THE MICROBIAL QUALITY OF SOME SELECTED ORAL LIQUID PHARMACEUTICAL PREPARATIONS

BY

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DECLARATION

I Hereby declare that this thesis has been compiled by me and is the record of my research work. It has not been accepted in any previous applications for a higher degree. All sources of information are specifically acknowledged by means of references.

BOLAJI, R. OLAJUMOKE (Mrs)

CERTIFICATION

This thesis titled "THE MICROBIAL QUALITY OF SOME SELECTED ORAL LIQUID PHARMACEUTICAL PREPARATIONS" by BOLAJI R.O. (Mrs) meets the regulations governing the award of the degree of Master of Science (Pharmaceutical Microbiology) of Ahmadu Bello University, and is approved for its contribution to scientific and literary presentation.

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DEDICATION

"TO GOD BE THE GLORY"

With gratitude to God Almighty for his love, mercy and faithfulness, I dedicate this work to my children- OLUWAFEMI, ADEBUKOLA and ADEDOYINSOLA and to my husband- ADEGBOYEGA for their love, encouragement and prayers.

ABSTRACT

The microbial quality of some commonly prescribed oral liquid preparations namely Paracetamol, Multivitamins and Cough mixtures from three different companies (1, 2, 3; 1, 2', 3' and 1, 2", 3") obtained from pharmaceutical chemists located within Kaduna and Zaria were analysed during the two prevalent seasons (harmattan and heat) in this part of the country. The analysis involved the determination of the microbial load in the syrups by viable cell counting, determination of pH at the beginning of harmattan season and at the end of heat season, and identification of some of the isolated organisms. The analysed samples were purchased as dispensed by the manufacturers ready for consumer use.

The bacteria flora detected include *Staph, aureus.. E. coli,* and *Bacillus* species, while the mould/yeast include *Aspergillus niger. Aspergillus flavus, Cladosporium Aautianux.. Cladosporiux wernikii.. Penicillium* species, *Curvularia* species, *Rhodotorulla* species, Mucor, yeast as well as *Ascoxycetes* species. Generally, the bacterial load was higher during the heat season compared to the harmattan whereas the mould/yeast count were higher during the harmattan season. The bacterial cell count obtained for the Paracetamol syrups ranged from 1.0×10^2 - 6.5×10^2 cfu/ml for the harmattan season and 1.5 - 5.2×10^3 cfu/ml for the heat season. Multivitamins syrups, the bacteria cell count range was 3.5×10^2 - 6.5×10^2 cfu/ml during the harmattan season and 5.0×10^3 - 6.5×10^2 cfu/ml during the heat season. The Cough syrups had a count range of 1.0×10^2 - $1.2\ 10^3$ cfu/ml during harmattan season and zero 2.6×10^3 during the heat season.

For the mould/yeast, however, the count obtained for Paracetamol syrups ranged from 0.5 x 10^2 spu/ml during the harmattan and 0.2 x 10^2 - 4.5 x 10^2 spu/ml during the heat season. For Multivitamins syrups, the count ranged from 3.0 - 7.0 x 10^2 spu/ml during harmattan season and 1.0 x 10^2 - 5.5 x 10^2 spu/ml during the heat season. The Cough syrups had a mould/yeast count range of 1.0 x 10^2 - 1.0 x 10^3 spu/ml during harmattan and 0.1 x 10^2 - 7.5 x 10^2 spu/ml during the heat season.

However, there were batch to batch differences recorded as three different batches from each company were analysed. The pH at the beginning of the analysis (harmattan season) were mainly on the acidic level and this shifted towards the alkaline level at the end of the period of analysis (heat season). It was observed that the microbial load was not directly associated with changes in pH.

The result of this work has demonstrated that, the samples analysed actually contained microbial contaminants right from the manufacturer and that the seasons do affect the microbial load due to the changes that occur in the storage temperature and the resulting effect of this microbial contaminants may lead to spoilage as well as unacceptability of products and subsequent health hazards on the consumers. Therefore, there is the need for manufacturers to adhere strictly to good manufacturing practices, and good storage facilities should be provided in each pharmaceutical premises.

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CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Microbial contamination is the term used to describe the presence of micro-organisms in products where their presence is undesirable. It has long been recognised that all pharmaceutical preparations for oral, topical and cosmetic use are subject to microbial attack. The amount of contamination in finished products depends on:-

(a) the microbial content of raw materials

(b) the conditions of manufacture and cleanliness of the equipment used

(c) the extent to which preparations are preserved through the normal constituents or by the additions of known chemical preservative agents.

(d) and conditions of storage by both the manufacturer and the users (Sykes, 1969).

The interest in microbial contamination has been aroused firstly because of our greater awareness of the general need for contamination control, secondly because of the much wider use of newer types of medicaments and formulation which are often susceptible to contaminations and thirdly, because of the unfortunate and sometimes tragic consequences of using contaminated materials. It is this third reason that brought the matter of contamination to a head when the reports from the Royal Swedish Medical Board incriminated certain

contaminated tablets as sources of an outbreak of typhoid infections (Kallings *et. al.*, 1965) and the occurrence of *Pseudomonas aeruginosa* in ophthalmic preparations which sometimes cause eye damage or even loss of sight.

Microbiological standards where they exist at all vary considerably from country to country e.g. in Britain, it is obligatory for all eye preparations to be dispensed sterile. It has also been reported by a group of Committees of Official Laboratories and Drug Control Services and the section of Industrial Pharmacist of the FIP (July 1975) the degrees of microbiological purity according to the categories of pharmaceutical preparations as shown in Table 1.

In the U.S.P. XX (1980), a standard for dried yeast was introduced of not more than 7,500 bacteria and 50 mould/g and in the U.S.P. XXI (1985), it was specified that the total bacteria count for gelatin should not exceed 10,000 cells/g with coliform bacteria totally absent from 10 g. Similarly, there is the introduction of specific tests e.g. test for the detection of *Salmonella* in carmine powder (B.P., 1980), while in Sweden, as a result of the Royal Medical Board report, a limit of 100 bacteria/g was proposed for all drugs including veterinary products, baby creams, powders and certain skin preparations for use in hospitals. Also, a limit of 10,000 bacteria/g for drugs taken orally and 100 bacteria/g for other types of preparations with the absence of certain specified pathogenic organisms from amounts of drugs ranging from 0.1 - 5 g (Sykes, 1969). These clearly show that there is no uniformity of opinion concerning the levels of contamination which might be permitted in pharmaceutical products generally and it is for this reason that various bodies in different countries are investigating the subject in details.

TABLE 1: Degree of Microbiological Purity According to Categories of Pharmaceutical Preparations

CATEGORY	PRODUCTS	REQUIREMENTS
1.a	Injectable	Sterility under the conditions of Pharmacopoeias
1.b	Ophthalmic preparations, and preparations to be applied in body cavities, on burns and severe ulceration are normally free from micro-organisms	Absence of revivable micro-organisms in 1 g or 1 ml
2	Preparations for topical applications e.g. on skin lesions in the nose, throat, ear etc. (High risk preparations)	Limiting level of revivable micro-organisms: 10^2 in 1 g or 1 ml and among these: - No Enterobacteria - No *Ps. aeruginosa* - No *Staph. aureus*
3	Other Preparations	Limiting level of revivable micro-organism: 10^3-10^4 aerobic bacteria/g or ml 10^2 yeast and moulds/g or ml Limits for specified micro-organisms: - absence of *E. coli* in 1 g or ml In certain cases: - absence of *Salmonella* in 1 g or ml - other Enterobacteria max. 10^2/g or ml - absence of *Ps. aeruginosa* in 1 g or ml - absence of *Staph. aureus* in 1 g or ml

*Adapted from *Pharmaceutica Acta Helvitiae*, 51, Nr 3 (1976)

1.2 SOURCES OF CONTAMINATION

The quality of pharmaceutical products is greatly influenced by the environment in which they are manufactured, the raw materials used, the conditions of equipment, the personnel involved and the packaging materials as well as other factors, hence the level of microbial contamination of the final product may thus represent the contaminants from these various sources. A review by Sykes (1969) related sources of microbial contamination in a product to the mentioned sources above as well as the air and final users. From the manufacturing standpoint, the most difficult to control are the raw materials and users. Contamination by users can be minimised however, even if not totally prevented by the inclusion of antimicrobial preservatives in the formulation. However, this should not be seen as a substitute for Good manufacturing Practice (GMP).

In considering raw materials, due attention must be given to water which has been shown to be exclusive source of contamination in some products. The particular susceptibility of some ingredients, the conducive physicochemical characteristics and often the delicately balanced stability of many medicaments can render them very sensitive to microbial destruction unless the problem is properly managed. The use of crude animal or vegetable extracts or drugs with their wide assortment of nutrients and inorganic components, or the reliance upon many modern synthetic ingredients often designed to be readily biodegradable can markedly raise a formulation's potential for gross microbial destruction. Initially, though limited attack by one group of organisms may modify a product sufficiently to render it more susceptible to a second group able to induce even more devastating damage.

1.2.1 RAW MATERIALS

The raw materials for the preparation of pharmaceutical products consists usually of active ingredients, the excipients and vehicles. Contaminants found in finished products have been traced back to the use of contaminated raw materials.

1.2.1.1 ACTIVE INGREDIENTS

The active ingredients may be of natural origin, synthetic or semi-synthetic. Varied microbial flora is usually associated with untreated raw materials derived from natural sources. Biological products such as thyroid, digitalis and carmine powder have high bacterial content and are most difficult to control due to their origin which may be pathogens of animal origin notably *Salmonella* (Kunz and Ouchterlong, 1955; Kormany *et. al.*, 1967). Also, *Salmonella* infections have arisen from tablets and capsules of yeast, carmine, pancreatin and thyroid extracts as well as from powdered vegetable drugs which have low level of pathogens in their finished states.

In a wide study of pharmaceutical raw materials, the microbial contamination of synthetic and semi-synthetic were discovered to be tolerably low with the exception of a few e.g. calcium lactate, Hydrocortisone (Pedersen and Ulrich, 1968). This implies that natural products such as minerals e.g. kaolin, talc and certain dyes from animal products e.g. cochineal or drugs of plant origin are generally more heavily contaminated.

1.2.1.2 EXCIPIENTS

Additives are sometimes of greater risk than active ingredients as sources of contamination. With solid forms, e.g. tablets in which contamination has been shown to arise mainly from the starch employed as a disintegrant where counts may run up to 1000 bacteria/tablet including coliforms. Kallings *et. al.* (1966) estimated the degree of contamination in potato starch and found it to be more heavily contaminated than maize starch. Also Kallings *et. al.* (1966) found that coliform contamination of tablets of barbiturates and tranquillisers were due to the starch used in their formulations. However, gelatin has generally displayed low count due to the fact attributed to its preparation i.e. dissolving connective tissue in a medium of either strong acid or strong base (Martindale, 1982).

1.2.1.3 VEHICLE

The most employed vehicle in pharmaceutical formulations is water. Water is the greatest potential source of contamination as it is used in the preparation of many products as well as for various washings and cooling processes. The microbial flora of water depends on the quality of raw water and any processing it received as well as the distribution system. Preparations containing water present an entirely different microbiological problem than do the dry preparations. This is because such products constitute a much more suitable environment for growth and survival of both bacteria and moulds. The nutritive requirements of many micro-organism are very small and even simple salt solutions have been known to support their growth (Baird, 1987).

