MICROBIOLOGY OF TRADITIONAL FERMENTED MILK PRODUCT (ROUB)

By

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DEDICATION

To my father and mother

To my dear small family wife and son

To all people in my heart
All grateful to Allah for the assistance, health, patience and will to accomplish this work.

I would like to express my gratitude and thanks to my supervisor Dr. Mohamed Osman Mohamed Abdalla for his supervision, help and proper guidance during all the stages of this research.

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MICROBIOLOGY OF TRADITIONAL FERMENTED MILK PRODUCT
(ROUB)
Salah Ibrahim Khider Hussain
Degree of Master of Science in Tropical Animal Production

ABSTRACT

This investigation was carried out to evaluate the microbial load of a Sudanese fermented dairy product, locally fermented (Roub) from different areas in Sudan.

Roub samples were collected from Nyala (South Darfur State), El Obied (North Kordofan State) and Abu Naama (Blue Nile State) and transported to the Faculty of Animal Production in sterile disposable plastic containers in ice box at 4°C. The samples were subjected to microbiological examination (total viable bacteria, *Staphylococcus aureus*, *Salmonella*, lactic acid bacteria and yeasts and moulds).

The results indicated the present *E. coli*, *Staphylococcus aureus*, lactic acid bacteria, yeasts and moulds in the Roub sample from Nyala, El Obied and Abu Naama, while *Salmonella* sp. was not detected in all samples tested. *E. coli* was not detected in all samples collected from Abu Naama area. Total viable bacteria were detected in all samples (100%), while *E. coli* was detected in 30% of the samples from El Obied, 40% from Nyala and was not detected in samples from Abu Naama area.

*Staphylococcus aureus* was detected in 40%, 60% and 40% of samples from El Obied, Nyala and Abu Naama, respectively while lactic acid bacteria were detected in all samples tested. Yeasts and moulds were detected in 100%, 90% and 90% of samples from El Obied, Nyala and Abu Naama areas, respectively.
كاز راشد. لفتا على العناصر المذكورة في درجة حرارة 40 درجة مئوية. استخدمت الأدوات والمستلزمات اللازمة لتحليل العينات. حيث تم استخراج الـ60% من العينات من منطقة حضرموت، والـ40% من منطقة عدن، والـ30% من منطقة مأرب.

(أو أي ملاحظات أخرى حسب الحاجة)
CHAPTER ONE
INTRODUCTION

Preservation of food is one of the oldest methods known to mankind. A typical example is the lactic acid fermentation which is used in the manufacture of fermented dairy products, like cheese and fermented milks (Robinson, 1985).

The nature of fermented products is different from one region to another. Thus is depending on the local indigenous microflora, which in turn reflected the climatic conditions of the area. Thus traditional fermented milk in regions with cold climate contained mesophilic bacteria such as Leuconostoc spp., whilst thermophilic bacteria, which include mostly Lactobacillus and Streptococcus, prevailed in regions with a hot, subtropical or tropical climate (Thomas, 1985; Tamai et al., 1988 and Kurmann, 1994).

"Roub" is the most important indigenous fermented milk product of considerable economic and dietary importance of the people of Sudan especially in the west and the east of Sudan (Abdel Gadir et al., 1998).

In Sudan favorite sour milk "Roub" is made from fresh raw cow's milk and to some extent goat's and sheep's. However, camel's milk is still the milk of choice for some people in Sudan. Since the existence of the art of the traditional manufacture of "Roub" in Sudan until present, the whole process depends entirely on natural fermentation of milk. This kind of fermentation process is the result of the presence of microorganisms and their enzymes in milk. In the dairy industry these microbes are known as "starter cultures". Attention is still going to be focused on the need of the dairy industry for new isolates of lactic acid bacteria as yet unused for dairy fermentations (Abdel Gadir et al., 1998).
Environmental conditions in each country affect the properties of the predominant native microflora and this limits the use of some universal starters. A rational solution for this problem might be to select starter cultures among the native flora that could be used successfully in the dairy industry.

The objectives of this study are:

1) To determine the bacterial load of traditionally made Roub collected from different areas.
2) To isolate some lactic acid bacteria from “Roub”.
3) Detect of some pathogenic bacteria from “Roub”.

CHAPTER TWO
LITERATURE REVIEW

2.1 Fermentation and fermented milk products:

2.1.1 Fermentation:

Definition of fermentation:

Campbell-Platt (1987) defined fermented foods as those foods which have been subjected to the action of microorganisms or their enzymes so that desirable biochemical changes cause significant modification to the food. However, to the microbiologist, the term “fermentation” describes a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is incompletely oxidised, and an organic carbohydrate acts as the electron acceptor (Adams, 1990). This definition means that processes involving ethanol production by yeasts or organic acids by lactic acid bacteria are considered as fermentations, but not the production of fish sauces in Southeast Asia, that still has not been shown to have a significant role for microorganisms, and not the tempe production since the metabolism of the fungi is not fermentative (Adams, 1990). Whichever definition is used, foods submitted to the influence of lactic acid producing microorganisms are considered fermented foods.

2.1.2 Origin of fermented milk:

Fermented milk was originated in the Near-East, perhaps before the Phoenician era, and spread through central and Eastern Europe. The earliest example of fermented milk was warm, raw milk from cow’s, sheep's, goat’s, camel’s or horse’s of the nomads roaming the area which was turned into clabber, or curd by bacteria and their end products (Abdel Gadir et al., 1998).
2.1.3 The fermentation of milk:

The term was first applied when the only known reaction of this kind was the production of wine. The early fermentation concepts stated that fermentation was strictly a chemical aspect of the process that the meaning was changed to signify the breakdown of sugars into ethanol and CO₂ (Abdel Gadir et al., 1998). Between 1856 and 1875, Louis Pasteur showed conclusively that fermentation was a result of microbial action and each type of fermentation was accompanied by the growth of a particular type of micro-organism (Kosikowski, 1982). Louis Pasteur also marked the birth of chemical microbiology with his association of microbes with the fermentation in 1857. The term "fermentation" thus became associated with the idea of cells, gas production and production of organic by-products (Abdel Gadir et al., 1998).

