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AN INVESTIGATION INTO THE MICROBIOLOGICAL POPULATIONS PRESENT IN COCOA PRODUCTS

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DECLARATION

I hereby declare that this project work was carried out by me at the Food Research Institute of the Council for Scientific and Industrial Research (CSIR), Accra, Ghana under the supervision of Mr. Jacob Kweku Otu, Chief Medical Laboratory Scientific Officer, of the Department of Microbiology, Ghana Medical School, Korle-Bu Teaching Hospital, Accra, Ghana.

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ABSTRACT

The investigation into the microbiological populations present in the cocoa products, Bournvita and Richoco, purchased from "Mobil Mart" Supermarket and the Open Market on the bacterial loads ranged between 1.4×10^4 and 1.7×10^4 cfu/g in Bournvita purchased from the Supermarket and the Open market respectively.

The bacterial loads ranged between 6.2×10^3 and 6.6×10^3 cfu/g in Richoco purchased from the supermarket and the open market respectively.

The mould counts also ranged between 15×10^1 and 22×10^1 cfu/g in Bournvita purchased from the Supermarket and the Open Market respectively. The mould counts in the case of Richoco from the Supermarket and the Open market ranged between <10 and 1×10^1 cfu/g respectively.

The bacteria isolated from Bournvita and Richoco from both sampling sites were *Bacillus* species and coagulase negative *Staphytococci* (CONS), while the mould isolates were *Mucor* species, *Aspergillus fumigatus* and *Aspergillus niger*.

The bacterial load in Bournvita from the Open market was slightly higher than that of Bournvita from the Supermarket. In the same trend, the bacterial load in Richoco from the Open market was higher than that of Richoco from the Supermarket.

The mould count followed the same trend. From the recommended microbial limits per gram of cocoa, all the products were of good quality.

CHAPTER ONE

1.0 INTRODUCTION

Cocoa beans are obtained from pods growing on cocoa trees (*Theobroma cacao*) in warm humid climates such as Ghana, Cote d'Ivoire, Brazil, Nigeria etc. with temperatures of 21^0 -32^0C and annual rainfall of 125-300 cm. Cocoa products means any form of chocolate, chocolate product, cocoa, or cocoa product (U.S. Department of Health, Education and Welfare, 1975 c); additional definitions are available (World Health Organization, 1976, 1978). In some countries, chocolate may legally contain a small percentage of non-cocoa lipid (Wolf, 1977).

Additionally, many products that simulate chocolate contain a large percentage of non-cocoa lipids (Durkee, 1977 a,b). These simulated products, however, contain significant quantities of cocoa powder and are used widely in confectionery.

The objectives of the project, therefore, are:

a) To compare and assess the microbiological hygiene of the environment of the sampling sites

b) The quality control of the products under different environmental conditions under which the products are sold, and

c) To advise on how best these products could be stored for safety and longer shelf life.

Cocoa products have a water activity (Aw) sufficiently low to prevent growth of intrinsic microorganisms or extrinsic contaminants. There are, however, stages during the production of cocoa when spoilage may occur or large numbers of bacteria, yeasts, and fungi may be introduced. These sometimes persist in the final product.

Cocoa beans are found in pods surrounded by pulp, which is normally sterile. To be converted to cocoa, the beans must be removed from the pods, fermented, dried, roasted, ground and pressed. Microbially generated changes decrease pH and increase temperature during fermentation. These changed conditions destroy germinative capability of the beans and produce desirable colour and some flavour. Nonmicrobial enzymatic activities in the beans effect desirable changes at the same time. Drying stabilizes the beans against microbial spoilage, and roasting destroys some microorganisms and develops the full flavour.

The pulp in the ripe pods contain 80-90% water; 6-13% sugar of which about one-third is sucrose and most of the remainder invert sugars; 0.5-1% citric acid, and small amounts of aspartic acid, asparagine and glutamic acid (Forsyth and Quesnel, 1963; Rohan, 1963). The pH is about 3.5. Pulp from unripe pods contain less sugar and is difficult to ferment (Roelofsen, 1958).

The beans consist of testa (shell or seed-coat) and cotyledons. The cotyledons contain about one-third water and one-third fat (cocoa butter), and the remainder consists of phenolic compounds, starch, sugar, theobromine, non-volatile acids, and many other components in small concentrations *(Forsyth and Quesnel, 1963). Before cocoa beans are fermented, the ripe pods are harvested and transported in baskets, sacks or boxes to a central location. In some plantations, pods are transported to the fermentation site, and then opened by breaking or cutting, and beans (30-40 per pod) are removed with accompanying pulp by scooping *(Ostovar and Keeney, 1973). The beans with residual pulp are then covered with plantain leaves to retain heat during fermentation.

The size of the fermenting pile, the time between removal of beans and putting into the heap for fermentation, and the type of container all may have an effect on the course of the fermentation and, to some extent, on the quality of the final product *(Rohan, 1958a; Howell, 1948).

The sources of microflora are varied. The surface of pods, hands, and matchets contain species in at least seventeen genera of bacteria as well as unidentified yeasts (Ostovar and Keeney, 1973). At a start of fermentation, 65% of microorganisms are from soil or air. However, some selection of microbes may be in effect before the start of fermentation because many microbes active in fermentation are on the walls of boxes, baskets and sacks that are used frequently to transport beans to central facilities for fermentation or are left in the sweat boxes from previous fermentation *(Ostovar and Keeney, 1973). *Drosophila* species also may introduce microorganisms. The microorganisms found in fermentation and spoilage of cocoa are fermentative, and probably oxidative: yeasts, bacteria that produce acetic and lactic acids, *Bacillus* species, and moulds. Many of the yeasts found in cocoa fermentation are also found in the soil and fruits. In fermenting fruits, they are often associated with lactic acid – and acetic acid – producing bacteria *(Last and Price, 1969).

The most frequently isolated and most numerous yeasts during fermentation are *Saccharomyces cerevisiae, Saccharomyces* spp.' *Candida krusei, Pichia fermentans, Hansenula anomola,* and *Schizosaccharomyces pombe.* These species have been critically reviewed *(Rombouts, 1953). The fermentative yeasts rapidly dominate and many persist through drying and storage *(Hansen and Welty, 1970).

Bacillus species are present in low numbers at the beginning of the fermentation. At the low pH of the fresh pulp (about 3.7), Bacillus species are unable to grow. However, as the fermentation progresses and the pH and temperature increase, they become the predominating organisms, numbering about 10^6 cfu/g *(Ostovar and Keeney, 1973), or 10^7-10^8 per bean *(Rombouts, 1952)

Unlike the yeasts and acid-producing bacteria, most moulds are potentially harmful during fermentation, drying, and storage. They vary with the crop, moisture content, and temperature. During storage, moulds may or may not grow depending on the moisture content. Moulds isolated from normal dried beans are *Aspergillus glaucus, Aspergillus*

flavus, Aspergillus niger, Aspergillus tamarii, Mucor pusillus, Penicillium spp., Mucor spp.' and Geotrichium candidum.

Obviously, mouldy beans contain large numbers of *Penicillium* spp., *Aspergillus* spp. (*A. flavus, A. tamarii*), and *Paecilomyces* spp (Hansen and Welty, 1970).

CHAPTER TWO

2.0 LITERATURE REVIEW

Prior to roasting, cocoa beans contain a wide variety of microorganisms. The bacterial population varies from 5x 10^5 to 5 x 10^7 *(as reported by Meursing and Slot, 1968; Hansen and Welty, 1970' Barrile et al. 1971). Over 90% of the bacteria are aerobic spore bearers – *Bacillus* species (Barrile et all., 1971). Non-spore-forming bacteria belonging to the genera *Enterobacter*, *Escherichia*, *Flavobacterium*, *Microbacterium*, *Micrococcus*, and *Streptococcus* are usually a small percentage of the total population.