Sykes (1972) also reported counts of the order of 10^5 bacteria/ml (all

Gram negative bacilli) in distilled water fresh from the still but on replacing plastic tubing daily, the level of contamination dropped to less than one organism/ml. De-ionised water has been reported to be microbiologically worst than original supply into the de-ioniser. This is because the column retains organic debris and such other nutrients that makes a good breeding for bacteria more especially when the column is only used intermittently. De-ionised water is therefore also not suitable for use in the preparation of pharmaceuticals especially creams and mixtures hence both tap and de-ionised water have to be treated either by boiling, filtering or passing through ultra-violet sterilisers immediately before use. Water from streams, ponds and surface water are more heavily contaminated than those from running rivers. Deep well water are relatively pure due to the filtration by the different strata of the earth. Rain water is inevitably contaminated as it descends from the atmosphere where it comes in contact with contaminated air.

Therefore, all raw materials to be used in preparing a product must be sampled and tested microbiologically to ensure the absence of pathogenic and objectionable micro-organisms in view of the intended use according to the current Good Manufacturing Practice (GMP).

1.2.2 AIR AND ENVIRONMENT

The environment under which pharmaceutical products are manufactured can influence the level of microbial contamination. The type of movement allowed into and out of the manufacturing area, the cleanliness and hygienic methods adopted also contribute to the final microbial count. Air on its own does not make a natural habitat for the

growth and proliferation of micro-organisms, bacterial cells and fungal spores are sometimes suspended and easily transported in air currents. Fall-outs loaded with such debris and settling on pharmaceutical products are a ready source of contamination especially if such debris carry non-exerting organisms (Denyer, 1987). Also air acts as vehicle carrying micro-organisms from one area to another especially the spore-bearing *Bacillus, Clostridium*, moulds, mucor and yeast.

Air in an area containing working machines and active personnel have higher microbial count. The microbial flora of air fluctuates with seasons:- dusty harmattan aid the spread of bacteria than wet rainy season which is humid and damp. The type of environment sometimes dictates the type of micro-organism in the air around the place.

1.2.3 EQUIPMENT

With regards to equipment, particular attention must be paid to items such as pipelines, valves and faucets. The variety of contaminants from these can be very wide and depend largely on the nature of the materials being processed through the plants. It is important for certain amounts of specialised production equipment to be made available in the manufacture of satisfactory products. Traces of products left in the transfer pipes or scratches on the inner surfaces of kettles can bring about rapid contamination of subsequent batches of the products.

The main offenders are usually the water-borne non-pigmented *Pseudomonads* but yeast and mould are also quite common. for instance, Basset (1971) traced the outbreak of pneumonia in the new born to the use of *Pseudomonads* contaminated suction apparatus. Joints and cross connections in pipes often provide a site for microbial growth, the

proper drill should be to flush through the whole plant with hot water immediately after a batch has been processed and just as the next batch is being handled. Unless precautions are taken, colonisation can take place in less accessible areas of the equipment and so act as continuous source of contamination to the products.

1.2.4 PERSONNEL

The microbial flora of air and environment in which pharmaceutical manufacturing takes place is influenced greatly by the personnel involved at each stage of production. Operators can transfer micro-organisms to products from their skin which is rich in various types of bacteria especially the undesirable *Staph. aureus* which is not easily eliminated by washing. Coughs and sneezes are prolific sources of air contaminations especially droplets infectious organisms. Talking has also been shown to increase the microbial flora of a clean enclosed area. Human carriers are always potential source of infection. *Staph. aureus* is widely spread and there is a risk of infection with this and other cocci unless proper hygienic precautions are taken.

Human-borne *Salmonella*, coliforms and other intestinal bacteria are possibilities to be reckoned with as well as *Pseudomonas aeruginosa* of which it has estimated that up to 12% of normal healthy people are carriers (Sykes, 1969). The risk of cross infection with these organisms is significant especially if one takes into account the common practice of the user dipping his or her fingers into a jar of cream or ointment. Bacteria other than skin flora can be transferred from operators as a result of poor personal hygiene, this include faecal organisms from the anal region, wounds and abscesses. Organism

from nasal passages often transmitted include *Staph. aureus* and limited quantity of saprophytic *Streptococcus* viridans group.

1.2.5 CONTAINERS AND CLOSURES

Containers and closures can also contribute contaminants to the product though not very high but vary according to the type of activity taking place. The container in which the product are packaged can introduce microbes into its content depending on the composition and storage conditions. Containers made of glass, metal and plastics have low microbial content but can be contaminated during the process of transportation in cardboard boxes or storage by spores of mould such as *Aspergillus*, *Cladosporium* and *Bacillus* species.

In the past, cork closures have been incriminated as carriers of Ps. aeruginosa in contaminated cetrimide solutions (Sykes, 1969) but nowadays such closures have generally been replaced with screw-caps. Nevertheless, there is still hazard of mould infection from liners of such closures particularly if they are made of cork or card hence unless they are treated specially with preservatives, are good source of contamination for liquid and semi-solid preparations.

1.2.6 STORAGE

The conditions under which raw materials and finished products are stored is also very important, this is more so for hygroscopic substances. Storage of pharmaceuticals in damp conditions will make the product exceed the water activity level (A_W) for mould (i.e. $A_W = 0.80$) and that of osmophilic yeast ($A_W = 0.60$) while that of bacteria (Aw = 0.71), thus this will enhance microbial growth. While microbial

contamination count may remain constant or even decrease in solid products e.g. tablets, it may likely increase in others e.g. mixtures, lotion and creams during storage in few days (Khante *et. al.*, 1979).

1.2.7 IN-USE CONTAMINATION

Higher rates of contamination are often associated with products after opening and use. This is more so with drugs dispensed from bulk containers in hospital pharmacies as compared with those in use in the general community. There are fewer opportunity for contamination in homes since smaller quantities are used by individual patients. Hugo and Wilson (1970) and Harte *et. al.* (1978) showed a higher proportion of eye drops and ointment preparations which had been contaminated during the period of use. The so called "Antiseptic" solutions in common use are also possible source of contamination particularly with organisms of the *Pseudomonads* group which can sometimes be present in very large numbers. This group of organism is well known for their resistance to chemical disinfectant and ability to adapt themselves to relatively unfavourable conditions, they can therefore grow without difficulty in situations which are lethal to most other organisms. This adaptation usually occurs with antiseptics which tend to be selective in their action. The use of unit-dose packs will eliminate the danger of microbial contaminations. The sources of microbial contamination can be summarised as shown in the figure below.

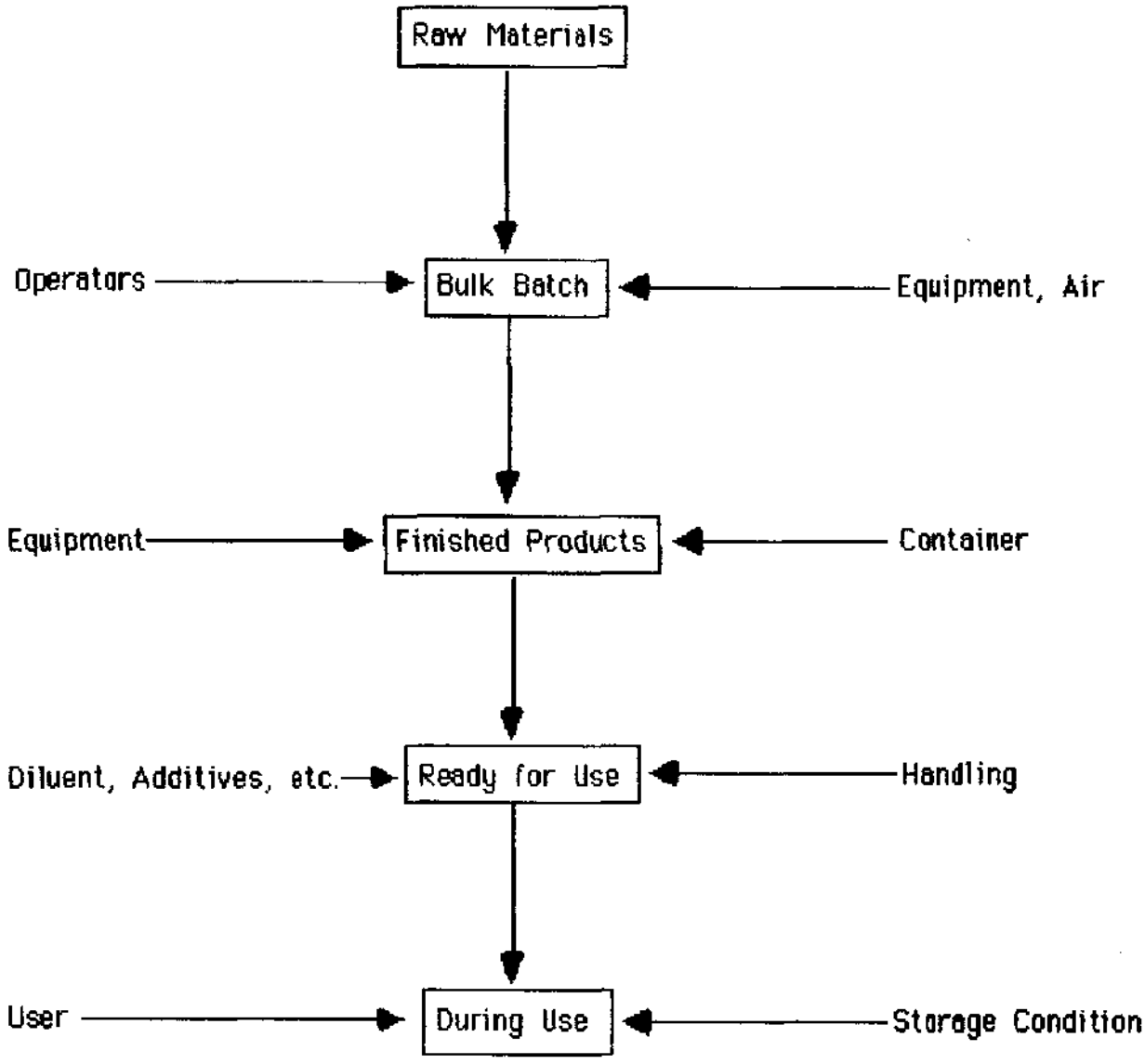


Figure 1: Sources of Microbial Contamination

1.3.0 TYPES OF CONTAMINATION

The types of contamination with which we are concerned can be classified into those that are pathogenic or potentially pathogenic e.g. *Salmonella* and those that are not necessarily harmful but can cause changes in physical characteristics of the preparation or otherwise cause its spoilage and disfigurement.

Every type of contaminant will not grow in every type of medicament; each formulation is particularly susceptible to certain types of contaminants. Similarly, different types of contaminants are not of equal significance in every type of product, some are less desirable and even more dangerous in some products than in others. For instance, few *Clostridia* in oral preparations are not likely to cause trouble but they could be dangerous in an ointment or cream if applied to an open wound. By the same rule, *Salmonella* are quite unacceptable in tablets but they are probably of no significance in topical products except that the user might spread the organism around and so cause trouble elsewhere by cross infection. Also animal products are more likely to carry organisms of human pathogens especially *Salmonella* (Sykes, 1972).

It is worth noting that *Staphylococcus*, Coliforms bacteria and *Ps. aeruginosa* are undesirable in all situation. Therefore, it is virtually impossible to designate with certainty any contaminant as being harmless.