The evolution of gas and presence of whole cells were invalidated as criteria for defining fermentation when it was discovered that in some fermentation such as the production of lactic acid no gas is liberated, while the fermentation processes could be obtained with cell-free extracts, indicating that the whole cell may not be necessary (Abdel Gadir et al., 1998). Fermentation is clearly needed to be redefined when the situation was further complicated by the discovery that the ancient process of vinegar production, which yielded considerable quantities of organic by-products was a strictly aerobic process. Fermentation came to be regarded as the anaerobic decomposition of organic compounds to products which could not be further metabolized by the enzyme systems of cells without intervention of oxygen.

The fermentation products differed with different microorganisms, being mainly governed by the enzyme complex of cells and the
environmental conditions. The economic values of these by-products lead to the development of industrial microbiology (Abdel Gadir et al., 1998).

2.1.4 Types of fermented milks:

A characteristic common feature to all milks is the presence of lactic acid, for example cultured buttermilk, yoghurt, acidophilus and Bulgarian milks. Other fermented milks are alcoholic in addition to acidic, for example, Kefir and Koumiss. The term "cultured" is frequently substituted for "fermented" in buttermilk, yoghurt and sour cream because, commercially, these foods were among the first to be produced on a large scale by pure bacterial cultures (Kosikowski, 1982).

2.2 Health aspects of fermented foods:

2.2.1 Probiotic effect:

One of the reasons for the increasing interest in fermented foods is its ability to promote the functions of the human digestive system in a number of positive ways (Sahlin, 1999). This particular contribution is called probiotic effect. Already early in 1900, Metchnikoff pointed out the use of fermented milks in the diet for prevention of certain diseases of the gastrointestinal tract and promotion of healthy day to day life. Since then a number of studies have now shown that the fermented food products have a positive effect on health status in many ways. The human intestinal microbial flora is estimated to weigh about 1000 grams and may contain $10^{16} - 10^{17}$ colony forming units (cfu) representing more than 500 strains. For physiological purposes, it can be considered to be a specialized organ of the body with a wide variety of functions in nutrition, immunology and metabolism (Gustafsson, 1983). Studies on mice have shown that the indigenous microorganisms in the stomach are Lactobacillus, Streptococcus and Torulopsis, while in the small intestine, ceacum and colon several
different species (*Bacteroides, Fusobacterium, Eubacterium, Clostridium*, etc.) coexist (Savage, 1983). The gastrointestinal microflora in humans is also known to contain hundreds of species. Even though there is a wide variation among individuals, the number of species and size of the population are usually kept stable in normal healthy subjects. There is a constant struggle in maintaining the desirable balance and a dynamic equilibrium between microbial populations within the intestinal flora (Robinson and Samona, 1992). The anaerobic organisms, which outnumber the Gram negative enteric bacteria by about 10000:1, are associated with the intestinal epithelium limiting adherence of potential pathogens by effective colonization (Van der Waaij *et al.*, 1972; Nord and Kager, 1984; Swank and Dietch, 1996). The stability of the intestinal microflora is affected by many factors including dietary habits. Decrease in the number of anerobic bacteria is associated with increase in the number of Gram negative pathogens in the intestinal tract and their translocation to extraintestinal tissues. Under normal conditions the intestinal wall prevents translocation of organisms both dead and live as well as microbial products like toxins from the gut to the blood. However, in patients with systemic insult like starvation, shock, injury and infection or specific insult of the gastrointestinal canal through inflammation, chemotherapy or radiation, the gut mucosal permeability will be increased leading to translocation of microbes (Alexander *et al.*, 1990; Wells, 1990 and Kasravi *et al.*, 1997). A fermented food product or live microbial food supplement which has beneficial effects on the host by improving intestinal microbial balance is generally understood to have a probiotic effect (Fuller, 1989).
2.2.2 Flatulence reducing effect:

During fermentation of the beans for preparation of tempe, the trypsin inhibitor is inactivated, and the amount of several oligosacharides which usually cause flatulence are significantly reduced (Hesseltine and Wang, 1983). Bean flour inoculated with *Lactobacillus* and fermented with 20% moisture content, showed a reduction of the stachyose content (Duszkiewicz-Reinhard *et al.*, 1994).

2.2.3 Anticholesterolemic effect:

Hepner *et al.* (1979) reported hypercholesteremic effect of yoghurt in human subjects receiving a one-week dietary supplement. Studies on supplementation of infant formula with *Lactobacillus. acidophilus* showed that the serum cholesterol in infants was reduced from 147 mg/ml to 119 mg/100 ml (Harrison and Peat, 1975). In an in vitro study, the ability of 23 strains of lactic acid bacteria isolated from various fermented milk products to bind cholesterol was investigated, and no cholesterol was found inside the cells (Taranto *et al.*, 1997). Poppel and Schaafsma (1996) have also reported the ability of yoghurt to lower the cholesterol in serum by controlled human trials. Possible role of lactic acid bacteria in lowering cholesterol concentration and various mechanisms by which it may be possible has been discussed by Haberer *et al.* (1997). Brigidi *et al* (1993) have cloned a gene encoding cholesterol oxidase from *Streptomycin lividans* into *Bacillus, Lactobacillus* and *E. coli*.

2.2.4 Effect on transit time, bowel function and glycemic index:

The transit time for 50% (t50) of the gastric content was significantly reduced for regular unfermented milk (42±10 min) in comparison with a fermented milk product indigenous to Sweden called "långfil" or ropy milk (62±14 min). Another study reported increase in transport time and
improved bowel function in patients with habitual constipation (Wilhelm, 1993). The number of defeacations per week increased from three during control period to seven using conventional fermented milk and fifteen when acidophilus milk was served. Regular unfermented milk also gave significantly higher increase in glycemic index curve than fermented milk product called långfil (Strandhagen et al., 1994). Liljeberg et al., (1995) showed that the presence of acid, specially acetic or lactic, lowered the glycemic index in breads to a significant level. Koji which is prepared from Aspergillus oryzae and beni-koji made from Monascus pilosus were found to express rises in blood pressure (Tsuji et al., 1992).