Mould and yeast populations in normal beans may vary widely depending on the sources, for example, moulds from ,<10^3 /g in beans from some countries *(Barrile et al., 1971) to 10^5 /g in a high percentage from other countries (Hansen and Welty, 1970). The yeasts are the commonest on dried beans and are dominant during fermentation *(Knapp, 1937).

Roasting is the main step in the process for destroying microorganism. If time and temperature of roasting are sufficient, all microorganisms except some bacterial spores are destroyed. Roasting at higher temperatures destroys more spores. After roasting for 40 minutes at 150°C, the Bacillus spp., are in order of frequency, Bacillus coagulans, *Bacillus stearothermophilus*, *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus subtilis* (Ostovar and Keeney, 1973). However, several additional species frequently are found in cocoa powder, indicating their survival during roasting.

The nibs are ground and milled to produce chocolate liquor with over 50% fat. Heat generated by milling increases the temperature to 60^0-80^0C or higher, but at the low water activity (Aw) of chocolate liquor (usually less than 2% water), mircroorganisms are not readily destroyed by heat. Chocolate liquor may be pressed to produce cocoa press cake and cocoa butter, mixed with fully or partially dehydrated milk and sucrose to produce chocolate crumb, or stored at temperatures of 60^0-80^0C, or occasionally higher, until used for chocolate, Bournvita, Drinking Chocolate, Richoco, etc. During storage, moisture is

reduced to about 1-2% and a slight reduction of asporogenous bacteria may occur. All the above products may be used in manufacturing chocolate confectionery.

Cocoa powder contain 9-36% fat and less than 8% moisture; its pH is 5.5 – 6.2 (natural cocoa) or 7.0 – 8.0 (alkalised cocoa). As the relative humidity (R.H.) increases, the moisture content also increases: at 60, 70 and 80% R.H., the moisture content of cocoa is approximately 8, 11 and 13%, respectively. Storage at 15^0 – 18^0C at less than 70% R.H., and with minimum fluctuation of temperature, is recommended (Meursing, 1976).
Adequately roasted beans that have been hygienically handled should yield cocoa with if any yeasts, moulds or asporogenous bacteria. Finding large numbers of organisms other than spore-forming bacteria is a signal that the beans or nibs were not roasted adequately or that the product experienced post-processing contamination and possibly growth during storage because of high water activity (Aw).

The mircroflora of cocoa consists mainly of *Bacillus* spp.' with variable numbers of yeasts and moulds. A small percentage of samples may contain *Enterobacteriaceae* (Meursing and Slot, 1968) and other non-spore forming bacteria (Gabis et al., 1970). *Salmonella* spp. have been found (Depew, 1968; Collins-Thompson et al, 1978). The examination for Salmonella spp. requires special mention. Cocoa contains some antibacterial compounds (Busta and Speck, 1968) that express anti *Salmonella* activity when cocoa is pre-enriched in the usual lactose or nutrient both at a 1:10 ratio. The use of skin milk as an enrichment medium (Park et al, 1976, 1979) or the addition of casein to lactose or nutrient broth (Zapatka et al., 1977) negates the anti *Salmonella* activity. The antibacterial effect is unlikely to compromise determinations for other bacteria (Park et al., 1979) because of lower ratios of cocoa to bacteriological medium.

The predominating bacteria are *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, and *Bacillus alvei*. These two thermophiles, *Bacillus coagulans* and *Bacillus stearothermophilus*, may be a potential cause of spoilage in some cocoa - containing products with high water activity (Aw) (Meursing and Slot, 1968; Gabis et al., 1970; Mossel et al., 1974).

The only pathogen of importance in chocolate is *Salmonella*. It has been found in cocoa and chocolate on several occasions (Depew, 1968; Goepfert and Foster, 1968; D'Aoust, 1977; Anonymous, 1978) and may survive in chocolate for years (Rieschel and Schenkel, 1971; Dockstader and Groomes, 1971; Tamminga et al., 1976). *Salmonella* in cocoa (WHO, 1973) and milk chocolate (D'aoust et al., 1975; P.C. Craven et al., 1975) have caused illness in a large number of consumers.

2.1 Sampling Sites

Three samples each of Bournvita (200g per pack) and Richoco (200g per pack) – cocoa products – manufactured by Cadbury Ghana Limited, Accra, Ghana, were purchased from 'Mobil Mart' Supermarket situated at Adenta, a suburb of Accra,

Another three samples each of the above products were also purchased from Makola market, an open market in the centre commercial area in Accra.

2.2 Sample Preparation

The three packs of Bournvita purchased from 'Mobil Mart' supermarket were aseptically opened in the inoculation room and pooled together as one sample. Then, a representative sample was taken from the bulk and sealed in a sterile stomacher bag.

A similar treatment was applied to the three packs of Richoco from'Mobil Mart' supermarket. Similar procedures were employed also for the same cocoa products purchased from the open market, Makola market.

2.3 Physical appearance of samples

The physical appearance of the samples purchased from both sampling sites was as follows:

a) External conditions of the packages – all seams were intact, there were no punches, leakages nor breakages. The expiry dates were December, 2001.

b) Internal conditions

The contents were well dehydrated, smell was typical of the products and the appearance was normal.

PLATE 1

COCOA PRODUCTS

(Left to right – Richoco and Bournvita)

CHAPTER THREE

3.0 MATERIALS

3.1 Aparatus and glassware

a) Stomacher 400 Lab. Blender (Seward, UK), for homogenisation of samples for microbiological examination

b) Stomacher bags (Seward, UK), for holding samples and diluents for homogenisation.

c) Autoclave (Astell, UK), for sterilization of culture media and decontamination of cultures

d) Hot-air Oven (Helios, UK), for sterilization of glassware, scalpels, forceps and spatulas.

e) Incubator for maintaining the incubated culture plates and broth at temperatures of $30 + 1^0C$ and $37 + 0.5^0C$

f) Water bath (Lauda, Germany), for heating and cooling solutions and culture media to the appropriate temperature

g) Siirrer (Janke & Kunkel Co., Germany), for mixing diluents containing culture

h) Colony counter (American Optical Corp., USA), for counting colonies on culture plates

i) Microscope (Olympus Optical Co., Japan), for microscopic identification of microorgnisms

j) Balance (Janke & Kunkel Germany) for weighing samples, dehydrated culture media and stains

k) Culture tubes and flasks (Scientific & chemical supplies Ltd, UK) for sterilization and storage of culture and dilution fluids

l) Micro pipettes with tips (Scientific & Chemical Supplies Ltd, UK), for transferring fluids and dilutions. (Range 100-1000)

m) Glass Petri dishes (Scientific & Chemical Supplies Ltd, UK), for culturing and media preparation

n) Microscope glass slides and cover slips, for microscopic examination

o) Inoculating loops, for inoculation

p) Lab. PH meter (Radiometer Analystical A/S, Denmark), for measuring hydrogen-ion concentration (pH) of culture media, and fluids.

3.2. Preparation of diluents and media

Culture media

Many factors are involved in the cultivation of microorganisms under laboratory conditions. It is therefore necessary to have a wide range of culture media, which must contain the necessary chemical substances at the correct hydrogen-ion concentration (pH), which an organism requires for growth and reproduction. The materials used in the preparation of culture media are generally those, which are readily assimilated by microorganisms.