1.2.1 PATHOGENS

With their fastidious nature, contaminants acutely pathogenic are unable to replicate in most medicament but could remain viable and

infectious for an appreciable length of time, although infrequently reported as pharmaceutical contaminants. In the reports by Public Health Laboratories Services Working Party (1972) five hundred and sixty-seven (567) distinct bacterial types were identified in three hundred and nine-three (393) samples of various pharmaceutical products. Results of the investigation of the various other workers revealed that the type and quantity of contaminants in most cases were invariably dependent on the type of preparations (White *et. al.*, 1968; Bowman *et. al.*, 1971; Beveridge and Hope, 1971).

Of the pathogens, *Staph. aureus*, *Ps. aeruginosa*, *Salmonella* and coliform bacteria have caused the greatest damage and have attracted the most attention. Others which merit consideration in some situations are the anaerobic bacteria including *Cl. welchii* and *Cl. tetani*. Also of great practical concern are a wide variety of common non-fastidious contaminants usually of limited pathogenicity to healthy individuals which may replicate readily within some aqueous medicines. To certain groups of unhealthy patients however, or to particularly damaged tissues, some of these opportunistic microbes can represent a serious health hazard e.g. whilst the intact cornea is resistant to infection, when scratched, scarred or surgically incised, it offers little resistance to *Pseudomonads* and other gram negative bacteria, hence many eyes have been lost following the use of improperly designed ophthalmic solutions containing organisms.

Gram negative microbes in ointments and creams have infected eczematous skin, *Klebsiella* contaminants in barrier creams have also induced fatal respiratory disease in neonates. Oral mixture and antacid suspensions have become colonised with gram negative bacteria with probably limited consequences to many but in patients of immuno-compromised as a result

of anti-neoplastic chemotherapy or as an aid to transplant surgery, life threatening infection have resulted and *Candida* species are a particular hazard to such patients and fatal septicaemia have been associated with these contaminants in oral and intravenous solutions.

1.3.2 MICROBIAL SPOILAGE

Microbial spoilage of a product is said to occur if low levels of acutely pathogenic micro-organisms or high levels of opportunistic pathogens are present. A product may also be spoiled by physical or chemical means such as through hydrolysis or oxidation of active components to therapeutically inactive products. Usually moderate contaminant levels do not show any sign of spoilage but the production of fishy amines, the rotten egg odour of hydrogen sulphide or the pungent odour of ammoniacals indicates that spoilage has set in. Aqueous products and even water itself sometimes develop earthy taste which is an early sign of microbial growth. The diffusion of microbial pigments which discolour the product gives an observable indication of microbial attack while the decrease in viscosity of viscous products may result from depolymerisation of the thickening or suspending agents. The growth of organisms may in addition result in the accumulation of metabolites which can cause change in pH of a product e.g. growth of yeast may raise the pH as to promote subsequent bacterial growth (Denyer, 1987).

The various additives are subject to microbial attack and spoilage e.g. the various surface active agents utilised in pharmaceutical formulations are all prone to degradation by various micro-organisms (Beveridge, 1987). In addition, organic polymers such as starches,

pectin, tragacanth and acacia, dextrans and proteins are liable to depolymerisation by bacteria providing the relevant degradative enzymes for the polymers. Humectants are also prone to microbial attack e.g. glycerol and sorbitol when used in low concentration. Sweetening, flavouring and colouring agents have also been shown to suffer microbial attack e.g. low level of syrups readily support microbial proliferation whereas very concentrated stock solutions of syrups i.e. with low water activity (A_w) are resistant to microbes except osmophilic yeast. Flavouring agents e.g. chloroform and peppermint waters can be attacked by bacteria and yeast (Beveridge, 1987) while colouring agents e.g. tatrazine and amaranth also do readily support bacterial and fungal growth. Preservatives and disinfectants themselves are known to be susceptible to attack, more so by certain species of *Pseudomonads* e.g. organo-mercurials such as thiomersal when used at concentration below their recommended levels.

Frequently, the contaminants of the synthetic and semi-synthetic drugs were found to fall under the Gram positive spore-forming bacteria with occasional Gram negatives. The level of such contaminants is mostly in the order of less than 10 bacteria/g but may occasional rise up to 100 bacteria/g or more (Fischer *et. al.*, 1968). These workers further demonstrated the absence of the coliform bacteria, *E. coli*, *Salmonella* from such products generally. However, Kallings *et. al.* (1966) demonstrated the presence of the faecal organisms whose origin was traced to the methods of manufacture. In Alumina, Magnesia and such preparation, the contaminants almost without exceptions are Gram negative bacteria originating from the water supply used during processing as the growth of these bacteria are quite rapid.

1.4.0 PREPARATIONS AFFECTED

A few preparations by the nature of their formulation e.g. those containing alcohol are self disinfecting and therefore any microbial contamination that is introduced with other constituents or during manufacture is of no significance because it is rapidly killed. On the other hand, there are many preparations in which bacterial growth can readily take place and it is here that special precautions have to be taken. The greatest difficulties arise with aqueous preparations, creams and emulsions, often the problem can only be overcome by use of a suitable antimicrobial preservatives. Dried products such as tablets, powders and capsules are not immune, however because they do not allow the actual growth of bacteria, they can maintain them in a viable state for a long period so that given suitable conditions, growth and spoilage can take place.

1.4.1 MIXTURES AND SUSPENSIONS

Mixtures are liquid dosage forms intended for oral administration and may contain one or more medicaments dissolved or suspended usually in a flavoured vehicle, and may also contain suitable preservatives. The official monographs such as the British Pharmacopoeia do emphasise that mixtures should be recently prepared since deterioration is likely to ensure if such products are kept longer than a few days usually 14 days. For this reason, mixtures are usually prepared and dispensed within a few days with a minimum of 5 days. There are various types of mixtures depending on the nature or physical properties of the ingredients:-

(i) Simple mixtures which are those in which the ingredients are

soluble or miscible with the chosen vehicle e.g. Potassium Citrate Mixture B.P.C

(ii) Simple mixtures containing diffusible solids in which the solids readily disperse in the vehicle e.g. Magnesium Trisillicate Mixture B.P.C

(iii) Mixtures containing indiffusible solids require the addition of a suspending agent such as tragacanth, starch or carboxymethyl cellulose to uniformly distribute the particles in the vehicle e.g. Aspirin Mixture B.P.C. Precipitate-forming liquids e.g. Myrrh Tincture also requires a suspending agent

(iv) Emulsions are mixtures of oil dispersed in aqueous vehicle with the help of an emulsifying agents such as acacia. Example is Liquid Paraffin Emulsion. For a good emulsion, the volume of the oil should not be less than 20% v/v of the total volume to be prepared.

The product of this group most susceptible to contamination are those containing magnesium and/or aluminium hydroxides or the natural earth as well as syrups and certain specific products such as Gripe water. *Micrococci*, *Staphylococci*, spore-forming bacteria, yeast and moulds have been found in different samples on the market and this can be corrected by good plant hygiene and strict attention to the quality of the raw materials used.

1.4.2 TABLETS, CAPSULES AND POWDERS

The microbial content of tablets, capsules and powders are generally low, but they vary in both numbers and types with the nature of the

drug and of the excipient used. With tablets, the method of granulation plays a role, with the moist granulation, some organisms can grow quite readily during mixing stages but fortunately, they tend to die out in the drying and compression stages. This is not always the case however and care must be taken in controlling the moist granulation stages. Tablets of synthetic drugs usually have viable counts of less than 100 bacteria/tablet, often it is less than 10 bacteria/tablet. Most of the bacteria are aerobic spore-formers of the *Bacillus subtilis* group, a few mould may be present along with a few cocci but no coliform or other gram negative bacilli.

1.4.3 OINTMENTS AND CREAMS

Several workers such as Ayliffe *et. al.* (1965) have found *Ps. aeruginosa* often in large number in a variety of creams but notably in those containing corticosteroids. These contaminants are not usually present in the material when supplied by the manufacturers are introduced mainly in hospitals either whilst being formulated or otherwise manipulated or during use in the wards or by patient. This again indicates the importance of ointments and creams having suitable preservatives.

1.4.4 EYE, EAR AND NASAL DROPS

Apart from the already mentioned Ps. aeruginosa as contaminant in eye preparations, *Staph. aureus*, *Bacillus subtilis* and *Proteus vulgaris* have also been involved and these along with other bacteria have been found in eye drops, lotions and irrigating fluids (Dale *et. al.*, 1959). Ear drops are not subjected to this type of contaminants because the

softeners used in these formulations possess antimicrobial properties, however care needs be taken to eliminate *Ps. aeruginosa* which can sometimes prove problemsome. Nasal drops do not have this natural protection and so are subject to bacterial and mould infection in the same way as eye preparations.

1.5 HAZARDS OF MICROBIAL CONTAMINATION

The consequences of microbial contamination of pharmaceutical products are very gross and fatal in some cases. The therapeutic efficacy of products are either changed or reduced to non-effective. For instance, the salicylic acid formation from acetylsalicylic acid (Aspirin) or the inactive products from Penicillin and certain other antibiotics can be demonstrated. Appreciable loss of potency of some alkaloids and degradation has been recorded. Localised transformation of steroids have been observed around fungal colonies growing on the surface of steroids, tablets and in creams containing steroids.

Also products become unappealingly discoloured by various microbial pigments, loss of viscosity and sedimentation of insoluble ingredients can be observed. The polymerisation of sugars or surfactants can produce viscous slimy masses in syrups and shampoos, aggregation in creams can produce gritty texture. Accumulation of acidic or basic metabolites can produce marked shifts in the pH of some products.

Ayliffe *et. al.* (1965) and Basset (1971) traced the following disease conditions:- meningitis, pneumonia, respiratory tract infection, urinary tract infection, septicaemia, arterial thrombosis and wound infections to the use of *Ps. aeruginosa* contaminated products and surgical equipment. Between 1960 and 1964, documented cases of food

poisoning in the food industry showed that the causative agents in most cases were *Staph. aureus* and *Salmonella typhi*.

1.6 PREVENTION AND CONTROL OF CONTAMINATION

Prevention and control of contamination can be achieved if the principle and rules of hygiene are strictly observed. Cleanliness of equipment to minimise infection during the manufacture of pharmaceutical products is very important. These rules are essential in both small and large scale operation units, including hospitals and other pharmacies, although the greatest precautions may be taken during processing, it is possible for contaminants to gain entrance and actually grow in certain types of preparations. Some indication has already been given of types of preparations in which these can occur and these are all those containing water either in form of mixtures, suspensions, creams or emulsions.

Both ethical and health reasons stresses the need for pharmaceutical procedures to ensure that products are adequately protected from microbial contamination which has often led to spoilage of products, loss of activity or even disease in the patient.

1.6.1 PRESERVATION

Mixtures in common with other aqueous preparations are liable to support microbial growth unless adequately preserved. It is for this reason that preservation of a pharmaceutical product is aimed at preventing the deterioration which may accompany the growth of these organisms. This deterioration may arise as a result of either physicochemical process or through microbiological means. The former

process is usually prevented by the inclusion of an antioxidant, pH adjustment or the use of containers which have been designed to screen off ultra-violet rays which triggers off photosensitive reactions. On the other hand, deterioration through microbiological means is normally prevented by the incorporation of antimicrobial agents which are drugs capable of preventing the growth and proliferation of micro-organisms. However, antimicrobial preservatives must not be used in masking unhygienic procedures while formulating, or the use of contaminated raw materials because the smaller the microbial load a preservative has to deal with, the more efficient it is, whereas if the challenge is too great, the preservative may fail totally.