2.2.5 Anticarcinogenic effect:

There are interesting data on anticarcinogenic effect of fermented foods showing potential role of lactobacilli in reducing or eliminating procarcinogens and carcinogens in the alimentary canal (Reddy et al., 1983; Shahani, 1983; Mital and Garg, 1995). The enzymes β-glucuronidase, azoreductase and nitroreductase “which are present in the intestinal canal” are known to convert procarcinogens to carcinogens (Goldin and Gorbach, 1984). Oral administration of Lb. rhamnosus GG was shown to lower the faecal concentration of β-glucuronidase in humans (Salminen and Gorbach, 1993) implying a decrease in the conversion of procarcinogens to carcinogens. Fermented milk containing Lactobacillus acidophilus given together with fried meat patties significantly lowered the excretion of mutagenic substances compared to ordinary fermented milk with Lactococcus fed together with fried meat patties (Lidbeck et al., 1992). The processes of fermentation of foods are also reported to reduce the mutagenicity of foods by degrading the mutagenic substances during the process. Lactic acid bacteria isolated from Dahi (traditional Indonesian
fermented milk), were found to be able to bind mutagens and inhibit mutagenic nitrosamines (Harun-ur-Rashid et al., 2007). Milk fermented with *Lactobacillus acidophilus* LA-2 was demonstrated to suppress faecal mutagenic activity in the human intestine (Sahlin, 1999). Studies on antimutagenic activity of milk fermented with mixed-cultures of various lactic acid bacteria and yeasts showed that, the fermented milk products with mixed cultures of lactic acid bacteria had a wider range of activity against mutagens than those produced with a single strain of lactic acid bacteria (Tamai et al., 1995). However, a review by McIntosh (1996) concluded that there is only limited data to support the hypothesis that probiotic bacteria are effective in cancer prevention. On the other hand, a study by Hosono and Hisamatsu (1995) on the ability of the probiotic bacteria to bind carcinogenic substances have reported that *E. faecalis* was able to bind aflatoxins B1, B2, G1 and G2 as well as some pyrolytic products of tryptophan.

**2.2.6 Immunoactive effects:**

Some lactic acid bacteria present in fermented milk products, are found to play an important role in the immune system of the host after colonization in the gut (De Simone, 1986). Oral administration of *Lactobacillus casei* caused an improvement of the function of the peritoneal macrophages and increased the production of IgA (Sato et al., 1988). The mechanism of this effect is not clearly known, but it is speculated that the lactobacilli, their enzymes or the metabolic products present in the fermented food product may act as antigens, activating production of antibodies. Marin et al. (1997) studied the influence of lactobacilli used in fermented dairy products on the production of cytokines by macrophages. The results indicated that for most strains, direct interaction with
macrophages caused a concentration dependent increase in tumour necrosis factor and interleukin. A study by Perdigon et al. (1995) showed that, *Lactobacillus casei* could prevent enteric infections and stimulate the secretion of IgA in malnourished animals but also translocate bacteria, while yoghurt could inhibit growth of intestinal carcinoma through increased activity of IgA, T- cells and macrophages. In a review by Marteau and Rambaud (1993), the authors concluded that there is a potential for using lactic acid bacteria for therapy and immunomodulation in mucosal diseases, especially in the gastrointestinal tract. Isolauri (1996) presented a study suggesting that *Lactobacillus spp.* strain GG could be used in the prevention of food allergy. It is suggested that dietary antigens induce immunoinflammatory response that impairs the intestine’s barrier function and that probiotic organisms could be a mean of introducing a tool to reinforce the barrier effect of the gut.

2.2.7 Important health properties of fermented milk:

Sometime between 1898 and 1908 a good deal of controversy existed over the special health-giving properties of fermented milk foods. Extremists claimed a larger life expectancy for the consumers where these foods are staple. They pointed to high percentage of centenarians in regions where fermented milks are consumed. Other saw nothing more in fermented milks than good basic food (Kosikowski, 1982). However souring of milk inhibits the growth of many pathogenic bacteria, so that outbreaks of intestinal diseases are much less likely than with fresh milks especially in hot countries. It is most likely that the healthfulness of fermented milks depends on their contents of protein, lactose, fat, calcium and other important minerals and vitamin B complex (Saarivirta, 1983). It has been claimed that the biological value of proteins of fermented milks is better than the ordinary
milk. There is, however, no evidence showing that simple protein fragments together with the unchanged protein have better nutritive value than the original proteins (Saarivirta, 1983). Since fermented milks have usually less fat, they contain less fat soluble vitamins than the ordinary milk. They are, however, excellent sources of B-vitamins; the amount of any single vitamin depends on the culture condition and the type of fermented milk in quest (Saarivirta, 1983).

It has been demonstrated that individual with limited ability to digest lactose (lactose intolerance) can usually consume nutritionally useful quantities of fermented milk (Saarivirta, 1983).

2.3. Traditional fermented milk products in Sudan:

2.3.1 "Roub":

It is the most common fermented milk product of Sudan. Milk is fermented overnight, and the resulting sour milk is churned to give butter; the remaining buttermilk is "Roub". The principal aim behind "Roub" production is the need to facilitate the extraction of butter from the milk. The butter (furssah) is later boiled to give butter oil or ghee, which can be stored for use in the lean season. "Roub" production is in the hands of animal-owning nomadic tribes, and the bulk of it is produced during the rainy season from July to October (Dirar, 1993).