3.2.1 Saline Peptone Water (Oxoid, UK)

Intended Use: for idole production and serial dilution

Formula:	gm/litre
Components	
Sodium Chloride	8.5
Peptone	1.0
Distilled Water	1.0 litre

PH 7.2 $\pm$ 0.1 at 25^0 C

Preparation of Medium

1. Suspend 1 gram of the peptone in 1 litre of distilled water
2. Add 8.5. grams of sodium chloride to the solution
3. Boil to dissolve completely
4. Dispense into appropriate containers –test-tubes
5. Sterilize by autoclaving at 121^0C for 15 minutes
6. Cool to room temperature

3.2.2 Standard Plate Count Agar (Difco, USA)

Intended Use: For enumerating bacteria in water, waste water, food and dairy products

Formula:	gm/litre
Components	
Bacto tryptone	5.0
Bacto yeast Extract	2.5
Bacto Dextrose (Glucose)	1.0
Bacto Agar	15.0
Distilled water	1.0 litre

PH 7.0 + 0.2 at 25^0C

Preparation of medium

1. Suspend 23.5g of the medium in 1000 ml of distilled water
2. Boil to dissolve
3. Sterilize by autoclaving at 121^0C for 15 minutes
4. Cool to 50^0C
5. Dispense into sterile petri dishes and screw-capped bottles
6. Allow to set

3.2.3 *Aspergillus flavus/parasiticus* Agar Base (Oxoid, UK)

Intended Use: A. selective identification medium for the detection of *Aspergillus flavus* and *Aspergillus parasiticus*

Formula	gm/litre
Components	
Peptone	10.0
Yeast extract	20.0
Ferric ammonium citrate	0.5
Dichloran	0.002
Agar	15.0
Distilled water	1.0 litre

PH 6.3 + 0.2 at 25^{0}C

Preparation of medium

1. Suspend 22.75g of the medium in 1000 of distilled water
2. Boil to dissolve
3. Dehydrate two vials of chloramphenicol supplement SR 78 as directed
4. Add to the AFPA Base
5. Sterilize by autoclaving at 121^{0}C for 15 minutes
6. Dispense into sterile petri dishes
7. Allow to set

3.2.4 Malt Extract Agar (Oxoid, UK)

Intended Use: A medium for the detection, isolation and enumeration of moulds and yeasts

Formula:	gm/litre
Components	
Malt extract	30.0
Mycological Peptone	5.0
Agar	15.0
Distilled Water	1.0 litre

PH 5.4 + 0.2 at 25^{0}C

Preparation of medium:

1. Suspend 50g of the medium in 1 litre of distilled water
2. Boil to dissolve
3. Dispense into screw-capped bottles
4. Sterilize by autoclaving at 115^0C for 10 minutes
5. Cool to 50^0C before use

3.2.5 Nutrient Agar (Difco, U.S.A.)

Intended Use: For cultivating a wide variety of micro organisms

Formula:	gm/litre
Components	
Bacto Beef Extract	3.0
Bacto Peptone	5.0
Bacto Agar	15.0
Distilled Water	1.0 litre

PH 6.8 + 0.2 @ 25^0C

Preparation of medium

1. Suspend 23g of the medium in 1 litre of distilled water
2. Boil to dissolve completely
3. Dispense into bottles
4. Sterilize by autoclaving at 121 – 124^0C for 15 minutes
5. Cool to 50^0C before use

3.2.6 Nutrient Broth (Difco, U.S.A.)

Intended Use: For cultivating non-fastidious microorganisms

Formula:	gm/litre
Components	
Bacto Beef Extract	3.0
Bacto Peptone	5.0
Distilled Water	1.0 litre

PH 6.8 + 0.2 @ 25^0C

Preparation of medium

1. Suspend 8g of the medium in 1 litre of distilled water
2. Boil to dissolve
3. Dispense into test tubes
4. Sterilize by autoclaving at 121-124^{0}C for 15 minutes
5. Cool to room temperature before use.

3.2.7 MacConkey Agar No.3 (Oxoid, U.K.)

Intended Use: For differentiating lactose – and non – lactose fermenting microorganisms

Formula:	gm/litre
Components	
Peptone	20.0
Lactose	10.0
Bile Salts No.3	1.5
Sodium Chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	15.0
Distilled water	1.0 litre

PH 7.1 + 0.2 @ 25^{0}C

Preparation of the medium:

1. Suspend 51.5g of the medium in 1 litre of distilled water
2. Boil to dissolve completely
3. Dispense into bottles
4. Sterilize by autoclaving at 121^{0}C for 15 minutes
5. Cool to 50^{0}C before use

3.2.8 MacConkey Broth (Oxoid, U.K.)

Intended Use: For detection of lactose-fermenting *Bacilli* in milk, water and other materials of sanitary importance

Formula: gm/litre

Components

Peptone	20.0
Lactose	10.0
Bile Salts	5.0
Sodium Chloride	5.0
Bromocresol purple	0.01
Distilled Water	1.0 litre

PH 7.4 $\pm$ 0.2 @ 25^{0}C

Preparation of the medium

1. Suspend 40g of the medium in 1000 ml of distilled water
2. Heat to dissolve
3. Dispense into test tubes containing Durham tubes
4. Sterilize by autoclaving at 121^{0}C for 15 minutes
5. Allow to cool before inoculating

For double strength broth suspend 80g.

3.2.9 Peptone Water (Oxoid, U.K.)

Intended Use: for indole test

Formula	gm/litre

Components

Trytone	10.0
Sodium chloride	5.0
Distilled Water	1.0 litre

PH 7.2 $\pm$ 0.2 @ 25^{0}C

Preparation of medium

1. Suspend 15g of the medium in 1000 ml of distilled water
2. Heat to dissolve
3. Dispense in 5 ml amounts into test tubes
4. Sterilize by autoclaving at 121^{0}C for 15 minutes
5. Allow to cool before inoculating.

3.3.1 Skim Milk Broth (Difco, U.S.A)

Intended Use: For pre-enrichment for *Salmonella* and other *Enterobacteriaceae*.

Formula:	gm/litre
Components	
Skin milk powder	100.0
Brilliant green solution	4.0
Distilled Water	1.0 litre

PH 6.3 $\pm$ 0.2 @ 25^0C

Preparation of the medium

1. Suspend 100g of the medium in 1000 ml of distilled water
2. Head to dissolve
3. Add brilliant green solution and mix
4. Dispense into flasks in 225 ml amounts
5. Sterilize by autoclaving at 121^0C for 15 minutes
6. Cool to room temperature.

3.3.2 Brilliant Green Bile Broth (Difco, U.S.A.)

Intended Use: For confirmation test for coliform bacteria

Formula:	gm/litre
Components	
Bacto Peptone	10.0
Bacto Oxgall	20.0
Bacto Lactose	10.0
Brilliant green	0.0133
Distilled Water	1.0 litre

PH 7.2 $\pm$ 0.2 @ 25^0C

Preparation of the medium:

1. Suspend 40g of the medium in 1 litre of distilled water

2. Heat to dissolve
3. Dispense into test tubes containing Durham tubes
4. Sterilize by autoclaving at 121^0C for 15 minutes
5. Cool to room temperature

3.3.3 Rappaport-Vassiliadis Enrichment Broth (Oxoid, U.K.)

Intended Use: A selective enrichment broth for the isolation of *Salmonella*

Formula:	gm/lire
Components	
Soya Peptone	5.0
Sodium Chloride	8.0
Potassium dihydrogen phosphate	1.6
Magnesium chloride $6H_2$ 0	40.0
Malachite green	0.04
Distilled Water	1.0 litre

PH 5.2 $\pm$ 0.2 @ 25^0C

Preparation of the medium

1. Suspend 30g of the medium in 1000 ml of distilled water.
2. Heat gently to dissolve completely
3. Dispense in 10 ml quantities into test tubes
4. Sterilize by autoclaving at 115^0C for 15 minutes
5. Cool to room temperature

