Use of the RAD-PCR Fingerprinting for The Clustering of Isolates of Lactic Acid Bacteria from Fermented Milk (Rob) Samples

By

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B.Sc. (honors) 1996
Faculty of Agricultural Studies
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A thesis submitted in partial fulfillment of the requirements for the degree of master of science in Agriculture

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University of Khartoum
2004
DEDICATION

To soul of my father, and my brother Omer and
to my wife Sara, and to every one of all of my family
ACKNOWLEDGEMENT

First of all, ultimate thanks are due to Allah without whose aid this work could not have been achieved.

I wish to express my warmest thanks to my supervisor Dr. Ahmed Ali Mahdi for his kind guidance, supervision and support throughout the whole process of conducting this project. Thanks are also due to Dr. Eisa Ibrahim El-Giali, director of the Commission of Genetic Engineering and Biotechnology, National Center for Research, for suggesting the problem and for providing the lab. facilities.

I would like to express my deep gratitude and thanks to prof. Dr. Nabeel Ibrahim Elmagdoub, professor of microbiology, Faculty of Agriculture, Ain Shams University (CAIRO-MIRCEN) for his kind supervision, precious help, continuous support and critical criticism during practical work in Egypt. Thanks are also due to the head and staff members of the National Agricultural Genetic Engineering Institute (NAGEI), Cairo for their generous advice, and support by all means.

I would also like to thank the following individuals from NAGEI: Ahmed Shoukry, Dr. Salah, researcher Sameh for providing the facilities of the RAPD-PCR analysis, and from the Sudan: Dr. Mutasim Mohamed, for his help. Miss Sara Salem and Mr. Abdelmonium Hamed.

Finally I am deeply grateful to my family for teaching me the value of hard work and patience, and for their unconditional support.
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Abstract
One hundred and sixty isolates of lactic acid bacteria were obtained from ten samples of Sudanese traditional fermented cow’s milk (Rob), the most popular fermented milk in the Sudan. The samples were obtained from different locations in the Sudan. Thirty-two (18%) of the isolates were rod-shaped, and the rest (82%) were cocci.

On the basis of a number of preliminary tests, which are commonly used for the identification of lactic acid bacteria, 42 homo- and heterofermentative representative isolates (14 rods and 28 cocci) were selected for further investigation and characterization. These were identified by utilizing morphological, physiological and molecular typing techniques.

Randomly amplified polymorphic DNA (RAPD-PCR) fingerprinting using four oligonucleotide primers (A-11, C-4, O-10 and O-16) was the technique of choice for investigating the genetic diversity of the isolated strains.

Fingerprinting the fourteen rod-shaped isolates resulted in five clusters based on numerical analysis of the RAPD-PCR profiles. Representative strains representing these clusters were identified by the API 50 STREP identification system. These were identified as *Lactobacillus delbreuckii* subsp. *bulgaricus* (one strain), *Lactobacillus rhamnosus* (9 strains), *Lactobacillus plantarum* (2 strains), *Lactobacillus casei* (one strain) and *Lactobacillus pentosus* (one strain).
The selected 28 isolates representing the cocci were separated into nine clusters, the representative strains of which were identified by the API 20 STREP identification system. These representative strains were identified as *Aerococcus viridans* (4 strains), *Enterococcus faecium* (2 strains), *Enterococcus gallinarum* (2 strains), *Lactococcus lactis* subsp. *lactis* (5 strains), *Leuconostoc* sp (5 strains), *Streptococcus acidominimus* (8 strains) and *Streptococcus bovis* (2 strains).

Some of the tested 42 strains produced exopolysaccharides from sucrose under the conditions used. These isolates can be utilized for the production of safe fermented milk products. Further studies on representative strains from these clusters can be put to use in the production of distinct rheologic and organoleptic traits in the production of Rob and other milk fermentation products.