The choice of preservative is restricted to a limited few compounds of which esters of p-hydroxybenzoic acid (PARABENS), the quaternary ammonium compounds (such as cetrimide and benzalkonium chloride), Bronopol (2-bromo-2-nitropropan 1,3-diol) and the phenyl mercuric preparation are the most commonly used. These compounds are not all suitable for every type of formulation and are not interchangeable. It is important that the chosen agent should be active in the particular type of formulation concerned and this is an aspect not always fully appreciated. Other constituents in the formulation can sometimes radically affect the activity of the preservative and this applies particularly with emulsions and creams, to a lesser extent with suspensions. It is essential therefore that a preservative shall be assessed in the actual formulation in which it is intended to be used.

1.7 GENERAL METHODS OF ISOLATION

There are different methods that can be employed in the determination of microbial contaminants present in pharmaceutical products. These

include both general methods such as

(a) Plating. (b) Dilution (c) Filtration.

Also specific test such as test for:-

(a) *Staph. aureus*

(b) *Ps. aeruginosa*

(c) *Salmonella* and *Shigella* species.

1.7.1 PLATING;

This method involves the placing of known volume of a suitable dilution of the sample on a solid medium and incubating at appropriate temperature and time interval. The resulting colonies are then counted. The commonly employed types of plating methods include:

1.7.1.1 POUR PLATING

This can be of single agar layer or double agar layer. In the single method, 1 ml of a suitable dilution of the sample is added to a known volume of melted culture medium that has been cooled to about 45°C gently mixed and poured into a sterile plate. It is allowed to solidify, cooled then incubated inverted at 37°C for 18-48 hours. for bacteria and at 25°C for 3-14 days for fungi. In case of double layer, known volume of culture medium is allowed to solidify in the plate, then 1 ml of the diluted sample is mixed with known volume of culture media and poured on the surface of the first layer, allowed to solidify and then incubated as described.

1.7.1.2 ROLL-TUBE

Plugged test-tubes containing known amount of culture media (melted and cooled) are used. With the aid of standard dropping pipettes, known amount of sample are dropped into the test-tubes directly to the bottom of the test-tubes. The tubes are then shaken slightly without introducing air bubbles and rolled under a jet of cold water so that a thin film of solidified medium uniformly covers the test-tubes. These are then incubated appropriately.

1.7.1.3 SURFACE VIABLE

In this method, a standard dropping pipette is used to drop, at most five drops of the sample solution on the surface of an over-dried culture media plate. The pipette tip is held at a distance from the agar surface, the drops are allowed to dry, care is taken not to let the drops run into each other. The plates are then incubated appropriately. The number of colonies represent the number of viable cells in the volume of five drops of the sample.

1.7.2 DILUTION METHODS

Dilution methods are based on the premise that counts are distributed in accordance with the POISSON THEORY which predicts that at any given level of mean count, there is a known probability of getting no organism in the sample i.e. no growth observed when sample is introduced into broth and incubated. The technique involves the preparation of a series of dilution and inoculating from each dilution into several tubes of nutrient broth. After incubation, the pattern of sterile and fertile tubes is recorded and the count determined by

reference to prepared tables called the "Most Probable Number" (MPN). Tube dilution methods are generally used when very low counts particularly of swarming organisms are expected or when the presence of insoluble solids would interfere with other methods (Ibrahim, 1985).

1.7.2.1 TUBE DILUTION

In this method, 14 (20 x 150 mm) test-tubes containing 9 ml of sterile nutrient broth are used. Twelve of the tubes are arranged in four sets of three tubes each. One set of these tubes is used to serve as negative control to prove sterility of the medium. The other two test-tubes are labelled A and B respectively.

1 ml of the diluted sample is pipetted into each of set number two and tube A. The contents are shaken thoroughly to effect mixing and labelled 1:10 dilution. From test-tube A, 1 ml is pipetted into a third set of three tubes as well as tube B. The contents are again mixed. This new set is labelled 1:100. From tube B, 1 ml is pipetted into the fourth set of three tubes labelled 1:1000 dilution and mixed.

All the four sets together with A and B are incubated at 37°C for 48 hours. The number and pattern of tube showing growth is recorded and from prepared tubes, the most probable number of organisms in the dilution of the sample used is calculated (Table 3).

Table 2: Most Probable Number (Multiple Tube) U.S.P. 1980.

Observed combinations of number of tubes showing growth in each set. No. of mg or ml of sample per litre (dilution level)			MPN of organisms per g or ml
1:10	1:100	1:1000	
3	3	3	1100
3	3	2	1100
3	3	1	460
3	3	0	240
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	93
3	1	3	160
3	1	2	120
3	1	1	75
3	1	0	43
3	0	3	95
3	0	2	64
3	0	1	39
3	0	0	23

1.7.3 MEMBRANE FILTRATION METHOD

A known volume of sample solution is passed through a sterile bacteria-proof membrane filter. The membrane containing the cells is aseptically transferred to freshly prepared nutrient agar plate or a sterile filter paper pad soaked in nutrient broth. After incubation at 37°C for 24-48 hours, the resulting colonies are counted. The number of colonies should not be more than 200/membrane. This number represents the sample filtered, usually a membrane filter of pore size 0.45 µm is used. This method is very suitable for relatively large volumes of solution containing small numbers of bacteria. For yeast and mould count, the same procedure should be followed and the membrane filter placed on a fungal medium containing antibiotics.

1.7.4 WHY THE PROJECT

It has long been recognised that all pharmaceutical preparations for oral use are subject to microbial contamination (Sykes, 1969). The risk of contamination and subsequent spoilage of such products during storage and through administration to patients is generally significant. It is in appreciation of this therefore, that the design of a product must be such that it is able to withstand the challenges micro-organisms may impose upon it both during storage and until it is completely used up. The type and extent of contamination determines the nature of spoilage which occurs as well as the ill-health effects on the patient.

Syrups are popular non-sterile dosage forms of administering active medicaments to babies, children and the elderly and constitute the most

convenient form. The preparation and storage of syrups requires strict adherence to Good Manufacturing Practice (GMP) regulations in order to eliminate contamination by micro-organisms both spoilage and pathogenic. Many documented reports in various parts of the world has indicated the hazards associated with the use of contaminated non-sterile products such as mixtures, tablets, lotions and creams (Baird and Shooter, 1976; Noble and Savin, 1960; Glencross, 1972).

The common sources of contamination have been identified to be from the raw materials, stages of production as well as the personnel (Sykes, 1969; Flaum, 1978).

This work intends to analyse certain syrups commonly prescribed for children and available in chemists as "Over The Counter drugs (OTC) in small volumes of 50-100 ml single packs from the manufacturers and sold directly to patients (without further dispensing into small units) for their microbial quality in terms of microbial load, types of contaminants present as at the time of opening. The complete identification of some organisms isolated will be attempted and pH of the syrups before and after the experimental periods will be observed to see if there is correlation between the level of contamination and the pH of the syrup.

Two seasons (harmattan and heat seasons) were considered as the experimental periods i.e. from October to February and March to June respectively. This is due to the great difference in the temperature often observed in this part of the country at these particular periods which affects the growth of microbes even in some preserved products. The temperature for the harmattan season is as low as 10°C and heat period is as high as 37 - 40°C.

CHAPTER TWO

MATERIALS AND METHODS

2.1 PHARMACIES

Most of the sampled drugs were obtained from pharmaceutical chemists located within Kaduna and Zaria environs. Generally, five bottles each from three different batches were used except in cases where only two bottles were available. All samples were within their shelf life cycle and were purchased at the time to avoid scarcity problem. The syrups were stored on shelves in an office with air conditioned facilities. It should be noted that companies 2, 2', 2", 3, 3' and 3" are different from one another.

2.2 SYRUPS

Three types of syrups were analysed in the work and they are:-

(a) Paracetamol syrups from three companies

(b) Multivitamins syrups from three companies

(c) Cough mixtures (expectorants) from four companies.

These three groups of syrups are commonly prescribed for children in this area.

2.3 CULTURE MEDIA

Nutrient agar (BIOTEC LTD)

Nutrient broth (BIOTEC LTD)

Sabouraud Dextrose agar (OXOID LTD)

Glucose (OXOID LTD)

Cetrimide agar (BIOTEC LTD)

Lactose (OXOID LTD)

Peptone water (OXOID LTD)

Mannitol Salt agar (OXOID LTD)

MacConkey agar (OXOID LTD)

Distilled water

All media were prepared according to the manufacturer's instructions i.e. weighing specified amounts in stated volumes of distilled water with application of heat where required as in the case of agar. The media were then distributed in small volumes of 20 ml into universal bottles and sterilised by autoclaving at 121°C for 15 minutes.

2.3.1 GLUCOSE/LACTOSE FERMENTATION BROTH

A 1% glucose medium was prepared by dissolving 2 g of glucose in 200 ml of peptone water. Andreas indicator (acid fuchsin) 2 g was added to the solution and 20 ml volumes were distributed into screw-capped universal bottles and sterilised by autoclaving at 115°C for 30 minutes. The indicator is colourless in alkaline medium but turns red in acid medium. Lactose medium was similarly prepared.

2.4 pH MEASUREMENT

The pH of each of the syrup was measured at the beginning, [illegible] as at

the end of test period on Philips pH-meter after standardisation using pH 4 and pH 9 buffer solution as standards.

2.5 STATEMENT ON THE EXPERIMENTAL

The project intends to ignore whatever preservative that might have been added to the product analysed, since the aim of this work is to determine the microbial load present in these products at the point of final usage by the consumer.

However, in products where no organisms were found in the undiluted form, 3% Tween 80 was added to the medium. All samples did not have their compositions stamped on their packaging.

2.6 EXPERIMENTAL

2.6.1 SURFACE VIABLE METHOD

The surface viable method often referred to as MILES and MITSRA method was adopted. The prepared nutrient agar were melted in water bath and poured into sterile plates allowed to cool and solidify. These were pre-dried at 37°C for 15 minutes in an incubator inverted. Using standard dropping pipette, 20 µl of the syrup samples or appropriately diluted samples were carefully placed on five spots on the surface of the dried agar and allowed to dry off (the drying off of some the syrups was not complete due to their viscous nature).

The plates were incubated at 37°C for 24-36 hours for bacteria. This process was repeated using Sabouraud Dextrose agar for fungi and incubated at 25°C for 3-7 days. The resulting colonies were counted on

Gallenkamp Colony Counter for bacteria. Staining and microscopical processes were carried out on these colonies. Selective media were used to identify some colonies. In order to inactivate the effect of any preservative that may be present in the samples, 3% of Tween 80 was added to nutrient agar.

2.6.2 STAINING AND MICROSCOPICAL PROCESSES

2.6.2.1 BACTERIAL

A thin film of the obtained colony was prepared on a slide by placing a loopful of sterile water at the centre of the slide, a small quantity of the colony was taken and thoroughly mixed with the drop of water on the slide. This was then fixed by passing the slide over a flame until dried. A 1 in 10 dilution of Strong Carbol fuchsin stain solution (Ziehl Neelsen's stain) was used to stain the slide for 20 seconds and was rinsed with distilled water and dried between sheets of blotting paper. The slide was then observed under the microscope.

2.6.2.2 FUNGI

A piece of fungal growth was transferred unto a slide with a little amount of the medium. It was wetted with Lactophenol reagent (cotton blue lactophenol reagent) which gave the cells a deep blue stain, a cover-slip was placed on the surface and observed under the microscope.