The traditional manufacturing procedures of "Roub" in Sudan are not different with different regions despite the disparities in climate and culture. However, differences are still there in the whole process. These differences are mainly confined to the containers used and their names and to some extent to the use of "Roub" and "Roub" products (Abdel Gadir et al., 1998).
2.3.2 "Gariss":

Camel's milk is produced in certain areas of Sudan, under nomadic conditions, amounting to about 33,000 tons/year, because camels in Sudan are reared under unstable nomadic roaming, their milk is not always available to urban or village residents. The camel's milk being abundant in remote localities, the herders have to prepare Gariss, a fermented product, on which they sustain living for several months as the sole source of various nutrients (Dirar, 1993). Gariss from camel's milk is made by a semi-continuous fermentation process where the fermentation is carried out in two leather bags of tanned goat skin embedded in green or wet grass carried on the back of camels and subjected to continuous shaking by the jerky walk inherent to camels. Whenever part of the product is withdrawn for consumption, a portion of fresh camel's milk is added to make up volume and this continues for months (Dirar, 1993).

2.3.3 Other dairy products:

Biruni, also called leben-gadim, which is fermented unchurned milk ripened for up to 10 years. A related product, but not ripened, is mish, which is made by prolonged fermentation to the extent that maggots thrive in it. The product is consumed whole, with the maggots included. These two products are closely related to Egyptian mish (Abdel-Malek, 1978).

Dairy products that have entered the Sudan from Egypt within the last century are jibna beida (white cheese), zabadi (yogurt), and black cumin flavored mish. These products are strictly confined to urban communities, where the Egyptian influence is more strongly felt (Dirar, 1993).

2.4.1 Procedures of "Roub" processing:

In areas where the survey was carried out, the practice of "Roub" making was almost the same and can be outlined as follows:-
Source of milk is normally cow's milk (in Kordofan) and in addition, goat's, sheep's and camel's milk can be used. Usually milk for "Roub" making is used as raw i.e. not boiled. However, there are still some people who prefer heating milk (boiling) before processing. It is clear that it is just a matter of taste and liking, people who prefer boiling of milk say that the resultant "Roub" is better in taste and they called it "Roub barid" (cold Roub i.e. moderately acidic).

They usually (unless the container is new and not used before) do not add any of previous "Roub" (as starter culture) to the raw milk under processing, however, still there are some producers who prefer adding part of previous "Roub" as starter culture. "Roub" has a short lifetime i.e. deteriorates rapidly; in addition they all agree that there is a need to add "Roub" so as to expedite the fermentation.

Milk for "Roub" making generally is left for 12 hours after which the fermentation is complete and some people leave it for 24 hours; but they all agree that the fermentation period can go up or down according to the ambient temperature.

Coagulation of milk (i.e. complete fermentation) is usually noticed by experience. There is no specific way of judging the milk other than normally by sight and sense.

Milk is shaken (churned) after being curdled. The shaking time is usually 10 minutes after which a separation of butter is obtained. Addition of water to ease the collection of butter is common to all Roub-producer. Water is added just at the breakpoint (of butter and butter milk) which is detected by experience.
2.4.2 Containers used in Roub making:

The common traditional container is called "Bukhsah" but for those who tend to boil the raw milk they use a container called "Burma" (which is made from clay). After milk is heated, it is transferred to the "Bukhsah" where fermentation takes place; it is also called "Berkaba" (Abdel Gadir et al., 1998).

2.4.2.1 "Bukhsah" preparation:

"Bukhsah" is some kind of gourd, which in its unshaped form is wholly closed object. They open it from one side (usually the tapering end) and then spin the opening with some kind of tree-fiber called "Saaf" until the opening becomes narrow and considered as the mouth of "Bukhsah". Usually from the same "Saaf" they make a mouth-lid as a cover which tightly closes the mouth. They then spin a handle for "Bukhsah" (usually made from skin of cattle), this handle is for hanging the "Bukhsah" during shaking process. "Bukhsah" is usually rinsed at least after each two "Roub" makings, and then exposed to the sun for drying (Abdel Gadir et al., 1998).

2.4.3 Uses of "Roub":

"Roub" is usually used as a drink and is considered a good nourishing food, and the nomads depend on it.

Sometimes people add what is called "waika" (a kind of sun-dried okra) to process "Mullah Roub" (kind of stew) and sometimes "Roub" is left to get sharply soured and then the curd is collected leaving the whey which is sweetened with table sugar and drunk (as in Bija tribes).

2.5 Microbiology of "Roub":

2.5.1 Lactic acid bacteria:

The lactic acid bacteria consisting of a number of diverse genera are grouped as either homofermenters or heterofermenters on the basis of
fermentation end products (Carr et al., 2002). Homolactic fermentation is known as Embden-Meyerhof-Parnas pathway for glycolysis (Teuber, 1995). The homofermenters produce only lactic acid as the major end-product by the fermentation of carbohydrates (Axelsson 1998 and Carr et al., 2002).

The homofermentative group of lactic acid bacteria includes *Streptococcus, Pediococcus, Lactococcus*, and *Lactobacillus* species. The heterofermenters on the other hand produce a number of other products such as carbon dioxide, acetic acid, ethanol besides lactic acid (Axelsson 1998 and Carr et al., 2002). Homofermentation and heterofermentation are differentiated by the production of aldolase which is the key enzyme for glycolysis. The homofermenters possess aldolase which ferments glucose to lactic acid. The heterofermenters do not produce this enzyme and follow pentose-monophosphate pathway instead. They cannot breakdown fructose 1, 6 diphosphate to triose phosphates, and only oxidize glucose-6-phosphate to 6-gluconate by phosphoketolase (Okuklu, 2005).

### 2.5.2 Yeasts:

*Debaryces hansenii* has been isolated from cheese and other foods. Cells appear spherical to short oval and are arranged singly, in pairs or in short chains and propagate by multipolar budding. On slide cultures pseudomycelium is absent or, if present, is very primitive. There are unable to ferment lactose (Lovell, 1990).

Yeasts can break down protein and fat and therefore cause quality defects in cheese and butter. Yeasts grow best in acid environment and therefore usually found in acid products as cultured milk. Yeasts develop most vigorously at about 30°C in the presence of air. Yeasts are generally undesirable organisms from the dairy point of view because they can cause
fermentation in milk and dairy products, adversely effecting both flavor and smell (Alfa-laval Dairy Handbook).

