *Candida albicans* in a single dilution. Similarly, in RUTF (MANA) from North West (Kano) *Citrobacter youngae* was found in all dilutions cultured on Blood agar and EMB, four dilutions on SDA and *Candida albicans* in all dilutions (Table 5.5). Specie identification was conducted using BD Phoenix and Biomérieux Vitek 2 machine.

Table 5.5: Microorganism Identified on different culture media

Region	Samples	Medium used	Concentration per dilution (cfu/ml)	Pathogen found
North East (Maiduguri)	**RUSF (USAID)**	Blood Agar	$17 * 10^{-1}$	*Citrobacter youngae*
			$5 * 10^{-2}$	*Citrobacter youngae*
			$4 * 10^{-3}$	*Citrobacter youngae*
			$1 * 10^{-4}$	*Citrobacter youngae*
			$1 * 10^{-5}$	*Citrobacter youngae*
		EMB	$2 * 10^{-1}$	*Citrobacter youngae*
			$1 * 10^{-2}$	*Citrobacter youngae*
			-	-
			-	-
			-	-
		SDA	$17 * 10^{-1}$	*Candida albicans*
			$4 * 10^{-1}$	*Citrobacter youngae*
North West (Kastina)	**RUTF (USAID)**	Blood Agar	$21 * 10^{-1}$	*Citrobacter youngae*
			$12 * 10^{-2}$	*Citrobacter youngae*
			$8 * 10^{-3}$	*Citrobacter youngae*
			$4 * 10^{-4}$	*Citrobacter youngae*
			$1 * 10^{-5}$	*Citrobacter youngae*
		EMB	-	-
			-	-
			$2 * 10^{-3}$	*Citrobacter youngae*
			$1* 10^{-4}$	*Citrobacter youngae*
			-	-
		SDA	$10 * 10^{-1}$	*Candida albicans*
			$2 * 10^{-1}$	*Citrobacter youngae*

			$7 * 10^{-2}$	*Candida albicans*
			$1 * 10^{-2}$	*Citrobacter youngae*
			$3 * 10^{-3}$	*Candida albicans*
			$1 * 10^{-3}$	*Citrobacter youngae*
			$1 * 10^{-4}$	*Candida albicans*
			-	-
North West (Kano)	**RUTF (MANA)**	Blood Agar	$12 * 10^{-1}$	*Citrobacter youngae*
			$9 * 10^{-2}$	*Citrobacter youngae*
			$1 * 10^{-3}$	*Citrobacter youngae*
			$10 * 10^{-4}$	*Citrobacter youngae*
			$64 * 10^{-5}$	*Citrobacter youngae*
		EMB	$4 * 10^{-1}$	*Citrobacter youngae*
			$2 * 10^{-2}$	*Citrobacter youngae*
			$1 * 10^{-3}$	*Citrobacter youngae*
			$1 * 10^{-4}$	*Citrobacter youngae*
			$40 * 10^{-5}$	*Citrobacter youngae*
		SDA	$11 * 10^{-1}$	*Candida albicans*
			$3 * 10^{-1}$	*Citrobacter youngae*
			$1 * 10^{-2}$	*Candida albicans*
			$1 * 10^{-2}$	*Citrobacter youngae*
			$2 * 10^{-3}$	*Candida albicans*
			$1 * 10^{-3}$	*Citrobacter youngae*
			$3 * 10^{-4}$	*Candida albicans*
			$8 * 10^{-5}$	*Candida albicans*
			$10 * 10^{-5}$	*Citrobacter youngae*

5.2 Discussion

Total aerobic and spoilage bacteria, yeast and fungi were identified in our investigation. Although, incidences of Aflatoxin contamination and salmonellosis are common among children under the therapy of RUTF and RUSF (Henry et al., 2014; Podolak & Enache, 2010) and RUF that will not go through any form of heating before eating are at great risk to occurrence of high microbial contamination (Piper et al., 2018). Our finding is in agreement with Piper et al. (2018) of no *Cronobacter* and (Campos et al., 2013; Mokhalalati et al., 2004) of no *salmonella,* and (Caponigro et al., 2010; Mokhalalati et al., 2004) of no *Escherichia coli* ; *Coliform;* pathogenic *Staphylococci and Salmonella.* Moreover, *Listeria* and *Aflatoxin* were investigated and also exhibit no growth. Whereas,

these organisms where found in the studies of Amualla et al (2010); Aruwa & Akinyosoye (2015); Caldera & Franzetti (2014); Campos et a.l (2015); Caponigro et al. (2010); and Gurler et al. (2015).

Furthermore, *Citrobacter youngae* identified in our study suggests secondary contamination from either food processing chain or food handlers. An extended monitoring study showed that most Gram-negative infections were caused by *Citrobacter* species. Pathogenesis links this organism to apparently invade the mouth, respiratory tract then transforms to several infections such as bacteremia and infection of the central nervous system these are nosocomial which are infection acquired in hospital (Geser et al., 2012). Invasive infections induced by *Candida albicans* found in our study can lead to a dangerous clinical complication since they usually affect vital organs like brain, liver and kidneys. In human, they are found on vaginal wall, gastrointestinal tract and vocal cavity. Most reported cases of candidiasis are in pet animals like dogs affecting the urinary tract and young cats resulting to gastrointestinal granuloma (Duchaussoy et al., 2015; Da Costa et al., 2016). *B. licheniformis* have also been identified and Ingestion may result to food poisoning since similarly incident related to consumption of this pathogen in the mid 1970's have been reported although the toxic agents have not yet been identified and most recently, an outbreak of food poisoning of play-group children from powdered milk containing toxigenic *BacillIus subtilis* and *B. licheniformis* (Pavic et al., 2005) and its presence suggest environmental contamination. *Lysinibacillus sphaericus* found in our study is known to be a carry-over pathogen and as a result of consuming food containing this pathogen, one or two cases of bacteremia, endocarditis, meningitis, pseudotumor and food poisoning have been reported clinically worldwide (Geser et al., 2012).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

The results obtained from the analysis clearly indicate that the claim 'Ready to-Use Therapeutic Food and Ready to-Use Supplementary Food do not support the growth of microorganism' is no longer true rather it harbor the growth of various bacteria and yeast such as *Lysinibacillus sphaericus, Citrobacter youngae, Candida albicans* and *Bacillus licheniformis.*

Since inadequate hygiene and sanitation have been indicated as a causative determinant in various outbreaks of foodborne disease, contamination associated to a poor constituent: control and handling. Moreover, even a well-designed machine operating with comprehensive precautionary support programs and detailed operational practices cannot overcome cross-contamination from bad selection, sourcing, and control of raw ingredients and materials (Podolak et al., 2010).

It is recommended that Nigerian government should encourage investors to establish home base Ready to Use Food production plants to reduce cost and contamination during handling, storage and distribution, Ready to Use Food manufacturer both foreign and indigenous should adhere to good manufacturing practice to avoid cross contamination, authorities concern should established microbial limit throughout shelf life and assign a strong monitoring team to oversee compliance with international standard bearing in mind minimization of infection and fight against malnutrition, and relief agencies should establish active surveillance network to monitor outbreaks, all outbreaks should be well investigated and proper documentation of cause should be conducted to prevent reoccurrence.

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APPENDICES

APPENDIX 1

Lysinibacillus sphaericus

APPENDIX 2

Citrobacter youngae

APPENDIX 3

Bacillus licheniformis

APPENDIX 4

Candida albicans