The present study demonstrates that the RAPD-PCR fingerprinting technique was more sensitive and reliable for differentiating between the isolates compared to the traditional and biochemical methods.
خلاصه البحث

تم عزل عدد 160 سلالة من بكتيريا حمض اللاكتيك من الروب كأحد نواتج تخمير الحليب التقليدية الشائعة في السودان. تم الحصول على هذه العزلات من عشر عينات من الروب من مناطق مختلفة من السودان. تم التحديد المبدئي لخصائص العزلات المختلفة من بكتيريا حمض اللاكتيك المتحصل عليها من هذه العينات باستخدام المجهر الضوئي اعتماداً على الخصائص المورفولوجية حيث أظهر الفحص المجهري نوعية من الاشكال هما: العصوية 32 عزلة (20 %) والكروية 148 عزلة (82%). تم اختيار 42 عزلة من هذه البكتيريا من خلال الاختبارات المورفولوجية والفيزيولوجية التي تم اجراؤها على مجموع العزلات. وكانت 14 من هذه العزلات عصوية و28 كروية وذلك لدراسة التباين الوراثي بين هذه العزلات عن طريق البصمة الوراثية بتقنية الاكتثار العشوائي RAPD-PCR النووي.

تم استخلاص المحتوى الكلي من حمض DNA لكل العزلات المختارة حيث تم اختيار مدِّي التباني الوراثي بين هذه العزلات. وقد أظهرت النتائج ما يلي:–
- أظهرت أربع بادنات (primers) معلومة تسلسل النيكليوتيدات, تم تصنيعها بمعهد بحوث الهندسة الوراثية الزراعية (القاهرة) قدرتها على تمييز مجموعات رئيسية من هذه العزلات. كانت هذه البادنات هي A-11, O-10, O-16, C-4.
- تمت المقارنة بين السلاسل المختلفة وأظهرت المقارنة وجود اختلافات في حجم وعدد الناتجة من عملية الاكتثار العشوائي للحمض النووي مما يدل على وجود بعض الاختلافات بين هذه السلاسل.
- أظهر تحليل النتائج المتحصل عليها من الاكتثار العشوائي للحمض النووي أن الأربعة عشر عزلة عصوية الشكل تقع في خمس مجموعات رئيسة (Clusters). وقد تم التعرف API 50 CHL STREP على السلاسل الممثلة لكل مجموعة باستخدام نظام identification system Lactobacillus (عزلة واحدة) , Lactobacillus rhamnosus  (عزلة واحدة) , Lactobacillus delbreuckii subsp. bulgaricus (عزلة واحدة) , Lactobacillus casei (9 عزلات) , Lactobacillus plantarum (عزلة واحدة) و Lactobacillus pentosos (عزلة واحدة).
- The API 20 STREP identification system.

  The seven bacterial strains isolated in this study were: Lactococcus lactis ss. lactis (5 isolates), Enterococcus faecium (Enterococcus gallinarum) (4 isolates), Aerococcus viridans (8 isolates), Streptococcus acidominimus (5 isolates), Streptococcus bovis (5 isolates), and Leuconostoc spp. (7 isolates).

- The API 20 STREP identification system is used to identify the isolated bacterial strains. This system is based on the biochemical reactions of the bacteria and their ability to produce certain enzymes or pigments.

  The results showed that the isolated strains were categorized into seven groups, with each group containing one or more strains. The seven groups were identified as: Lactococcus lactis, Enterococcus faecium, Aerococcus viridans, Streptococcus acidominimus, Streptococcus bovis, and Leuconostoc spp.

  In addition, the study also showed that the isolated strains produced exopolysaccharides (EPS), which are polysaccharides that are extracellular and can be used as a source of energy by the bacteria. The EPS production was found to be higher in some strains than in others.

- RAPD-PCR (random amplified polymorphic DNA) is a molecular technique that is used to fingerprint bacterial strains. In this study, RAPD-PCR was used to differentiate the isolated strains from each other.

  The results showed that RAPD-PCR was more sensitive than classical biochemical tests in differentiating the isolated strains. This indicates that RAPD-PCR is a useful tool for identifying and characterizing bacterial strains.