2.5.3 Moulds:

Moulds are commonly found in dairy products. On mould extract agar, colonies are white in colour and appear yeast-like and butyrous, particularly when older. The commonest species of moulds in milk and milk products, do not in practice survive high temperature short time pasteurization (HTST). The presence of mould in heat treated product is therefore sign of recontamination. Many different families of moulds exist; those which are of the greatest importance in the dairy industry are *Penicillium* (Alfa-laval Dairy Handbook).

2.5.4 *Staphylococcus aureus*:

*Staphylococcus* is a genus of Gram-positive bacteria. Under the microscope they appear round (cocci), and form in grape-like clusters (Ryan *et al.*, 2004).

The *Staphylococcus* genus includes just thirty-three species (Holt, 1994). Most are harmless and reside normally on the skin and mucous membranes of humans and other organisms. Found worldwide, they are a small component of soil microbial flora (Madigan and Martinko, 2005).

2.5.5 *Escherichia coli*:

*Escherichia coli* is a Gram negative bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms). Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2 (Bentley and
Meganathan, 1982) or by preventing the establishment of pathogenic bacteria within the intestine (Reid et al., 2001).

*E. coli* is not always confined to the intestine, and its ability to survive for brief periods outside the body makes it an ideal indicator organism to test environmental samples for fecal contamination (Thompson, 2007). The bacterium can also be grown easily and its genetics are comparatively simple and easily-manipulated or duplicated through a process of metagenics, making it one of the best-studied prokaryotic model organisms, and an important species in biotechnology and microbiology (Feng, 2002).

### 2.5.6 *Salmonella* sp.:

*Salmonella* is a Gram-negative facultative rod-shaped bacterium in the same proteobacterial family as *Escherichia coli*, the family Enterobacteriaceae, trivially known as "enteric" bacteria. *Salmonella* is nearly as well-studied as *E. coli* from a structural, biochemical and molecular point of view, and as poorly understood as *E. coli* from an ecological point of view. *Salmonellae* live in the intestinal tracts of warm and cold blooded animals. Some species are ubiquitous and other species are specifically adapted to a particular host. In humans, *Salmonella* is the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the blood stream, and acute gastroenteritis, resulting from a food borne infection/intoxication (Todar, 2007).

### 2.6 Effect of lactic acid on pathogenic organisms

Lactic acid inhibits many pathogenic bacteria, and the undissociated form of the acid is considered to be the active component (Robinson and Samona, 1992). The amount of undissociated lactic acid depends on both the concentration of lactic acid and the pH. The pKa of lactic acid is 3.86, giving that at pH 4.8 only 10% while at pH=3.86 half of the lactic acid is in the
undissociated form. In contrast to the lactate ion, the uncharged undissociated form of lactic acid can penetrate the cell membrane of the bacteria. The cytoplasm of the bacteria has a much higher pH than the surrounding, which provokes the dissociation of the lactic acid molecule inside the bacteria, thus liberating $H^+$. This will lower the pH of the cytoplasm. The amount of undissociated lactic acid passing through the cell membrane at a point near pH 4 is too high for the mechanisms in the cell dealing with the regulation of pH in the cytoplasm. The pH inside the bacteria will drop and eventually cause the destruction of the pathogen (Abdel Gadir et al., 1998).

2.7 Microbiological properties of some fermented dairy products:

El Sadic et al (1972) studied the microbiological properties of 50 samples of "Zabady" from Cairo market. They found that the total microbial count averaged $1.2 \times 10^9$ cfu/ml, the dominant species being streptococci (mainly *Strep thermophilus*) followed by micrococci *Lactobacillus* count averaged $7 \times 10^8$ cfu/ml, with *Lactobacillus bulgaricus* predomination. Yeasts and moulds count averaged $7 \times 10^5$ cfu/ml and they mainly *Candida spp*. Yeasts were selected in 84% of samples. Colifirm count ranged from 0.0 to $2.4 \times 10^6$ cfu/ml with an average $15 \times 10^4$ cfu/ml, these organisms being detected in 58% of samples.

Bhat and Fernande (1984) examined the microbial flora of "Mawa". From 5 representative "150" isolates were obtained. These isolates were mainly: (8) species of each of *Micrococcus*, *Staph. citoreous* and *Microbacterum lacticum*, (6) species of *Bacillus*, *Clostridium perfringens* (the only anaerobic Bacillus) and *Actinomyces rutger sensii* and (3) species of moulds (*Asperigillus*, *Penicilliuim* and *Pachytrichum*). The average colony
count/gm of "Mawa" on 2% agar (same samples) was: 33,664,000 (after 24 hrs), 385,621,020 (after 72 hrs) and 1,643,018,200 (after 144 hrs).

Savadogo et al. (2004) found that total plate count agar (aerobic mesophilic bacteria) ranges of counts were $8.12 \times 10^5 - 3.6 \times 10^8$ and mean counts for all samples $6.71 \times 10^7$, Rogosa agar (lactobacilli) ranges of counts (cfu ml$^{-1}$) $0.30 \times 10^6 - 1 \times 10^8$ and mean counts (cfu ml$^{-1}$) for all samples $24.44 \times 10^6$ and yeast and moulds ranges of counts (cfu ml$^{-1}$) $1.83 \times 10^3 - 3.7 \times 10^6$ n=25 and mean counts (cfu ml$^{-1}$) for all samples.

Al-Tahiri (2005) studied the microbiological conditions between traditional dairy products sold in Karak in the south of Jordan and same products produced by modern dairies. He found the microbiology of unpasteurized milk, yoghurt, lebnah (concentrated yoghurt) and white soft cheese produced by farmers as total count ($5 \times 10^5 - 3 \times 10^3$, $6 \times 10^2 - 2 \times 10^4$), S. aureus count ($3 \times 10^2 - 4 \times 10$, less than $10 - 5 \times 10^3$) and yeast and moulds count ($15 \times 10^3 - 15 \times 10$, $4 \times 10 - 5 \times 10^5$), but in products produced by modern dairies he found the total count (zero- $6 \times 10^6$, $8 \times 10^5 - 5 \times 10$), yeasts and moulds count (zero- 10) and no Staph. aureus were detected in all samples.