3.3.4 Bismuth Sulphite Agar (Oxoid, U.K.)

Intended Use: A selective medium for the isolation of *Salmonella*

Formula	gm/litre

Components

Peptone	5.0
'La-Lemco' powder	5.0
Glucose	5.0
Di-sodium phosphate	4.0
Ferrous sulphate	0.3
Bismuth sulphite indicator	8.0
Brilliant green	0.016
Agar	12.7
Distilled Water	1.0 litre

PH 7.6 $\pm$ 0.2 @ 25^0C

Preparation of the medium

1. Suspend 20g of the medium in 1000 ml of distilled water
2. Heat gently with frequent agitation to dissolve completely
3. Allow to simmer for 10 minutes
4. Cool to 50^0C and mix well to dispense suspension
5. Dispense 25 ml medium into Petri dishes and allow to set

3.3.5 Brilliant Green Agar (Oxoid, U.K.)

Intended Use: A selective and diagnostic medium for the isolation of *Salmonella* from food and fees

Formula	gm/litre
Components	
'Lab-Lemco' Powder	5.0
Peptone	10.0
Yeast Extract	3.0
Disodium hydrogen phosphate	1.0
Sodium dihydrogen phosphate	0.6
Lactose	10.0
Sucrose	10.0
Phenol red	0.09

Brilliant Green	0.7
Agar	12.0
Distilled Water	1.0 litre

PH 6.9 $\pm$ 0.2 @ 25^0C.

Preparation of the medium

1. Suspend 52g of the medium in 1000 ml of distilled water
2. Heat gently with occasional agitation to dissolve completely
3. Cool to 50^0C and mix well
4. Pour into sterile Petri dishes and allow to set.

3.3.6 Xylose Lysine Deoxycholate Agar (Oxoid, U.K)

Intended Use: A selective medium for the isolation of *Salmonella* and *Shigella* from clinical specimens and foods

Formula:	gm/litre
Components	
Yeast extract powder	3.0
L-Lysine HCl	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium deoxycholate	1.0
Sodium chloride	5.0
Sodium Thio sulphate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Distilled Water	1.0 litre

PH 7.4 $\pm$ 0.2 at 25^0C

Preparation of the medium:

1. Suspend 53g of the medium in 1000 ml of distilled water
2. Heat gently with frequent agitation to dissolve completely
3. Cool to 50^0C and mix
4. Dispense asetically into sterile Petri dishes
5. Allow to set.

3.3.7 Buffered Peptone Water (Oxoid, U.K)

Intended Use: a pre-enrichment medium for *Salmonella* species contained in water supplies, food and dairy products

Formula:	gm/litre
Components	
Peptone	10.0
Sodium Chloride	5.0
Dosodium phosphate	3.0
Potassium dihydrogen phosphate	1.5
Distilled Water	1.0 litre

PH 7.2 $\pm$ 0.2 at 25^0C

Preparation of the medium:

1. Suspend 20g of the medium in 1000 ml of distilled water
2. Heat to dissolve
3. Dispense into flasks in 225 ml amounts
4. Sterilize by autoclaving at 121^0C for 15 minutes
5. Cool to room temperature before use

3.3.8 Violet Red Bile Agar (Oxoid, U.K.)

Intended Use: A lactose containing selective medium for the detection and enumeration of *coli-aerogenes* in water, food and dairy products

Formula:	gm/lire
Components	
Yeast extract	3.0
Peptone	7.0
Sodium chloride	5.0
Bile Satts No.3	1.5
Lactose	10.0
Crystal violet	0.002
Neutral red	0.03
Agar	12.0
Distilled Water	1.0 litre

PH 7.4 $\pm$ 0.2 at 25^0C

Preparation of the medium

1. Suspend 35.5g of the medium in 1000 ml of distilled water
2. Heat to dissolve completely
3. Cool to 50^0C before use
4. Use within 3 hours

3.3.4.0 STAINS

3..3 4.1 Loeffler's Methylene blue

Intended Use: A simple stain for bacteria

Formula: per 100 ml

Components

Methylene Blue (B.D.H., U.K.	0.5g
Ethanol absolute (B.D.H., U.K.)	30.0 ml
Potassium hydroxide, (BDH)	1.0 ml
(200g/l – 20% w/v)	
Distilled Water	100.0 ml

3.3.4.2 Gram stain

Intended Use: compound stain for differentiating between Gram-positive and Gram-negative microorganisms

(a) Crystal violet

Formula: To make 250 ml

Components

Crystal violet (BDH, UK	5.0g
Ammonium oxalate (BDH, UK)	2.25g
Ethanol, absolute (BDH, UK)	23.7 ml
Distilled Water	250.0 ml

(b) Lugol's iodine solution

Formula: to make 250 ml

Components

Potassium iodide (BDH, UK)	5.0g
Iodine (BDH, UK)	2.5g
Distilled Water	250.0 ml

© Acetone-alcohol decolorizer

Formula: To make 250 ml

Components

Acetone (BDH, UK)	125.0 ml
E thanol, absolute (BDH, UK)	118.75 ml
Distilled Water	6.25 ml

(d) Carbol fuchsin stain

Formula: To make 250 ml

Components

Basic fuchsin (BDH, U.K.)	2.5g
E thannol, absolute (BDH, UK)	25.0 ml
Phenol (BDH, UK)	12.5 g
Distilled Water	250.0 ml

3.3.4.3 Lactophenol cotton blue

Intended Use: for staining moulds

Formula: to make about 45 ml

Components:

Phenol	10.0 g
Cotton blue (aniline blue) water soluble	0.04 g
Lactic acid	10.0 ml
Glycerol	20.0 ml
Distilled water	10.0 ml

3.3.4.4 Reagents

Intended Use: for Biochemical test of microorganisms

a) Kovac's indole reagent

Intended Use: For testing indole

Formula: to make about 40 ml

Components

4-Dime thylaminobenzaldehyde (BDH, U.K.)	2.0 g
(Paradime thylaminobenzaldehyde)	
Iso-amyl alcohol	30.0 ml
Hydrochloric acid, concentrated	10.0 ml

b) Sodium Chloride (0.85% w/v)

(Physiological saline)

Intended Use: For emulsifying colonies for smears

Formula: to make 100 ml

Components

Sodium Chloride (BDH, U.K.)	0.85 g
Distilled Water	100.0 ml

Preparation of the saline:

1. Dissolve the sodium chloride in 100 ml of distilled water
2. Heat to dissolve completely
3. Dispense in 5 ml amounts into MacCarthney tubes
4. Sterilize by autoclaving at 121^0C for 15 minutes
5. Cool to room temperature before use

c) Hydrogen perodixe, for catalase test

d) Rabbit plasma, for coagulase test

(e) Oxidase test strips, (Merck, Germany), for testing of cytochromoxidase

CHAPTER FOUR

4.0 METHODS

4.1 Preparation of serial dilutions

10g of the prepared sample were weighed out aseptically into a sterile stomacher bag. Then 90 ml of the diluent sterile Saline peptone water (3.2.1) were added and placed in the stomacher 400 Lab-Blender (3.1.a). Then, the mixture was homogenized at normal speed for 30 seconds. This represented 1:10 dilution, that is, 1 ml contains 0.1g.

With a micro-pipette (3.1 l), 1 ml was pipetted out from the 1:10 dilution and delivered into the first test-tube containing 9 ml amount of sterile diluent. The tip was ejected and discarded. The test-tube was vortex mixed. This represented 1:100 dilution.

With a fresh sterile micropipette tip fixed, 1 ml was transferred from the first tube (1:100) into the second test-tube containing 9 ml sterile diluent. This represented 1:1000 dilution. The pipette tip was discarded.