2.6.3 IDENTIFICATION OF GRAM POSITIVE RODS

Colonies from gram positive rods were first subjected to acid fast staining to detect any spores. Colonies of spore-bearing cells were

grown on nutrient agar slope and further subculture in nutrient broth at 37°C for 24 hours. The broth culture was heated at 80°C for 10 minutes in a water bath to detect heat resistant spores (Collins and Lyne, 1976) and then cooled to room temperature. A loopful was then used to streak the surfaces of a nutrient agar and incubated at 37°C for 24 hours. Characteristic colonies were further gram stained and tests carried out according to Cowan (1974) for identification of *Bacillus* species as shown below.

(i) CATALASE TEST

Colonies of the rod shaped organism were picked with a sterile loop and emulsified in 0.5 ml of a 1% solution of Tween 80 in a MacCartney bottle. A volume (0.5 ml) of 20 volume hydrogen peroxide was added. The bottle was securely fastened and observed for effervescence which indicates the catalase positive reaction.

(ii) GLUCOSE FERMENTATION

A 10 ml volume of glucose medium was inoculated with suspected culture and incubated at 37°C for 24 hours. The medium was observed for a change of colour from pale yellow to a magenta red (or pink) indicating the production of acid from glucose.

2.6.4 IDENTIFICATION OF GRAM NEGATIVE RODS

Colonies of gram-negative rods were subcultured in nutrient broth at 37°C for 24 hours. The broth culture was then used to test for the presence of various gram-negative organisms using the criteria by Cowan

(1974).

2.6.4.1 TEST FOR *ESCHERICHIA COLI*

About 1 ml of broth culture was added to 5 ml MacConkey broth containing a Durham's tube and incubated at 37°C for 48 hours. The culture was then examined for acid and gas production. Those positive for acid and gas production were then subjected to the following tests:-

(i) EIJKMAN'S TEST

A volume of 0.1 ml of the positive MacConkey broth culture was added to another 5 ml MacConkey broth containing a Durham's tube. This was incubated at 44°C in a water bath for 24 hours and then examined for acid and gas production. Change in colour of the broth from red to brown was positive of acid production while gas was observed in the Durham's tube.

(ii) LACTOSE FERMENTATION

A 10 ml volume of lactose medium was incubated with the colonies and incubated at 37°C for 24 hours. The medium was observed for a change in colour from pale yellow to magenta red indicating the production of acid from lactose.

2.6.4.2 TEST FOR *PSEUDOMONAS*

A volume (0.1 ml) of the broth culture was inoculated into 10 ml of 0.1% cetrimide broth and incubated at 37°C for 3 days. A loopful of

this culture was used to streak onto the surface of a 0.1% cetrimide agar and further incubated for 48 hours as described by Cowan (1974) and Collins and Lyne (1976). Characteristics colonies from cetrimide agar plates were used to streak onto two nutrient agar plates as described by Skinner and Lovelock (1979). At the end of each incubation period, characteristics colonies from both plates were Gram stained and tested for oxidase reaction as follow.

OXIDASE TEST

Two drops of a 1% aqueous p-aminodimethyl alanine oxalate and 1% α-naphthol in ethanol were dropped on the same spot on filter paper (Skinner and Lovelock, 1979) and smeared with the suspected colony. The spot was observed for the appearance of a deep blue colour which is a positive oxidase reaction.

2.6.5 MYCOLOGICAL EXAMINATION

Simple mycological examination of the fungal mycelia was used for the identification of the organisms.

2.6.5.1 MOULDS

A small portion of a representative mycelium was teased out with forceps and wet mounts prepared using the method described by Bradshaw (1979). A cover slip was carefully placed and the slide examined under the microscope. Characteristic of mycelium for mucor, *Aspergillus*, *Penicillium* and other species isolated were observed with reference to Koneman (1979) and Bryan (1977).

2.6.5.2 YEAST

A little portion of the fungal growth was picked with a sterile loop and smeared in a drop of sterile distilled water on a microscope slide. The film was air-dried, lightly fixed by gentle heating, flooded with 40% ethanol solution for 1 minute and then washed with sterile distilled water. It was again flooded with 0.1 ml potassium hydroxide solution and left aside for 1 hour before washing with sterile distilled water. A drop of 0.1% toluidine blue solution in 10% ethanol was added and allowed to react for 2 minutes. The dye was then washed off with 10% ethanol, air dried and then examined under oil-immersion. Relatively large yeast cells with large central vacuoles were observed.

CHAPTER THREE

RESULTS

The results of microbial load in the various samples obtained are presented in Figures 2-7.

3.1 BACTERIAL CELL COUNT

It was observed that the bacterial viable cell count in the different products differ from one company to another as illustrated in Figures 2-4.

The results obtained for Paracetamol syrups from the three companies that were analysed showed generally that the bacterial load ranged from 1.0×10^2 to 6.5×10^2 cfu/ml for the heat season.

For Company 1, there was a batch range of 1.5×10^2 - 6.5×10^2 cfu/ml Company 2 had a batch range of 2.5×10^2 - 5.0×10^2 cfu/ml while Company 3 had a batch range of 1.0×10^2 - 4.0×10^2 cfu/ml during the harmattan season.

During the heat season however, Company 1 had a batch range of 1.0×10^3 - 5.2×10^3 cfu/ml, Company 2 had a batch range of 3.9×10^3 - 4.7×10^3 cfu/ml and Company 3 had a batch range of 1.5×10^2 - 4.2×10^3 cfu/ml (Figure 2).

The result obtained for Multivitamins syrups are shown in Figure 3. During the harmattan season, only one company's product was available for analysis as most companies products were not in stock at the

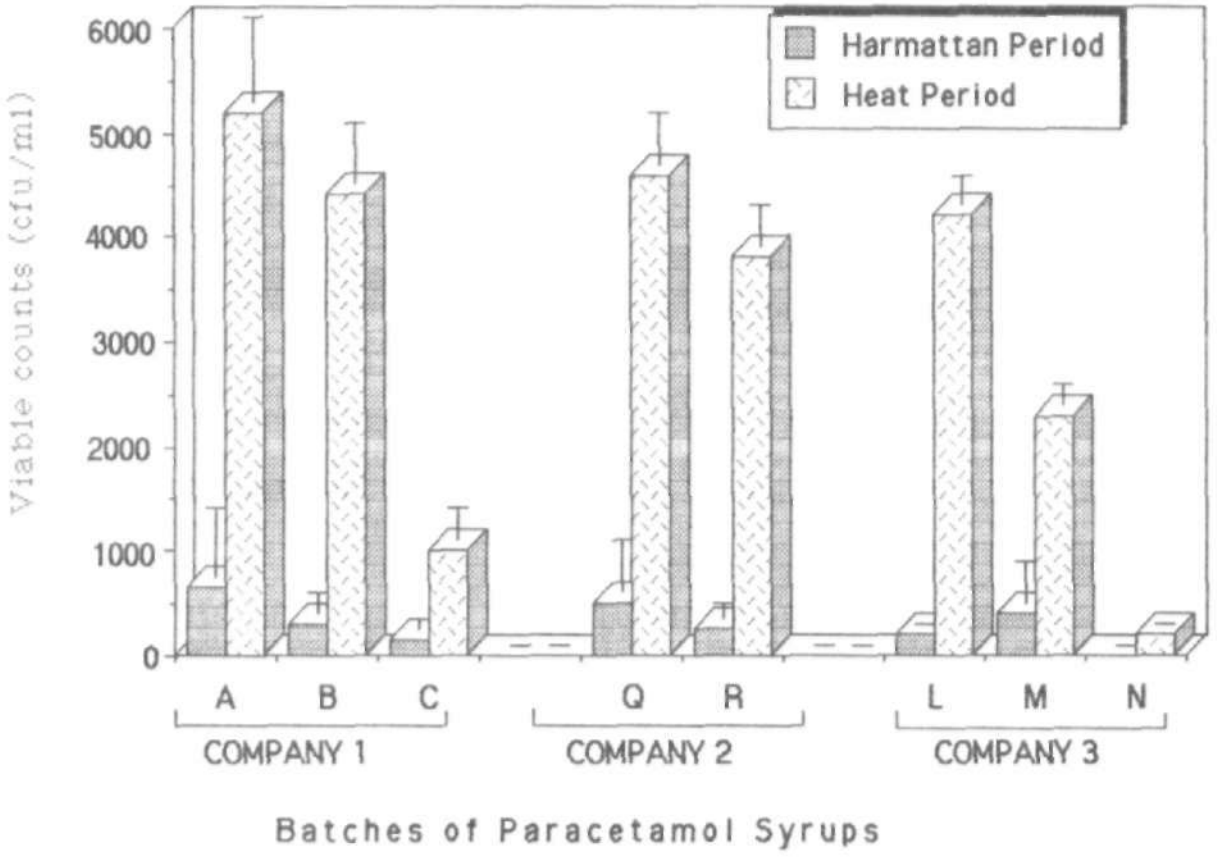


Fig. 2: Bacterial Load of Different Batches of Paracetamol Syrups in Harmattan and Heat Seasons

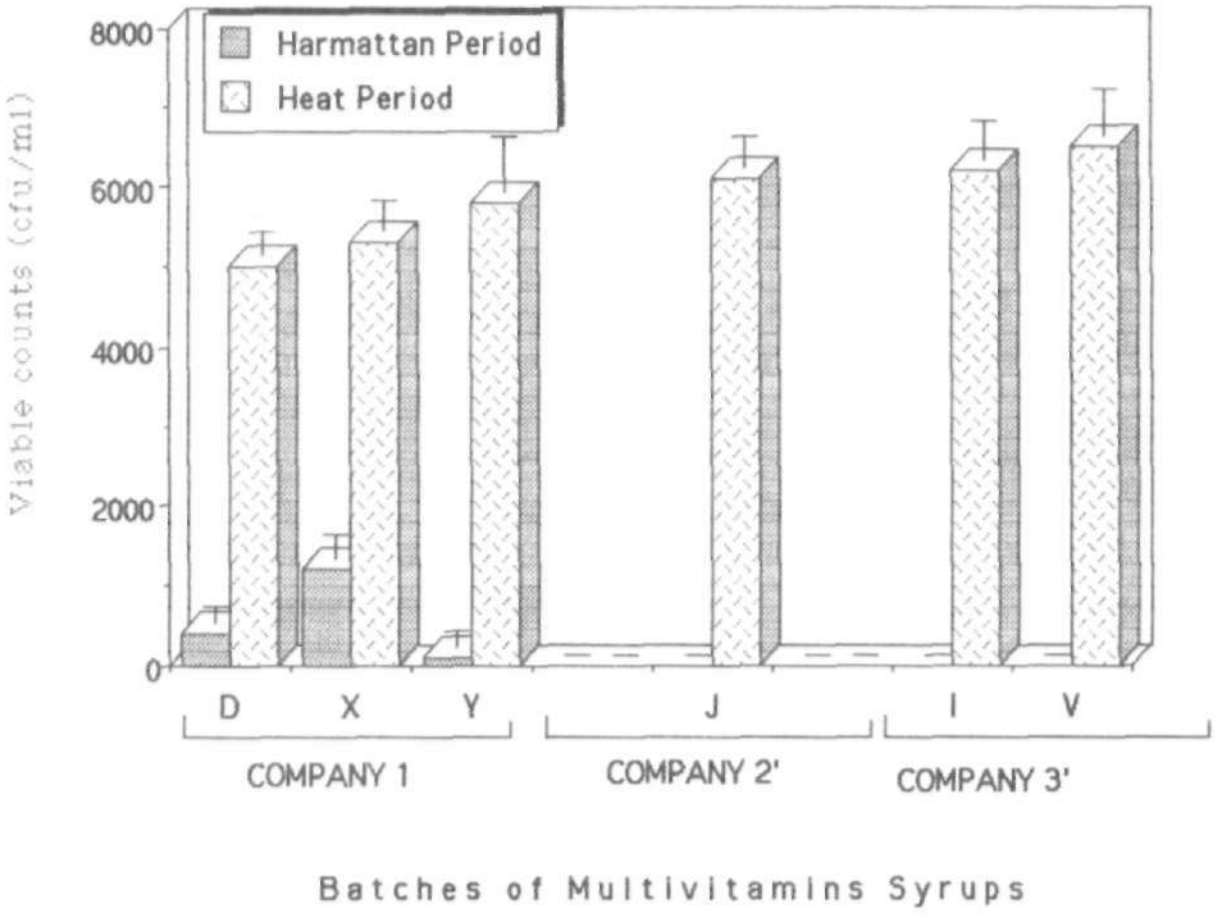


Fig. 3: Bacterial Load of Different Bactches of Multivitamins Syrups in Harmattan and Heat Seasons