Abdalla and El Zubeir (2006) found high count of E. coli, Staphylococcus aureus, and Salmonella spp. in yogurt and their mixture and reported that these organisms are potential for food spoilage. Escherichia coli, Listeria monocytogenues and Yersinia enterocolitica are three of the most important food-borne bacterial pathogens and can lead to food-borne diseases through consumption of contaminated milk and fermented milk products (Morgan et al., 1993; Mead et al., 1999).
CHAPTER THREE
MATERIALS AND METHODS

3.1.1 Source of samples:

A total of 30 "Roub" samples were obtained from three regions in Sudan ("Nyala", "Al Obied" and "Abu Namma"). "Roub" samples were collected from the market in sterile "50 ml" plastic flasks and transported in cooled containers (4°C) to the laboratory of the Department of Dairy Production, Faculty of Animal Production, University of Khartoum. The samples were stored at the same temperature (4°C) till the microbiological examination was carried out during 48 hours.

3.1.2 Preparation of media:

3.1.2.1 Plat Count agar medium (Scharlawu 01-161):

The medium consisted of 5 gm casein peptone, 2.5 gm yeast extract, 1.0 gm dextrose and 15 gm agar. The medium was prepared by suspending 28 gm of the powder in one liter of distilled water, then boiled until dissolved completely and sterilized by autoclaving at 121°C for 15 minutes.

3.1.2.2 MRS agar medium (7543):

The medium consisted of 10 gm casein peptone tryptic digest, 10 gm meat extract, 5 gm yeast extract, 20 gm glucose, 5 gm sodium acetate, 1 gm polysorbate “80”, 2 gm potassium phosphate, 2 gm ammonium citrate, 0.1 gm magnesium sulfate, 0.05 gm manganese sulfate and 15 gm agar.

The medium was prepared by suspending 70 gm of the powder in one liter of distilled water, then boiled until dissolved completely and sterilized by autoclaving at 121°C for 15 minutes.
3.1.2.3 Mannitol salt agar medium (7143A):

The medium consisted of 10 gm proteose peptone, 1 gm beef extract, 75 gm sodium chloride, 10 gm D- mannitol, 0.025 gm phenol red and 15 gm agar.

The medium was prepared by suspending 111 gm of the powder in one liter of distilled water, then boiled until dissolved completely and sterilized by autoclaving at 121°C for 15 minutes.

3.1.2.4 Salmonella Shigella (SS) agar medium (7152A):

The medium consisted of 5 gm beef extract, 2.5 gm enzymatic digest of casein, 2.5 gm enzymatic digest of animal tissue, 10 gm lactose, 8.5 gm bile salts, 8.5 gm sodium citrate, 8.5 gm sodium thiosulfate, 1 gm ferric citrate, 0.00033 gm brilliant green, 0.025 gm neutral red and 13.5 gm agar.

The medium was prepared by suspending 63 gm of the powder in one liter of distilled water, boiled until dissolved completely.

3.1.2.5 MacConky agar medium:

The medium consisted of 17 gm pancreatic digest of gelatin, 3 gm peptones (meat and casein), 10 gm lactose monohydrate, 5 gm NaCl, 1.5 gm dehydrated bile, 1 gm crystal red, 30 gm neutral red and 13.5 gm agar.

The medium was prepared by suspending 51.5 gm of the powder in one liter of distilled water, then boiled until dissolved completely and sterilized by autoclaving at 121°C for 15 minutes.

3.1.2.6 Malt extract agar medium:

The medium consisted of 12.75 gm maltose, 2.75 gm dextrin, 2.35 gm glycerol, 0.78 gm peptone and 15 gm agar.

The medium was prepared by suspending 50 gm of the powder in one liter of distilled water, boiled until dissolved completely and sterilized by autoclaving at 121°C for 15 minutes.
3.1.3 Preparation of serial dilutions:

Eleven milliliter of "Roub" were dissolved in 99 milliliter of sterile distilled water to make 10\(^{-1}\) dilution, then one ml from the above mentioned dilution was specially transferred to 9 milliliter sterile distilled water. This procedure was repeated to make serial dilutions of 10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\), 10\(^{-5}\) and 10\(^{-6}\). From the last dilution, 1 ml was transferred to Petridish in duplicate (Houghtby et al., 1992).

3.2 Examination of culture:

Growth on solid media was examined visually with naked eyes for colonies appearance and change in media.

3.2.1 Total viable bacterial count

Plate count agar was used for the enumeration of total bacterial count, 1 ml quantities of each sample decimal dilutions, 10\(^6\) was streaked in dried plate with plate count agar and incubated at 32 \(\pm 1\)°C for 48 \(\pm 3\) h for enumeration of total plate count. They were counted by colony counter (Houghtby et al., 1992).

3.2.2 Lactic acid bacteria count:

MRS agar was used for the enumeration of Lactobacilli, 1 ml quantities of each sample decimal dilutions, 10\(^6\) was streaked in dried plat of MRS agar. The culture was incubated at 35°C for 48 hours (DeMan et al., 1960). They were counted by colony counter.

3.2.3 Staphylococcus aureus count:

Mannitol salt agar was used for the enumeration of staphylococci, 1 ml quantities of each sample decimal dilutions, 10\(^6\) was streaked in dried plate of mannitol salt agar. The culture was incubated at 37°C for 48 hours (Harrigan and McCance, 1976). They were counted by colony counter.
3.2.4 *Salmonella spp. count:*

Salmonella Shigella agar (SS agar) was used for the enumeration of *Salmonella spp*, 1 ml quantities of each sample decimal dilutions, $10^6$ was streaked in dried plate of SS agar. The culture was incubated at 35°C for 48 hours. They were counted by colony counter.