With a fresh sterile pipette tip, 1 ml from the second test-tube (1:1000) was transferred into the third test-tube containing 9 ml sterile diluent. This represented 1:10000 dilution. The pipette tip was ejected and discarded. This process was continued for the required number of dilutions.

The dilutions were

Tube No	1	2	3	4
Dilution	1:100	1:1000	1:10000	1:100000

Weight of Original Sample	0.01 g (10^{-2})	0.001g (10^{-3})	0.0001g (10^{-4})	0.00001g (10^{-5})

4.2 Total Viable Count (Pour Plate Technique)

Sterile Petri dishes were labelled with the dilution numbers above (4.1) and set out in duplicate per dilution to be tested. 1 ml of each of the above serial dilutions was transferred aseptically into the centre of the appropriate dishes, using a fresh sterile pipette tip for each dilution.

Quickly and aseptically, 10-15 ml of the molten Standard Plate Count Agar (3.2.2) that has been previously melted and cooled to 45^0C were poured into each dish in turn and mixed by moving the dish gently six times in a clockwise direction, six times anticlockwise direction, back-and-forth six times and side-and-side six times to spread the inoculum so as to grow discrete colonies. The medium was allowed to set at room temperature. The Petri dishes were inverted and incubated at 30^0C for 48-72 hours.

Enumeration

Plates with between 30 and 300 colonies (1:10 dilution) were selected. The open dish was placed, glass side up, over the illuminated screen of the colony counter (3.1,h). The colonies were counted using a 75 mm magnifier and a hand-held counter. The glass was marked above each colony with a felt-tip pen. The mean of the two countable plates was recorded. The result was express in colony forming units per gramme (cfu/g).(As referred in Table 1)

4.3 Mould and Yeast Count (Using Malt Extract Agar)Sterile, Petri dishes were labelled with the dilution numbers (4.1) and set out in duplicate per dilution to be tested. 1 ml of each of the above serial dilutions was transferred aseptically into the centre of the appropriate dishes, using a fresh sterile pipette tip for each dilution.

Quickly and aseptically, 15-20 ml of the molten Malt Extract Agar (3.2.4) that has been previously melted and cooled to 45^0C were poured into each dish in turn and mixed by moving the dish gently six times in a clockwise direction six times in an anticlockwise direction, back-and-forth six times and side-and-side six times to spread the inoculum so

as to grow separate colonies. The medium was allowed to set at room temperature. The Petri dishes were incubated univerted at 30^0C for 5 days.

Enumeration

Plates with countable colonies (1:100 dilution) were selected and counted, using the colony counter (3.1.h), a 75 mm magnifier and a hand-held counter. The mean of the two countable plates (1:100 dilution) was recorded and expressed in colony forming units per gramme (cfu/g). (As referred in Table 2)

4.4 Coliform Count, (Using Violet Red Bile Agar)

Sterile Petri dishes were labelled with the dilution numbers (4.1) and set out in duplicate per dilution to be tested. 1 ml of each of the above serial dilutions were transferred aseptically into the centre of the appropriate dishes, using a fresh sterile pipette tip for each dilution.

Quickly and aseptically, 10-15 ml of the molten Violet Red Bile Agar (3.3.9) that had been previously melted and cooled to 45^0C were poured into each dish in turn and mixed by moving the dish gently six times in a clockwise direction, six times in an anticlockwise direction, back-and-front six times and side-and-side six times to spread the inoculum so as to grow discrete colonies. The medium was allowed to set at room temperature. After solidification of the medium, 5-10 ml of the same medium were poured into each Petri dish as an overlay, and allowed to set. The plates were inverted and incubated at 37^0C for 24-48 hours.

Enumeration

There was no bacterial growth on the Violet Red Bile Agar, that is the reaction was negative (As referred in Table 3).

4.5 Coliform Count – Most Probable Number (MPN) Method – The Dilution Tube Technique.

One tube of 50 ml of double-strength Mac-Conkey broth (3.2.8) with inverted Durham tube was filled with equal volume of 50 ml 1:10 dilution of the test sample. 5 (five) tubes of 10 ml each of double-strength MacConkey broth (3.2.8) with inverted Durham tube each were filled with equal volume of 10 ml 1:10 dilution each of the test sample.

5 (five) tubes of 1 ml each of single-strength MacConkey broth (3.2.8) with inverted Durham tube each were filled with 1 ml 1:10 dilution each of the test sample.

Enumeration

There was no bacterial growth in any of the broth in the tubes that is the reaction was negative. (As referred in Table 4)

4.6 *Pathogenic microorganism*

4.6.1 Detection of *Salmonella*

25g sample each of the cocoa products – Bournvita and Richoco – were weighed aseptically into separate sterile stomacher bags. 225 ml of sterile Skin milk broth (3.3.1), a pre-enrichment medium, were added to each sample and allowed to dissolve completely. The broth cultures were incubated at 37^0C for 16-20 hours.

Then 0.1 ml each of the broth cultures was aseptically transferred into tubes containing 10 ml of Rappaport-Vassiliadis (RV) broth (3.3.3), a selective enrichment broth, and incubated at 42^0C for 18-24 hours.

Using a sterile loop, a loopful of the RV broth culture was streaked onto the surface of previously poured and dried plates of each of the selective media-Bismuth Sulphite Agar – BSA (3.3.4), Brilliant Green Agar – BGA (3.365) and Xylose Lysine Deoxycholate Agar – XLD (3.3.7) and incubated at 37°C for 24 hours, after which the plates were examined for growth.

4.6.2 IDENTIFICATION OF BACTERIA

Bacteria isolates were identified according to Cowan and Steel (1974) in a scheme as referred in Appendices 1, 3, 5 and 7. The solates were subjected to morphological and biochemical tests.

a) Gram Staining (Compound-staining method)

Procedure

1. A thin smear was prepared from an 18-24 hours culture and heat-fixed.
2. Stain with cystal violet solution (3.3.4.2,a) for 1-2 minutes
3. Rinse off with Lugol's iodine solution (3.3.4.2.b) and allow the iodine to act for 1 minute
4. Pour off the iodine, blot dry and wash the slide with Acetone-alcohol decolorazer (3.3.4.2.c) until no more violet stain runs from the slide.
5. Rinse under the tap and stain with dilute carbol fuchsin solution (3.3.4.2.d) for 10 secopnds.
6. Wash the slide well and blot dry.

b) Oxidase Test (Cytochrome Oxidase)

A loopful of the suspected colony was smeared onto the reagent zone of the oxidase test strip (3.3.4.4.e).

Bacterial colonies having cytochrome oxidase, activity develop a deep blue colour at the inoculate site within 10 seconds.