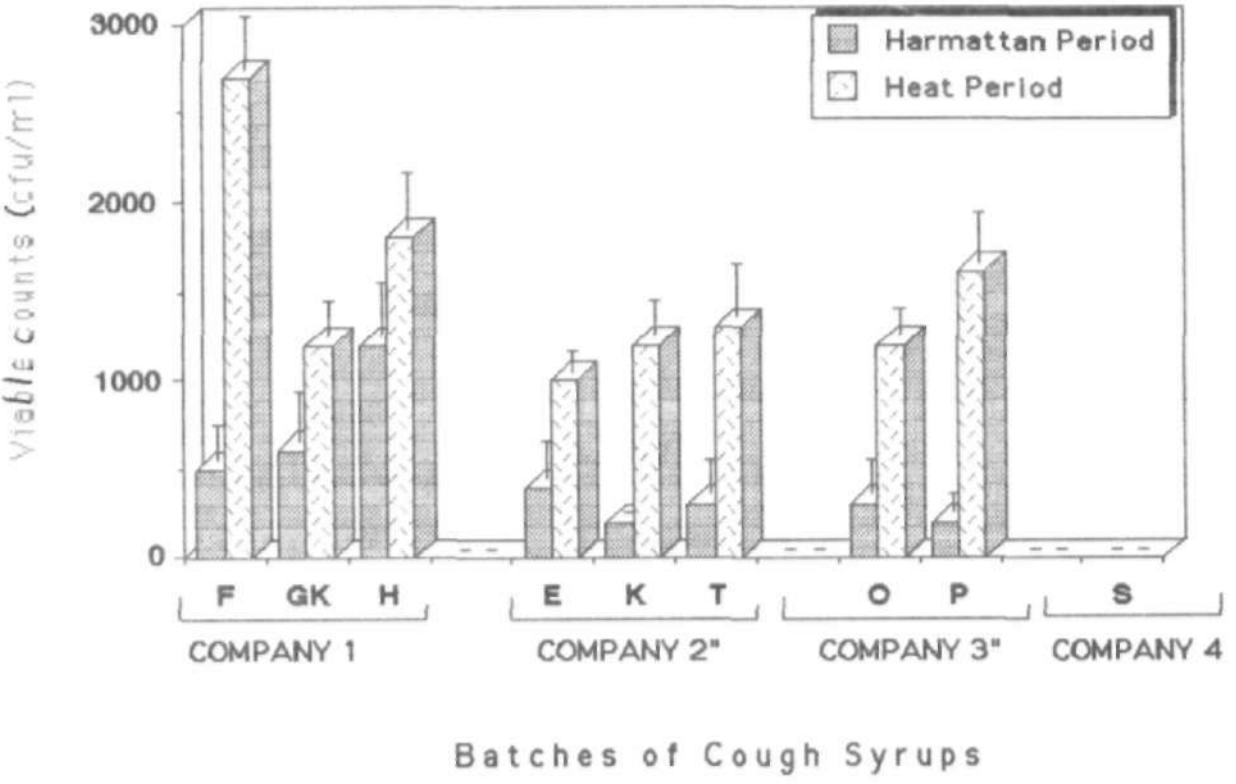


Fig. 4: Bacterial Load in Different Batches of Cough Mixtures in Harmattan and Heat Seasons

designated chemist chosen for the project, hence for the harmattan season, the bacterial load for Company 1 had a range of 3.5 x 10^2 - 6.5 x 10^2 cfu/ml.

For the heat season, three companies products were analysed and a general bacterial load range of 5.0 x 10^3 - 6.5 x 10^3 cfu/ml was obtained. Company 1 had a batch range of 5.0 x 10^3 - 5.7 x 10^3 cfu/ml, Company 2' had only one batch available with a count of 6.2 x 10^3 cfu/ml and Company 3' had a batch range of 6.3 x 10^3 - 6.5 x10^3 cfu/ml (Company 3' had two batches available for analysis).

The result obtained for the Cough mixtures analysed are as shown in Figure 4. For the harmattan season, bacterial load range for Company 1 was 5.0 x 10^2 - 1.2 x 10^3 cfu/ml, Company 2" had a batch range of 2.0 x 10^2 - 4.0 x 10^2 cfu/ml and 3" had a batch range of 1.5 x 10^2 - 2.5 x 10^2 cfu/ml (only two batches of Company 3" were available) and Company 4 showed no bacteria load and only one batch was available.

For the heat season, a general bacterial load range of zero- 2.6 x 10^3 cfu/ml was recorded. Company 1 had a batch range of 8.0 x 10^2 - 2.6 x 10^3. Company 2" had a batch range of 5.0 x 10^2 - 7.5 x 10^2 cfu/ml. Company had a batch range of 7.0 x 10^2 - 1.2 x 10^3 cfu/ml and Company 4 showed no bacterial load.

3.2 FUNGAL COUNT

The results obtained for fungi are presented in Figures 5-7. The fungi/mould count ranged from 0.5 x 10^2 - 7.0 x 10^2 spu/ml for Paracetamol syrup with batch range for Company 1 being 0.5 x 10^2 - 4.5 x 10^2 spu/ml, Company 2 had a batch range of 3.5 x 10^2 - 7.0 x 10^2

spu/ml and Company 3 had a batch range of 2.0×10^2 - 3.0×10^2 spu/ml during the harmattan season. During the heat season, a general fungi/mould count of 0.2×10^2 - 4.5×10^2 spu/ml for Company 2 and 1.0×10^2 - 2.0×10^2 spu/ml for Company 3.

For the Multivitamins syrups, the harmattan season fungi/mould count range of 3.0×10^2 - 7.0×10^2 spu/ml was recorded. Only a company's product was available at the time of sampling, thus the above figure also represent the batch range. However, during the heat season, a general fungi/mould count range of 1.0×10^2 - 5.5×10^2 spu/ml was recorded. Company 1 had a batch range of 2.0×10^2 - 5.0×10^2 spu/ml and Company 2' had only one batch available with a count of 1.5×10^2 spu/ml while Company 3' had a batch range of 1.0×10^2 - 5.5×10^2 spu/ml (Figure 6).

The results obtained for Cough mixtures is shown in Figure 7. During the harmattan season, fungi/mould count generally ranged from 1.0×10^2 - 1.0×10^3 spu/ml. Company 1 had a batch range of 3.0×10^2 - 9.0×10^2 spu/ml, Company 2" had a batch range of 1.0×10^2 - 5.0×10^2 spu/ml while Company 3" had a batch range of 1.0×10^2 - 9.5×10^2 spu/ml but Company 4 with only one batch had a count of 1.0×10^3 spu/ml.

For the heat season, a general fungal/mould range of 0.1×10^2 - 7.5×10^2 spu/ml was recorded with Company 1 having a batch range of 2.0×10^2 - 6.5×10^2 spu/ml while Company 2" had a batch range of 0.5×10^2 - 4.0×10^2 spu/ml, Company 3" with a batch range of 0.1×10^2 - 7.5×10^2 spu/ml and Company 4 has a batch count of 7.5×10^2 spu/ml.

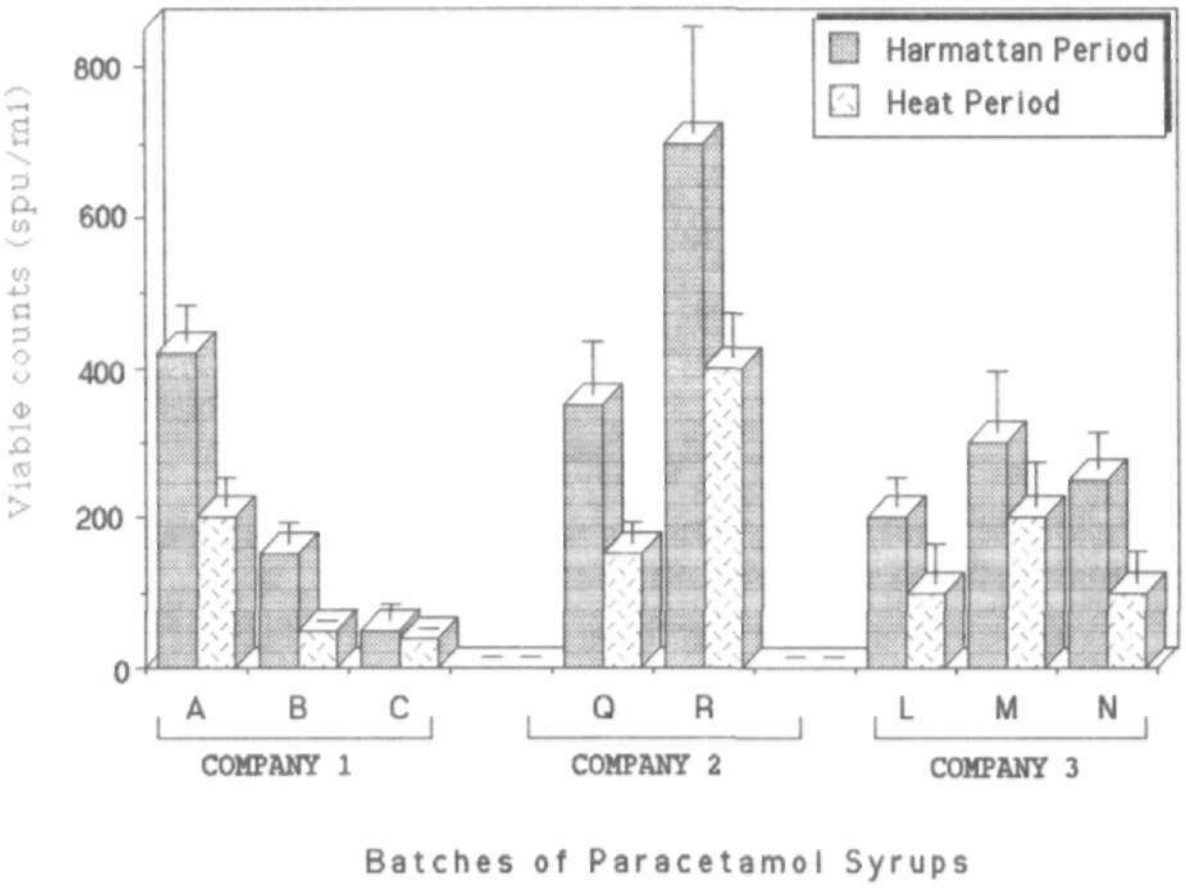


Fig. 5: Fungal/Mould Levels of Different Batches of Paracetamol Syrups in Harmattan and Heat Seasons