3.2.5 *E. coli count:*

MacConky agar was used for the enumeration of *E. coli*, 1 ml quantities of each sample decimal dilutions, $10^6$ was streaked in dried plate of MacConky agar. The culture was incubated at 35°C for 48 hours. They were counted by colony counter.

3.2.6 *Yeasts and moulds counts:*

This was determined by plating suitable dilution of sample in malt extract agar plate which was acidified to pH 3.5 using 10% acid as indicated in Harrigan and McCance (1976). Plates were incubated at 30°C ± 2°C for up to 5 days.

3.3 *Biochemical tests:*

Biochemical tests were performed according to Barrow and Feltham (1993) as follows:

3.3.1 *Gram stain*

A discrete colony was carefully picked with a sterile wire loop. The colony from fresh culture (18-24 hr) was emulsified in a drop of saline and spread evenly on clean slide to make a thin film. The slide was allowed to dry and fixed by slight flaming and stained. The smear was stained with crystal violet solution for seconds, then rinsed rapidly with water and Gram’s iodine solution was added and left to dry for seconds. The iodine was poured off and the slide was washed with 95% ethanol for 5-15 seconds.
The smear was then washed with tap water and stained with safranin solution for 20 seconds, again washed with water and allowed to dry. On microscopic examination, the Gram positive organisms appeared purple while Gram negative ones appeared pink (Harrigan and Mc Cance, 1976).

3.3.2 Catalase test:

The organisms to be tested were put in sterile slides. A drop of 3% hydrogen peroxide (H₂O₂) was added to the colony and emulsified. Production of gas immediately or after 5 minutes indicated a positive result.

3.3.3 Oxidase test:

The oxidase test was performed using oxidase test paper. The colonies to be tested were removed by a platinum wire or glass rod and smeared across the surface the oxidase test paper. The positive reaction was showed by the development of a dark purple color within 10 seconds.

3.3.4 Indole test:

Indole test was performed to detect the ability of an organism to break down tryptophan to indole. The colonies to be tested were inoculated into tube containing tryptophan water and incubated at 37°C for 48 hrs then added 1ml of Kovac's reagent was added and read immediately. The result was indicated by the appearance of bright pink color in the top layer.

3.4 Statistical analyses:

The samples were analyzed for total viable bacterial count, count, *Staphylococcus aureus* count, *Salmonella spp.* count, *E. coli* count and yeasts and moulds count. Means were separated using Duncan multiple range test with P≤0.05.
CHAPTER FOUR

RESULTS

The result in tables 1, 2 and 3 indicate that the mean total viable bacterial count in EL-Obeid, Nyala and Abu Naama areas was 8.14, 7.56 and 8.07 $\log_{10}$ cfu/ml respectively. These values showed significant different ($P<0.05$) between EL-Obeid and Nyala samples but no significant different ($P>0.05$) between El-Obeid and Abu Naama samples and between Nyala and Abu Naama samples were observed (Table 4). Out of the number of samples tested, it was found that all samples were positive (100%) for total viable bacterial count (Table 5).

Means of $Staphylococcus aureus$ count were 6.15, 6.18 and 5.30 $\log_{10}$ cfu/ml for EL-Obeid, Nyala and Abu Naama areas respectively (Tables 1, 2 and 3), and these means were not significant different ($P>0.05$) between the three areas under study (Table 4). The percentage of positive samples for $Staphylococcus aureus$ were 40% in El-Obeid, 60% in Nyala and 20% in Abu Naama areas (Table 5).

$Escherichia coli$ showed a mean value of 5.60 $\log_{10}$ cfu/ml in El-Obeid area (range: <1 est - 6.30 $\log_{10}$ cfu/ml), 5.70 $\log_{10}$ cfu/ml in Nyala (range: <1 est – 6.60 $\log_{10}$ cfu/ml), while in Abu Naama area the organism was not detected in samples tested (Tables 1, 2, 3 and 4). There were no significant different ($P>0.05$) between El-Obeid and Nyala areas (Table 4). Thirty percent of the samples tested were positive in El-Obeid and 40% in Nyala area, while all samples tested in Abu Naama area were negative.

$Salmonella sp$ was not detected in all samples tested in the three areas under study (Tables 1, 2, 3 and 4).
The mean values for yeasts and moulds were 5.53, 4.64 and 5.50 Log_{10} cfu/ml for EL-Obeid, Nyala and Abu Naama areas respectively and these means were not significant different (P>0.05) between the three areas under study (Table 4). All samples tested were positive (100%) for yeasts and moulds in El-Obeid area and 90% of samples were positive in Nyala and Abu Naama areas (Table 5).

The mean count of lactic acid bacteria was 7.80, 7.09 and 7.51 Log_{10} cfu/ml in EL-Obeid, Nyala and Abu Naama areas respectively (Table 4). These values showed significant different (P<0.05) between El-Obeid and Nyala samples but no significant different (P>0.05) between El-Obeid and Abu Naama samples and between Nyala and Abu Naama samples were observed (Table 4). All samples tested for lactic acid bacteria were positive (100%) in the areas under study (Table 5).
Table (1). Microbiological profile of “Roub” from El-Obeid area, North Kordofan State (Log\textsubscript{10} cfu/ml)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable bacterial count</td>
<td>7.53±0.32 - 8.47±0.32</td>
<td>8.14±0.40</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>&lt; 1 est - 6.30±2.95</td>
<td>5.60±2.62</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>&lt;1 est - 6.90±3.30</td>
<td>6.15±3.15</td>
</tr>
<tr>
<td>Salmonella sp.</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>7.04±0.42 - 8.29±0.42</td>
<td>7.80±0.57</td>
</tr>
<tr>
<td>Yeasts and moulds</td>
<td>4.00±0.62 - 5.97±0.62</td>
<td>5.53±1.41</td>
</tr>
</tbody>
</table>