Control – Postive control – *Pseudodomonas aerogenosa*

Negative control – *Escherichia coli*

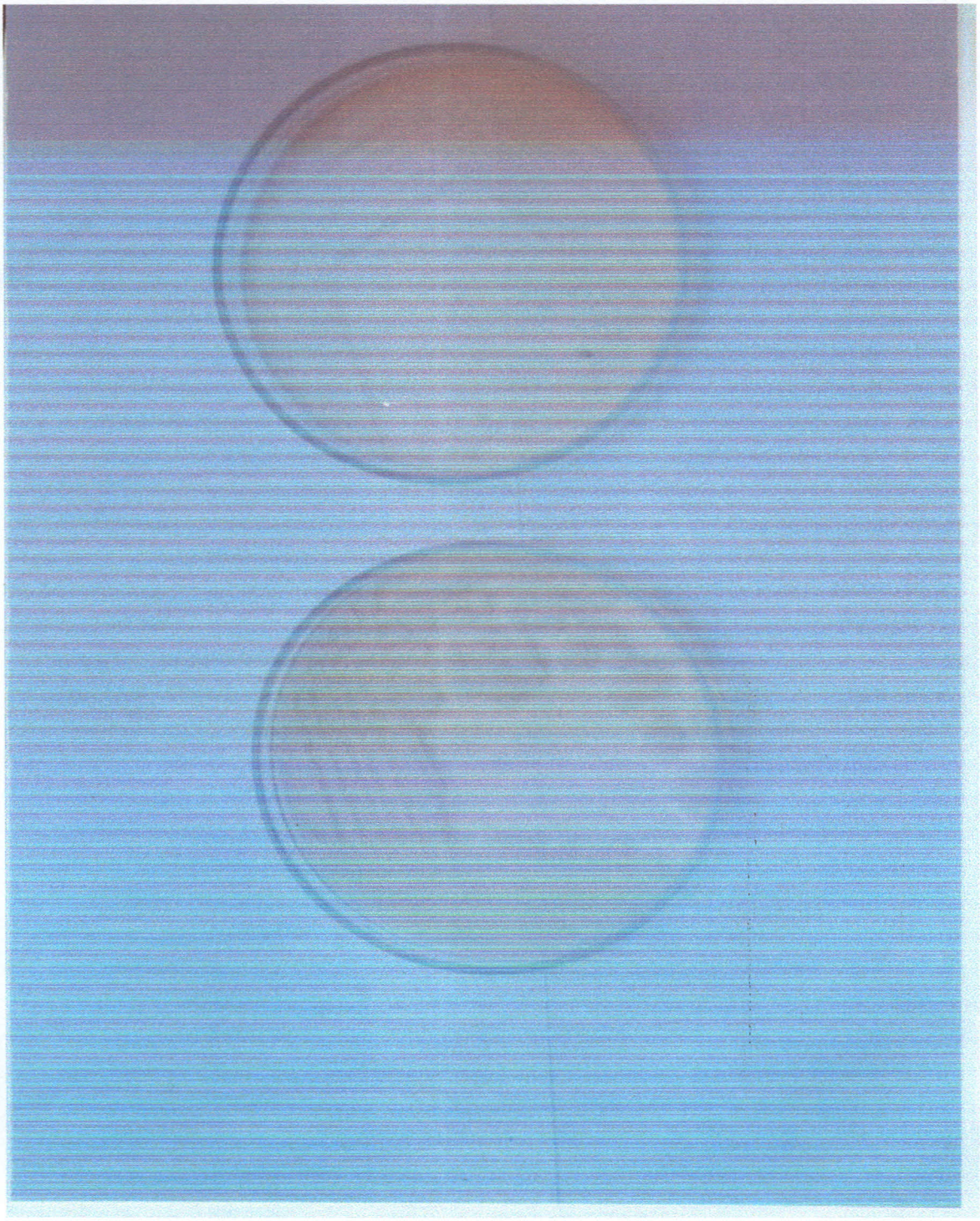
c) Catalase Test – (Tube method)

The surface of the colony of the test organism was touched with a microhaematocrit tube loaded with hydrogen peroxide (3.3.4.4.c). Rapid effervescence was of gas bubbles were seen.

Control – Positive control – *Staphylococcus aureus*

Negative control – *Steptococcus* spp.

PLATE 2



Bacterial isolates on NA plates

(Top) *Cocci* Species. (Bottom) *Bacillus* Species

d) Coagulase Test

A drop each of sterile distilled water and physiological saline (3.3.4.4 b) was placed on a glass slide. A suspension of the test organism was gently emulsified in the drop of physiological saline using an inoculating loop (3.1.0). A drop of reconstituted coagulase plasma was placed immediately adjacent to the drop of the bacterial suspension. The two suspensions were mixed together by tilting the slide back-and-front to observe an immediate formation of a granular precipitate of white clumps.

A positive reaction is usually detected within 5 to 20 seconds by the appearance of a ganular precipitate of white clumps.

Control – Positive control – *Staphylococcus aureus*

Negative control – *Staphylococcus epidermidis*.

4.6.3 Identification of Moulds

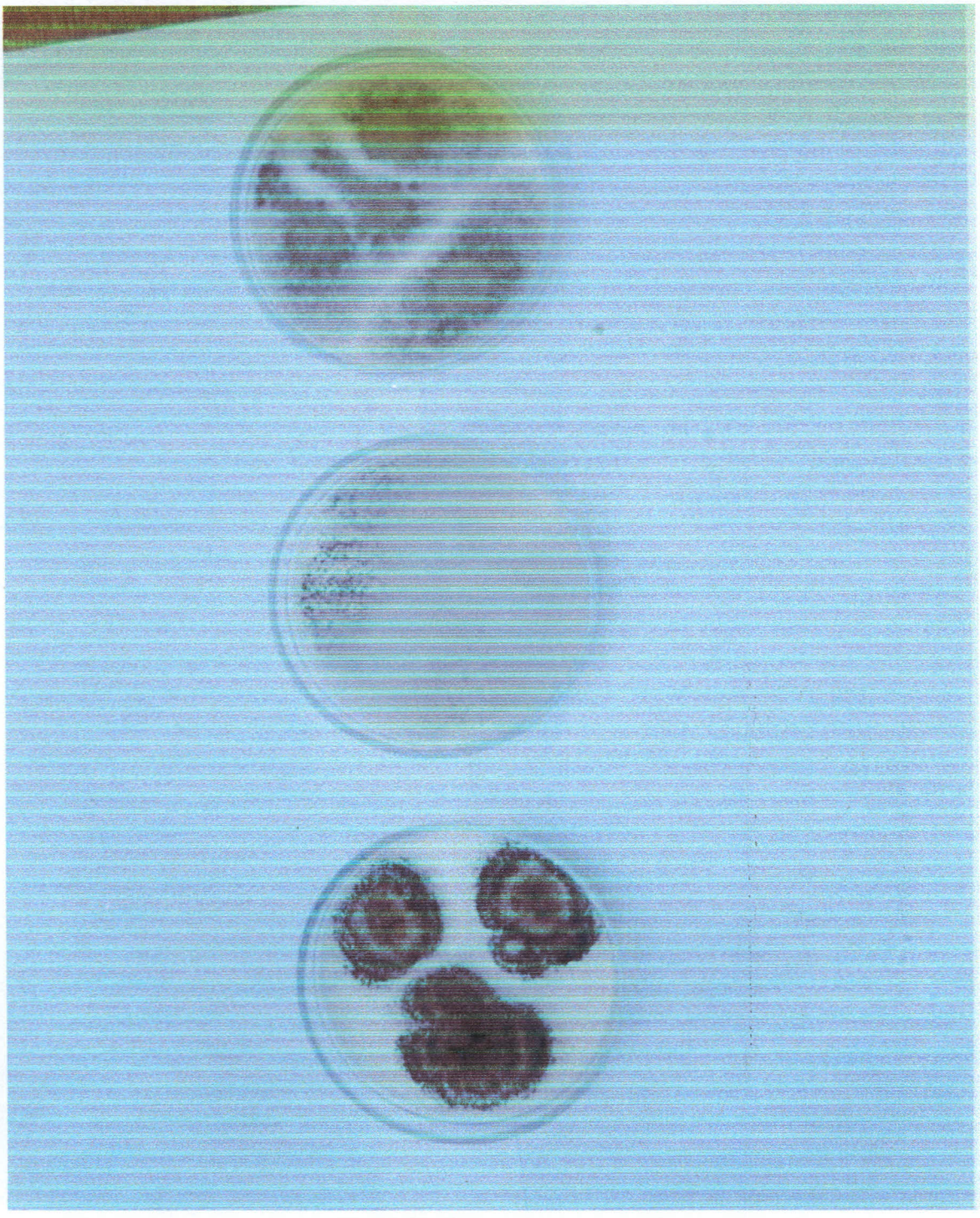
(a) Mould isolates were examined under the low-power objective of the microscope for the colonial morphology.