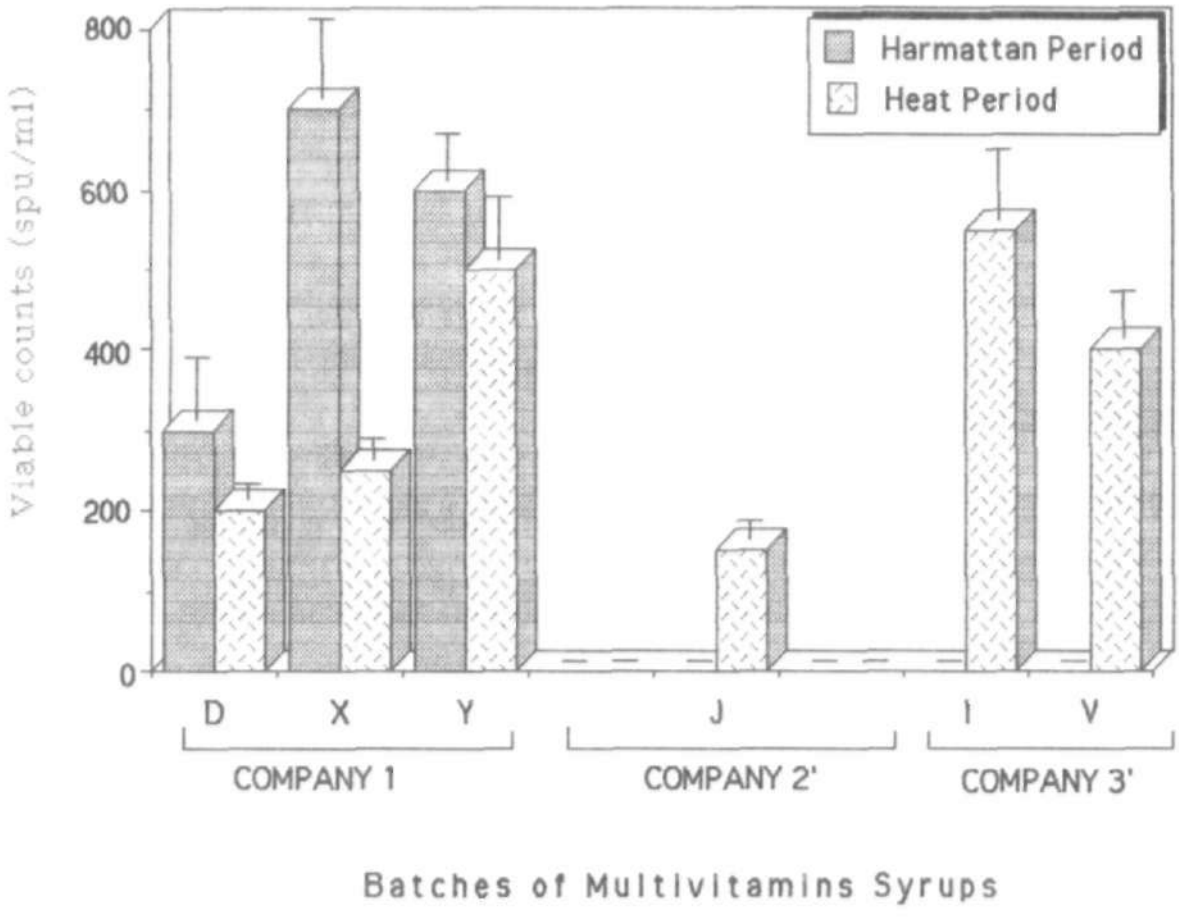


Fig. 6: Fungal/Mould Levels of Different Batches of Multivitamins Syrups in Harmattan and Heat Seasons

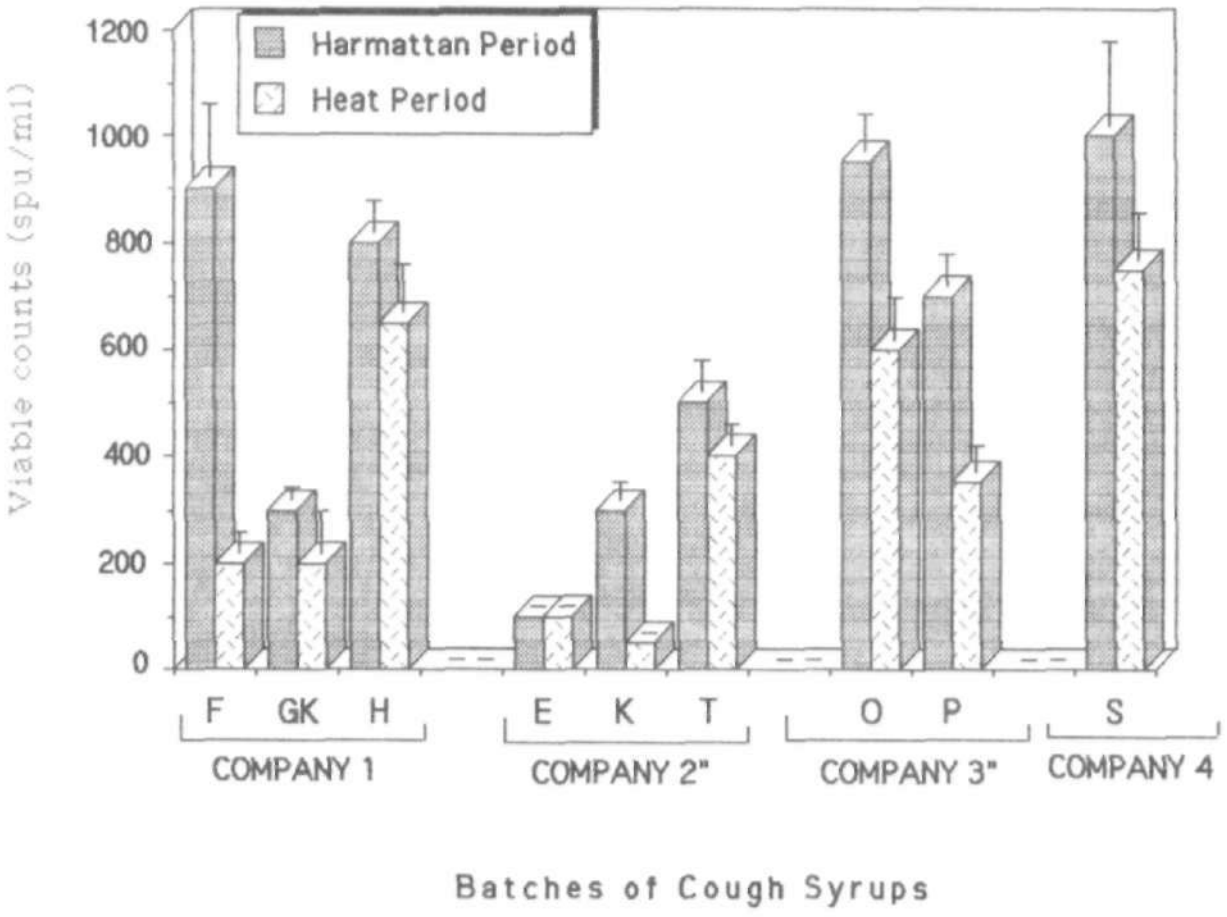


Fig. 7: Fungal/Mould Levels of Different Batches of Cough Syrups in Harmattan and Heat Seasons

3.3 pH READINGS

The pH values recorded at the beginning and at the end of the analysis period are shown in Table 3. For measurements, a bottle from each batch was used to record the pH values at the beginning of harmattan season and kept separately throughout the period of analysis and at the end of the heat season, these bottles were used to measure the pH values recorded as at the end of analysis period (Table 3).

Table 3: The Mean pH values of Samples Analysed

Product	Batch	pH-reading at the beginning (Harmattan season)	pH-reading at the end (Heat season)
Paracetamol			
Company 1	A	5.16	6.50
	B	5.26	6.20
	C	5.16	6.41
Company 2	Q	6.74	6.40
	R	5.20	5.86
Company 3	L	5.48	6.00
	M	5.57	6.10
	N	6.01	6.50
Cough Mixtures			
Company 1	F	3.91	4.80
	GK	3.80	5.71
	H	3.82	4.83
Company 2"	E	5.31	6.52
	K	5.25	6.34
	T	5.23	6.32
Company 3"	O	6.81	8.60
	P	6.71	8.57
Company 4	S	5.66	5.91
Multivitamins			
*Company 1	D	4.06	4.40
	X	4.08	4.50
	V	4.02	4.42
Company 2'	J	3.80	3.90
Company 3'	I	4.70	5.51
	Y	3.60	3.80

* For Multivitamins syrups, only Company 1 was available for analysis during the harmattan season but other two companies were analysed during the heat season hence the pH values shown indicate the pH reading recorded at the beginning and end of their analysis period.

3.4 IDENTIFICATION OF THE CONTAMINANTS

In the harmattan season, the bacteria species found in Paracetamol syrup include the milky to brown coloured colonies which on staining showed clusters of spherically shaped cells which were gram positive that are likely to be *Staph. aureus*. A few rod shaped organisms that stained gram-negative *E. coli* were also observed.

In Multivitamins syrups however, few cream coloured, mostly joined rods were observed likely to be *Bacillus* species.

In the Cough mixtures, majority of the colonies observed were the spore-forming bacillus species and a few *Staphylococcus* species.

During the heat season, bacteria found in Paracetamol syrups were the single short rods that are likely to be *E. coli* while in the Multivitamins syrup, bacteria colonies that range from yellow to orange colours which on staining showed clusters of spheres that are likely to be *Staphylococcus* species. In the Cough mixtures, clusters of rod shaped pink colonies likely *E. coli* that were confirmed by culturing on MacConkey agar and specific tests carried out as outlined in 2.6.4 (See also Table 4).

The fungi/mould identified during the harmattan season in Paracetamol syrup were *Penicillium* species. In Multivitamins syrups, a lot of different fungi/mould were observed. These include *Aspergillus niger*, *Cladosporium* species, *Rhodotorulla* species as well as *Mucor*. In Cough mixture, fungi/mould such as *Penicillium* species, *Aspergillus flavus*, and yeast were observed, whereas during the heat season, fungi/mould found in Paracetamol syrup include *Aspergillus* species. In Multivitamins syrup, *Curvularia*, *Penicillium* and *Aspergillus* species

were found, but mucor, *Ascomycetes* and *Cladosporium bautianum* were identified in the Cough mixtures.

3.4.1 COMPANY VARIATION

In comparing the companies for each product, Company 1's products showed the highest level of contamination in all products analysed with both Paracetamol syrup and Cough mixture products showing significant bacteria count as well as the presence of pathogenic *E. coli* as major contaminant. Also, some fungi/mould contaminants such as yeast, *Rhodotorulla* species as well as *Curvularia* species were found to be present in Company 1's samples. Therefore, the order of degree of contamination for the companies sampled is as shown for Paracetamol syrups:-

Company 1 > Company 2 > Company 3.

For Multivitamins syrups:-

Company 3' > Company 2' > Company 1.

These results are however based on the results obtained during the heat season as only Company 1's products were available for analysis during the harmattan season.

For Cough mixtures:-

Company 1 > Company 2" > Company 3" > Company 4.

Company 4 showed no contamination in form of bacteria but had the highest level of fungi/mould count.