ND = Not detected

est = Estimated
Table (2). Microbiological profile of “Roub” from Nyala area, South Darfur State (Log<sub>10</sub> cfu/ml)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable bacterial count</td>
<td>7.34±0.12 – 7.72±0.12</td>
<td>7.56±0.40</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>&lt;1 est - 6.60±3.14</td>
<td>5.70±2.62</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>&lt;1 est - 6.60±3.28</td>
<td>6.18±3.15</td>
</tr>
<tr>
<td><em>Salmonella sp.</em></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>6.78±0.16 – 7.28±0.16</td>
<td>7.09±0.57</td>
</tr>
<tr>
<td>Yeasts and moulds</td>
<td>&lt;1 est – 4.90±1.48</td>
<td>4.64±1.41</td>
</tr>
</tbody>
</table>

ND = Not detected  
est = Estimated
Table (3). Microbiological profile of “Roub” from Abu Naama area, Blue Nile State (Log$_{10}$ cfu/ml)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable bacterial count</td>
<td>7.24±0.51 – 8.68±0.51</td>
<td>8.07±0.40</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>&lt; 1 est – 6.00±2.53</td>
<td>5.30±3.15</td>
</tr>
<tr>
<td><em>Salmonella sp.</em></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>5.70 ±0.77 – 8.21±0.77</td>
<td>7.51±0.57</td>
</tr>
<tr>
<td>Yeasts and moulds</td>
<td>&lt; 1 est – 6.22±1.78</td>
<td>5.50±1.41</td>
</tr>
</tbody>
</table>

ND = Not detected

est = Estimated
**Table (4).** Microbiological profile of “Roub” from three areas under study (mean ±SD)

<table>
<thead>
<tr>
<th>Organism</th>
<th>El-Obeid Mean</th>
<th>Nyala Mean</th>
<th>Abu Namma Mean</th>
<th>SL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable bacterial</td>
<td>8.14±0.40 a</td>
<td>7.56±0.40 b</td>
<td>8.07a±0.40 b</td>
<td>*</td>
</tr>
<tr>
<td>count</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>5.60±2.62 a</td>
<td>5.70±2.62 a</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6.15±3.15 a</td>
<td>6.18±3.15 a</td>
<td>5.30±3.15 a</td>
<td>NS</td>
</tr>
<tr>
<td>Salmonella sp.</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>7.80±0.57 a</td>
<td>7.09±0.57 b</td>
<td>7.51±0.57 ab</td>
<td>*</td>
</tr>
<tr>
<td>Yeasts and moulds</td>
<td>5.53±1.41 a</td>
<td>4.64±1.41 a</td>
<td>5.50±1.41 a</td>
<td>NS</td>
</tr>
</tbody>
</table>

Means within each row bearing the same superscripts are not significantly different (P<0.05).

* = P<0.05
ND = Not detected
SL = Significant Level
NS = Not Significant
SD = Standard Deviation
Table (5). Percentage of positive samples from the areas under study

<table>
<thead>
<tr>
<th>Organism</th>
<th>El-Obeid</th>
<th>Nyalá</th>
<th>Abu Naama</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total viable bacteria count</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>30</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>40</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Yeasts and moulds</td>
<td>100</td>
<td>90</td>
<td>90</td>
</tr>
</tbody>
</table>
Due to absence of heat treatment of milk prior to fermentation in addition to utilizing natural fermentation, it is expected that total bacterial count is high in all areas sampled.

The results of microbiological examination indicate that this product is highly contaminated with microorganisms of public health concern. The high number of total bacterial count, *Staphylococcus aureus* and *E. coli* indicates unhygienic conditions during production of milk and further processing into “Roub” without heat treatment (Uzeh et al., 2006). Similar results of total bacterial count were reported for different traditional dairy products (Beukes et al., 2001; Mathara et al., 2004; Savadogo et al., 2004; Lore et al., 2005; Al-Tahiri, 2005 and Hassan et al., 2008 ). The detection of *E. coli* and *Staphylococcus aureus* in high number is a public health concern since it indicates faecal contamination during production or processing of this product. These organisms were isolated by different researches in other fermented dairy products (Savadogo et al., 2004; Al-Tahiri, 2005, Lore et al., 2005; Uzeh et al., 2006).

The results of Lactic acid bacteria count show that fermentation is mainly carried out by lactic acid bacteria in uncontrolled conditions of fermentation. Similar results were reported by Beukes et al., (2001), Mathara et al. (2004), Savadogo et al. (2004), El-Baradei et al. (2008), Hassan et al. (2008) and Jokovic et al. (2008).
Salmonella spp. was not detected in all samples tested. This result was in disagreement with the results reported by Abdalla and El-Zubeir (2006) in mish and roub.

“Roub” samples were highly contaminated with yeasts and moulds. This might be possible due to poor processing conditions and/or uncontrolled fermentation which lead to contamination with yeasts and moulds, and this is obvious by alcoholic fermentation resulting in alcohol production in addition to lactic acid. Ali et al. (2002), Mathara et al. (2004), Savadogo et al. (2004), Al-Tahiri (2005), Lore et al. (2005) and Uzeh et al. (2006) detected yeasts and moulds in different traditional fermented dairy products.
CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion:

Total viable bacteria were counted in all samples in the three areas under study, while half of samples were tested positive for *Staphylococcus aureus* in two areas and in the third area about 20% of samples were positive. *E. coli* was fawned in 35% of the samples under test two areas while there is no detection in the third area. *Salmonella spp.* was not detected in all samples tested, while yeasts and moulds were detected (100%) in all samples of El Obeid area and 90% of the samples from Nyala and Abu Naama were infected with yeasts and moulds. Lactic acid bacteria were detected in all samples under study.

6.2 Recommendations

1. More study of “Roub” microbiology is needed on the following:
   a. Isolation and identification of lactic acid bacteria responsible for Roub fermentation from different areas of Sudan.
   
   b. Production of Roub using improved method to meet the standards
   
   c. More research is needed to improve the quality of the product using selective starter culture.
   
   d. Isolation and identification of pathogenic bacteria involved in Roub contamination.
REFERENCES


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