(b) Slides of the mould isolates were prepared for microscopic examination in the following way.

A portion of the growth was picked off with a sterile needle and teased out in a drop of lactophenol-cotton blue solution (3.3.4.3) placed on a microscope slide and covered with a clean cover slip, taking care to exclude air bubbles.

The prepared slides were examined under the miscroscope, first using the low-power objective and then using high-power dry objective for a closer examination`

PLATE 3



Mould isolates on MEA plates

(Top) *Aspergillus fumigatus*, (centre) *Mucor* Species and (bottom) *Aspergillus niger*

CHAPTER FIVE

5.0 RESULTS AND DISCUSSION

5.1.Microbiological quality of Bournvita and Richoco from 'Mobil Mart Supermarket" and open market.

Tables 1,2,3,4, 5 and 6 show the results of the microbial loads of cocoa products sampled from the above sampling sites.

In Table 1, the total bacterial count ranged between 1.4×10^4 and 1.7×10^4 cfulg in Bournvita from the Supermarket and Open market respectively. The total bacterial count in Richoco ranged between 6.2×10^3 and 6.6×10^3 cfu/g from the supermarket and open market respectively.

In Table 2, the mould count ranged between 15×10^1 and 22×10^1 cfu/g in Bournvita from the supermarket and open market respectively.

The mould count in Richoco also ranged between <10 and 1 x 101 cfu/g from the supermarket and open market respectively.

In Tables 3, 4, 5 and 6, there were no bacterial growths, which indicated that there were no coliform groups, no gas-formers and no *Salmonella*.

However, the microbial loads indicated in Tables 1 and 2 were low and fell within the accepted microbial limits as recommended in Table 7.

5.1.0 Methods of Sterilization

a) Flaming (Red heat) – Inoculation needles and loops were sterilized by heating in the Bunsen flate until red-hot.
b) Dry heat – Petri dishes, bottles, other glassware, spoons, spalulae and other metal instruments were sterilized in a thermostatically controlled oven, Hot-Air Oven for 60 minutes at 160^0C

c) Mosist heat

Boiling – Media, like VRBA, XLD, BGA and BSA were sterilized by steaming for 20 minutes at 100^0C.

Autoclaving (steam under pressure) – All other culture media, except those which would be destroyed by great heat, and pipette tips, were sterilized by autoclaving at 103kPa (121^0C) for 15 minutes.

d) Fumigation – The inoculation room was sterilized by fumigating the room with a mixture of formalin and potassium permanganate crystals.

5.1.1 Safety

1. Clean laboratory coat was always worn.
2. Eating, drinking and smoking were not done in the laboratory
3. Inoculation needles loops were sterilized before and after use by heating in the Bunsen flame until red-hot.
4. Spattering of materials was avoided by introducing the instruments into the Bunsen flame.
5. Test-tubes containing cultures were always kept upright in test-tube racks. Test-tubes were never laid on the bench top
6. Hands were washed thoroughly before leaving the laboratory
7. Used pipette tips, glass slides and cover slips were discarded into a discard jar filled with disinfectant for overnight and autoclaved before washing
8. Micro Pipettes with tips were used to prevent accidental ingestion of microorganisms in liquid media
9. The mouths of media containers and culture tubes were flamed before and after pouring.
10. Bench tops and other surfaces were disinfected with 70% ethanol.
11. Contaminated samples, discarded culture plates and tubes were put in leak-proof containers, disinfected and autoclaved to render them harmless. The plates and tubes were washed for reuse.
12. Masks were worn during the identification of moulds in order to reduce inhalation of spores

13. Thermal protective gloves were worn to prevent burns when removing autoclaved items as well as those from the Hot-air Oven.

6.0 CONCLUSION AND RECOMMENDATIONS

This study has established that the cocoa products being sold in both Supermarkets and Open markets in Ghana are of good quality and they can stand the test of time since the expiry date on the packages exceeds one year.

Therefore, in conclusion the products have long shelf-life, the microbiological hygiene of the environment of the sampling sites was satisfactory and lastly, the quality of the products is the prime concern of the manufacturers, Cadbury Ghana Limited.

It is therefore recommended that the hygienic way of handling the cocoa products should continue in the same direction for a healthy nation.

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Table 1

Bacterial Count of Bournvita and Richoco from"Mobil Mart" Supermarket and Open market in Accra, Ghana. Using Standard Plate count Agar at 30^0C

Splitting Site	Supermarket Mean cfu perplate	Open Market Mean cuf per plate
Bournvita	1.4×10^4	1.7×10^4
Richoco	6.2×10^3	6.6×10^3

Key: Cfu – Colony – forming units

Table 2

Mould and yeast Count of Bournvita and Richoco from "Mobil Mart" Supermarket and Open market in Accra, Ghana. Using Malt Extract Agar at 30^0C

Splitting Site	Supermarket Mean cfu perplate	Open Market Mean cuf per plate
Bournvita	15×10^1	22×10^1
Richoco	<10	1×10^1

Key: Cfu – Colony – forming unit

Table 3

Coliform Count of Bournvita and Richoco from "Mobil Mart" Supermarket and Open market in Accra, Ghana. Using Violet Red Bile Agar at 37^0 C

Splitting Site	Supermarket Mean cfu perplate	Open Market Mean cuf per plate
Bournvita	0	0
Richoco	0	0

Key: Cfu – Colony – forming units
0 - No Bacterial growth

Table 4

Gas-Formers

Most Probable Numbers (MPN) per 100 ml. Using 1 (One) tube of 50 ml, 5 (five) tubes of 10 ml and 5 (five) tubes of 1 ml. Using MacConkey Broth at 37^0C
Mobil Mart" Supermarket

Product	Expiry Date	50 ml Tube	10 ml Tubes	1 ml tubes	MPN per 100ml
Bournvita	December 2001	0	0	0	0
Richoco	December 2001	0	0	0	0

Key: MPN – Most Probable Numbers
0 = No Bacterial growth

Table 5

Gas-Formers
Most Probable Numbers (MPN) per 100 ml. Using 1 (One) tube of 50 ml, 5 (five) tubes of 10 ml and 5 (five) tubes of 1 ml. Using MacConkey Broth at 37^0C.
Open Market

Product	Expiry Date	50 ml Tube	10 ml Tubes	1 ml tubes	MPN per 100ml
Bournvita	December 2001	0	0	0	0
Richoco	December 2001	0	0	0	0

Key: MPN – Most Probable Number
0 - No Bacterial growth

Table 6

Detection of *Salmonella* from bornvita and Richoco from "Mobil Mart" supermarket and Open market in Accra, Ghana.