Table 4: Microscopic and Colonial Morphology of Some identified Organisms

Colonial Morphology (Size is that of diameter expressed in mm)	Microscopical Character (staining)	Pathogens Identified
Bright yellow, circular, convex, entire, slightly translucent, smooth and shinning colony of size 1-2 mm	Gram positive cocci in clusters	*Staph. aureus*
Dark orange, circular, raised, entire, opaque, smooth and shinning colony of size 2-3 mm	Gram positive cocci in scattered groups	
Creamy white, circular, convex, entire, opaque, smooth and shinning colony of size 2-3 mm	Gram positive cocci mostly in clusters, few in chains	*Staph. aureus*
Faint pink, circular, raised, entire, opaque, smooth and shinning colony of size 2-2.5 mm	Gram positive cocci mostly single and in small groups	
Milky white, circular, convex, entire, smooth and shinning opaque colony of size 1 mm	Gram positive rods in diplo and in small groups	*Bacillus subtilis*
Light yellow, circular, convex, entire, smooth, shinning translucent colony of size 1 mm	Gram negative short rods in chains	*E. coli*

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 DISCUSSION

The products analysed i.e. Paracetamol, Multivitamins and Cough syrups are considered on the basis that they are oral liquid forms classified under the same standard as for non-compulsory non-sterile preparations. Thus, these samples analysed fall within the bacterial count range given by the official books. The British Pharmaceutical Codex 1979 records an acceptable bacterial count of 10^3-10^4 cells/g or ml as standard for aerobic bacteria, 10^2 cells/g or ml for mould/yeast in non-sterile pharmaceutical preparations with complete absence of some *Enterobacteriacese* like *E. coli*, *Shigella*, *Ps. aeruginosa* and *Staph. aureus*.

The highest count observed is 6.5 x 10^3 cfu/ml, but samples did not comply with the mould count of 10^2 cells/g or ml as the highest count observed was 7.0 x 10^2 spu/ml. However, some samples showed presence of some specific organisms such as *E. coli* and *Staph. aureus* which are meant to be completely absent according to the British Pharmaceutical Codex 1979 specifications.

The basic comparative discussion on these products are the effect of seasonal studies carried out and possibly the different companies from which they were manufactured and pH changes that were recorded. Generally, some of the micro-organisms found in these samples include for Paracetamol syrups mainly *Bacillus* species with centrally placed

spores; in Multivitamins syrups, mould and yeast and some *Bacillus* species while in Cough mixtures, *Staph. aureus*, *Bacillus* species and a lot of mould were isolated. Specific organisms include *Bacillus subtilis*, *Staph. aureus*, *E. coli*, *Cladosporium bautianum*, *Cladosporium wernikii*, *Penicillium* species, *Curvularia* species, *Rhodotorulla* species as well as *Ascomycetes* species.

Seasonally as indicated in the histograms, there were higher bacteria counts during the heat season for all the samples with counts as high as 5.15×10^3 cfu/ml for Paracetamol syrups, while the highest count during the harmattan season was 6.5×10^2 cfu/ml (Figure 2). This shows that increase in temperature increases the rate of proliferation of these organisms (temperature range recorded during the harmattan season was 10-17°C). There was batch difference in the bacterial count among products from the same company e.g. batches A, B and C from Company 1 showed viable counts of 5.15×10^3, 4.4×10^3 and 1.0×10^3 cfu/ml respectively for the heat season and 6.5×10^2, 3.0×10^2 and 1.5×10^2 cfu/ml respectively during the harmattan season. This may be due to the manufacturing process as well as the personnel and environmental conditions available at the time of manufacture. Also, the cleanliness of the equipment used as well as the microbial count of the starting raw materials.

It is worth noting that in Figure 2, Company 3 with batches L, M and N, during the harmattan season, the bacterial viable count recorded was 2.0×10^2 cfu/ml and 4.0×10^2 cfu/ml for L and M respectively while batch N had no bacterial count at all, but during the heat season, the viable count recorded was as high as 4.2×10^3, $2.25 \times 10^3$and 0.5×10^2 cfu/ml respectively for batches L, M and N. This result obtained for batch N could be explained in terms of the fact that these organisms

were in their dormant phase and the increase in temperature probably energised them and sensitise their growth process and this was confirmed since the isolated organisms were identified to be mainly *Bacillus* species with centrally placed spores.

For Multivitamins syrups, during the harmattan season, only Company 1's products were available and as shown in Figure 2, there was considerable increase in viable count of 5.7×10^3 cfu/ml during the heat season as compared to 6.5×10^2 cfu/ml during the harmattan season. There was batch to batch differences observed.

For Cough mixtures, the bacterial count for the two seasons considered were close, during harmattan season, highest bacterial viable count was 1.2×10^3 cfu/ml while 2.5×10^3 cfu/ml was recorded during the heat season. But it was clearly observed that there was great variation between the different batches as shown in Figure 4, where Company 1 products (batches F, GK, and H with bacterial count for harmattan season of 5.0×10^2, 5.5×10^2 and 1.2×10^3 cfu/ml respectively but had counts of 2.5×10^3, 8.0×10^2 and 1.8×10^3 cfu/ml respectively during the heat season). It can be seen that the effect of changes in temperature on storage can adversely affect products and that the rate of proliferation of different types of organisms differs and that change in temperature does not necessarily increase the viable count astronomically.

The fungi count obtained shows that change in seasons i.e. from harmattan to heat (corresponding to change in temperature) lowered the mould count of most products but certain species that were not isolated during the harmattan season were isolated during the heat season e.g. *Mucor*, *Ascomycetes* whereas fungi species such as *Curvularia*, yeast and

Rhodotorulla species were isolated in the harmattan season. This could be due to the change in temperature as some fungi require higher temperature to effect their growth. Others that were isolated during the two season are *Aspergillus*, *Cladosporium* and *Penicillium* species.

From the pH values recorded, there was the shifting of pH from the acidic level to alkaline level. There was also corresponding increase in bacterial load in the products, this is not surprising since acidic pH is known to be bactericidal but the change in pH values could also be due to the break down of the constituents of these products resulting in the metabolites causing the products to shift pH to the alkaline level. Also, this could be due to the effect of the packaging material which is mainly plain coloured glass bottles and these glass containers could also leach some alkaline substances into the product. There could also be adsorption as well as absorption of the constituents of these products that can lead to the change in pH as well as change the microbial load of the products. The change in pH from acidic level to alkaline level also affected the growth of mould/yeast pattern as evident in the two seasons, acidic conditions favours their growth while alkaline condition does not.

Some companies also use recycled containers and this pose great dangers to products in terms of microbial contamination considering the fact that most of these containers are usually not properly cleansed, sterilised before re-use, and they might be harbouring some contaminants from the previous use.

The optimum pH range for bacterial growth is 6.8-7.2, but there are some acidophilic organisms which grow even at pH values of 3, this was demonstrated in some of the products analysed as shown in Table 3.

Multivitamins syrup from Company 3' had pH value at the beginning of analysis of 3.6 and 3.80 for batches V and I respectively and only a slight increase at the end of analysis to 3.80 and 3.90 respectively.

The different companies from which samples analysed were obtained are from southern part of the country where the environmental conditions such as low temperature, high humidity etc. are quite different from the northern environmental factors such as high temperature, low humidity, dusty and windy harmattan periods. Also, the different personnel, hygienic conditions available during the manufacturing processes differs for the North and South. The above are not the only contributing factors, also the sources of raw materials, the handling and processes through which these materials undergo differs from company to company. The formulation designs, the different constituents and the equipment as well as in-process testing of these products are subjected to differs widely.

4.2 CONCLUSION

There is a general agreement on the need to limit the types and overall number of microbes in non-sterile medicaments to reduce infectious or spoilage risk. Also, there is no proper agreement as to what constitute an acceptable microbial content for various medicinal products or whether the type of contaminant is more important than its quantity. Although a sensible formulator will endeavour to design a medicine to be as unfavourable to microbial survival as possible, the only reliable way of confirming success is to confront the new complete formulation with experimental realistic microbial challenges. The use of accelerated testing procedures with extreme temperature, pH etc., is not

generally applicable to microbiological testing. However, temperature cycling (i.e. intermittent storage at two or more temperature) may detect localised weak spots in the microbial resistance of some formulations especially those which form dilute moisture film on the surface of a product.

We know that products with a high viable counts have been used satisfactorily for many years, but at the same time, there should be some control on the total bacterial count not only to prevent spoilage of the products but also to avoid further injury to the patient.

Event of recent years have clearly demonstrated that some microbiological control is necessary not only on sterile products for parenteral and ophthalmic use but also on all pharmaceuticals for oral or topical administration.

Investigation carried out by earlier workers (Report, 1971; Sykes, 1969; Fischer *et. al.*, 1968) in various parts of the world have clearly shown that certain types of preparations are more susceptible to microbial contamination than are others and it is these products that need the most attention. Also, total bacteria and mould count gives some indication of the cleanliness with which a product has been manufactured and of its degree of preservation and this information is useful but not conclusive. Most importantly is the need to check against the presence of pathogenic types of bacteria which even in small number can sometimes be quite dangerous. Good quality raw materials as well as good house keeping and manufacturing processes are most essential.

In considering what microbiological standard to be applied to pharmaceutical preparations, three aspects need to be borne in mind:-

1. the numbers of micro-organisms ingested daily from food, and the numbers picked up on the skin during day to day activities;

2. the hazard from pathogenic organisms sometimes in only small number;

3. the physiological state of the patient bearing in mind that if he or she is under medication, his or her resistance to infection may be less then that of normal patient.

To attempt to eliminate microbial contamination completely i.e. to make all preparations sterile (if this were possible) would be both unnecessary and excessively expensive for these reasons:-

a It is well recognised that the types of bacteria present are far more important than the total number

b It is very difficult to determine actual counts with some preparations especially powders and viscous products such as creams and emulsions because bacteria growth is almost certain to be concentrated in or near the surface of these materials and will not be evenly distributed.

Some possible standards have been suggested by Sykes (1969) that for oral preparations, the standard need not be as high as those for parenteral, ophthalmic as well as for surgical applications. Some tablets can be made with counts less than 10 bacteria/g but others may contain 10^3 bacteria/g or more and some liquid preparations may have similar high counts. There is no point in specifying low counts for certain types of oral preparation and allowing much higher counts for others hence it is essential that there should be one standard throughout.

For the samples analysed, it was not possible to obtain the composition used in their formulation as this was not indicated on the packaging, hence it was not ascertained if preservatives were included or not but to neutralise (inactivate) the effect of any preservative that may be present, 3% Tween 80 was added to the media used and since the objective of this study is not to verify the presence and efficacy of preservatives but the microbial quality of the products as at the time of purchase by final consumer, the samples in which no bacteria were isolated does not mean that the products were sterile but that probably, the manufacturers took care during manufacturing processes.

Thus it can be concluded from this study that even at the time of purchase and subsequent usage of some pharmaceutical products by consumers, there is the presence of micro-organisms and that the level differs from one product to the other, from one batch to another within a company as well as from one company to another. Therefore, it is suggested that:

i Pharmaceutical companies should try as much as possible to maintain Good Manufacturing Practice (GMP) for all their products irrespective of the route of administration.

ii Each raw material should be tested and purified accordingly before use.

iii The environment, equipment as well as the personnel involved in the manufacturing processes, be in the highest hygienic condi tions at all times during and after manufacturing processes.

iv If possible, all products be terminally sterilised to avoid re-contamination.

The inclusion of preservatives should not be taken for granted and final tests for quality assurance be conducted for all products before sending them into the market.

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