Sampling Site	Supermarket					Open Market				
	Media					Media				
Product	SMB @ 37^{0}C	RV @ 42^{0}C	BSA @ 37^{0}C	BGA @ 37^{0}C	XLD @ 37^{0}C	SMB @ 370^C	RV @ 42^{0}C	BSA @ 37^{0}C	BGA @ 37^{0}C	XLD @ 37^{0}C
Bournvita	NBG	NBG	NBG	NBG	NBG	NBG	BG	NBG	NBG	NBG
Richoco	NBG	NBG	NBG	NBG	NBG	NBG	NBG	NBG	NBG	NBG

Key: SMB - Skim Milk Broth
RV - RapportVassiliadius Broth
BSA - Bismuth Sulphite Agar
BGA - Brilliant Green Agar
XLD - Xylose Lysine Deoxycholate Agar
NBG - No Bacterial Growth

Table 7

Recommended Microbial Limits Per Gram of Cocoa

Source	Aerobic	*Escherichia coli*	Coliform Group	Yeasts	Moulds	*Salmonella*
Minifie (1970)	20,000	<0.1	-	<50	<50	-
Meursing (1976)	5,000	0	-	<50	<50	<0.1
I.C.P. Cocoa (1977)	5,000	0	<50	-	-	0
Van Houten (Undated)	5,000	-	<0.1	<50	<50	<0.0025
Woodward and Dickerson (Undated)	10,000.00	0	0	<50	<50	-
Meursing and Slot (1968)	20,000	-	0	<50	<50	-
Collins-Thompson et al .,(1978)	100,000	-	<1.8	<2000	<2000	-
ICMSF (1974)	10,000	-	-	-	<100	-

Appendix 1

Morphological and biochemical identification of bacterial isolates from Bournvita from "Mobil Mart" Supermarket

Isolate No	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**
Gram Stain	+	+	+	+	+	+
Oxidase	+	-	-	-	-	-
Oxidase	+	+	-	-	-	-
Catalase	+	-	-	-	-	-
Coagulase	-	-	-	-	-	-
Colonial Morphology	5.0 mm in diameter, irregular, flat, undutlate, dull, brettle	1.0 mm in diameter, single white, opague	1.0 mm in diameter, spindle and opaque	1.0 mm in diameter, spindle and granular creamy	1.0 mm in diameter, spindle and raised, opaque	0.5-1.0 mm slightly moist raised, white
Cell Morphology (Microscopy)	Sporing rods, non motile	Round non-motile in pairs and cluster	Round in pairs and clusters	Round in singles and some in clusters	Round in clusters	Round in singles
Microflora	*Bacillus* spp.	*Cocci* spp.	*Cocci* spp.	*Cocci* spp.	*Cocci* spp.	*Cocci* spp

Key: B1 B2, B3, B4, B5, and B6 = Number of isolates from Bournvita
+ = Positive - = Negative

Appendix 2

Morphological and microscopical identification of mould isolates from Bournvita from "Mobil Mart" Supermarket

Isolate No	B1	B2	B3
Colonial Morphology	Colonies were covered with a white, fluffy, aerial mycelium. As colony matures, a salt and pepper effect was noted with the surface covered with black spores. The reverse of the colony was tan coloured.	Colonies were covered with white, fluffy, aerial cotton wool-like mycelium	Mature colonies had a distinct margin and were some shade of green, blue-green and green-brown. Surface had a powdery and granular appearance from profuse production of pigmented spores. A white apron seen at the edge in the zone of active growth
Microscopic Features	Hyphae were hyaline and distinctly septate. Conidiophores were long and vesicle was covered with a thick ball of spores. Spores were spherical and black.	The hyphae were broad, non-septate and ribbon like. Sporangia contain small spores. No. rhizoids	Hyhae were hyaline and distinctly septate. Conidiophores were long terminating in a large club-shaped vesicle. Chains of spherical conidia were borne from a single row of sterigmata.
Microflora	*Aspergillus niger*	*Mucor* spp.	*Aspergillus fumigatus*

Key: B1, B2 and B3 = Number of Isolates from Bournvita.

Appendix 3

Morphogical and biochemical identification of bacterial isolates from Richoco from "Mobil Mart" Supermarket

Isolate No	R1	R2	R3	R4	R5	R6
Gram Stain	+	+	+	+	+	+
Colonial Morphology	2-5 mm in diameter, moist, flat, irregular, white and brittle and opaque	3-8 mm in diameter, flat, dry, with slimy centre and creamy in colour	1.0 mm in diameter, moist, creamy, raised and entire margin	1.0 mm in diameter, slightly raised, moist and creamy entire.	1.0 mm slightly raised, granular, creamy, and entire	1.0 mm, slightly raised, creamy, granular and entire
Oxidase	+	+	-	-	-	-
Catalase	+	+	-	-	-	-
Coagulase	-	-	-	-	-	-
Cell Morpology (Microscopy	Sporing rods Singles, some in clusters	Sporing rods In singles and other end to end	Round In pairs some in clusters	Round In singles	Round, some in pairs, others in clusters	Round In clusters
Microflora	*Bacillus* spp.	*Bacillus* spp.	*Cocci* spp	*Cocci* spp	*Cocci* spp	*Cocci* spp.

Key: R1, R2, R3, R4, T5, and R6 = Number of isolates from Richoco
+ = Positive - = Negative

Appendix 4

Morphological and microscopical identification of mould isolate from Richoco from"Mobil Mart" Supermarket

Isolate No	R1
Colonial Morphology	Colonies were initially covered with a white fluffy, aerial mycelium. As colonies matured the surface changed to black and the reverse of the colony remained tan coloured
Miscroscopic Features	Hyaphae were hyaline and distinctly septate. Conidiophores were long and vesicle was covered with a thick ball of spores were spherical and black.
Microflora	*Aspergillus niger*

Key: R1 = mould isolate from Richoco

Appendix 5

Morphological and biochemical identification of bacterial isolates from Bournvita from Open Market

Isolate No	B1	B2	B3	B4	B5
Gram Stain	+	+	+	+	+
Colonial Morphology	2-6 mm, dry irregular, white, opaque and flat	10.0 mm., moist, smooth, raised and entire	1.0 mm., moist, convex and entire and creamy	1.0 mm, moist,convex, entire and creamy	1.0 mm, moist, convex entire and creamy
Oxidase	+	-	-	-	-
Catalase	+	-	-	-	-
Coagulase	-	-	-	-	-
Cell Morpology (Microscopy)	Rods	Round in clusters	Round in clusters	Round in clusters	Round in clusters
Microflora	*Bacillus* spp.	*Bacillus* spp.	*Cocci* spp	*Cocci* spp	*Cocci* spp

Key: B1, B2, R3, B4, and B5 = Number of isolates
0 + = Positive - = Negative

Appendix 6

Morphological and microscopical identification of mould isolates from Bournvita from Open market

Isolate No	**B1**
Colonial Morphology	Colonies were initially covered with a white fluffy, aerial mycelium. As colonies matured, a salt and pepper effect was noted, with the surface ultimately covered with black spores. The reverse of the colony remained tan coloured.
Miscroscopic Features	Hyaphae were hyaline and distinctly septate. Conidiophores were long and vesicle was covered with a thick ball of spores. Spores were spherical and black.
Microflora	*Aspergillus niger*

Appendix 7

Morphological and biochemical identification of bacterial isolates from Richoco from Open market

Isolate No	R1	R2	R3	R4
Gram Stain	+	+	+	+
Colonial Morphology	2-5 mm in diameter, moist, flat, irregular, white and brittle and opaque	3-8 mm in diameter, flat, dry, with slimy centre and creamy in colour	1.0 mm in diameter, moist, creamy, raised and entire margin	1.0 mm in diameter, slightly raised, moist and creamy entire.
Oxidase	+	+	-	-
Catalase	+	+	-	-
Coagulase	-	-	-	-
Cell Morpology (Microscopy	Singles, some in clusters	Rods in singles and others end to end	Round in pairs some in clusters	Round in singles
Microflora	*Bacillus* spp.	*Bacillus* spp.	*Cocci* spp	*Cocci* spp

Key: R1, R2, R3, and R4 = Number of isolates from Richoco
+ = Positive - = Negative

Appendix 8

Morphological and microscopical identification of mould isolate from Richoco from Open market

Isolate No	R1
Colonial Morphology	Colonies were initially covered with a white fluffy, aerial mycelium. As colonies matured the surface changed to black and the reverse of the colony remained tan coloured
Miscroscopic Features	Hyaphae were hyaline and distinctly separate. Conidio phores were long and resicle was covered with a thick ball of spores were spherical and black.
Microflora	*Aspergillus niger*

Key: R1 = mould isolate from Richoco