- The study also showed that the isolated strains produced different exopolysaccharides, which could be used as a source of energy by the bacteria.
In fermentation, organic matter is converted by microorganisms (bacteria, yeasts and molds) to products that have acceptable qualities. In a natural fermentation the conditions are conducive to the preferential desirable microorganisms to produce metabolic byproducts which give the unique characteristics of the product. The consumption of fermented foods has increased greatly since the 1970s. This includes common foods like yogurt, buttermilk and fermented sausages as well as ethnic foods such as kefir, kumiss and fufu. One of the reasons for the increase in the consumption of a fermented food is that consumers consider these foods to be healthy and natural. The consumption of billions of living cells of desirable microorganisms and their metabolic products in fermented foods does not cause any panic or distress in the safety-concerned consumers. These foods have been around for thousands of years, and therefore have Withstood the test of time (Ray and Daeschel 1992). A natural next step would be to incorporate the same antimicrobial compounds naturally found in fermented foods into other foods in lieu of "chemical" preservatives.

Fermented products contain a variety of strains belonging to different genera and species, each with the major common characteristic of producing lactic acid. The metabolism of lactic acid bacteria leads to products with improved shelf-life and different flavors from the original food and may contribute to possible nutritional and health benefits as well. Among them, those belonging to the genera *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus* are common in many fermented foods and feeds.
The isolation and characterization of strains of lactic acid bacteria (LAB) from traditional fermented foods would obviously contribute to providing starters that allow standardization without changing the fundamental properties of the product (Herrero et al. 1996). Such LAB strains isolated from natural ecosystems can be a source of genetic variation from which desirable characteristics can be selected and used to improve the existing starter cultures (Klijn et al. 1995) or develop new products (Gillilond, 1990).

Prominent among the main fermented foods are the fermented milk products which are known for their taste, nutritive value and therapeutic properties. Fermented milks are products prepared from raw milk, whole, partially or fully skimmed, concentrated, or milk substituted from partially or fully skimmed dried milk. The milk can either be homogenized or non-homogenized, pasteurized or sterilized and is usually fermented by means of specific microorganisms. Milk from eight species of domesticated mammals (Cow, buffalo, sheep, goat, horse, camel, yak and zebu) has been used to make traditional fermented milk products throughout the world, (Kroger et al. 1989).

Among the fermented milks, lactic as well as lactic–yeast fermentations are considered to be beneficial to mankind. From a biological standpoint, fermented milk is characterized by the accumulation of microbial metabolic products. It was realized very early, that such microbial metabolites include lactic acid, ethyl alcohol and various other secondary metabolites. Traditional phenotypic characterization has been the method for characterization and identification of stains of lactic acid bacteria. Presently
there is an increasing need for isolation and characterization of strains of lactic acid bacteria with unique properties. Definitive characterization of these strains calls for adoption of more subtle and refined techniques.

The main objective of this study is to apply such new techniques for identification of strains of lactic acid bacteria picked up from “Rob” as one of the popular fermented milk products in the Sudan. This objective included:

1- Isolation and characterization of strains of LAB from traditional fermented milk (Rob) samples for possible use as industrial starters. These strains may prove to have characteristics that lead to production of unique flavour profiles for development of new fermented dairy products with different flavors and tastes.

2- Utilization of kit identification microsystems complemented with appropriate computer programs, in lieu of the conventional identification systems, in an attempt to characterize the isolated LAB strains.

3- Development of fingerprint profiles for the different groups of the isolated LAB strains to serve as genetic markers for their characterization.
2.1. Fermented milk products:

The bulk of milk in the Sudan is produced by transhumant tribes in the rainy season. The souring of milk into one or other of certain dairy products is a widespread practice. Some of these products are widespread in the country whereas others are confined to certain geographical locations.

Fermented milks have been claimed for being more nutritious and health promoting than fresh milk. Platt (1964) stated that fermented milks are good sources of B vitamins, including vitamin B12, the original amount of which may be increased by the process of fermentation. Shahani and Chandan (1979) mentioned that certain cultured milk products contained higher levels of folic acid, niacin, biotin, pantothenic acid, vitamin B6 and vitamin B12 than fresh milk. It is not always true, however, that vitamins are increased by fermentation, apparently that depends on the microorganisms present. Moreover, It has been shown that while some vitamins were reduced in quantity by fermentation of milk, others were not affected.

It has been claimed that the digestibility of milk proteins is improved by fermentation (Marshall, 1986; Doeth and Tamime, 1981). In addition, there are also claims that fermented milks contain some chemical factor that reduces the level of serum cholesterol in humans (Mann and Spoery, 1974: Mann, 1977; Grunewald, 1982; Pulusani and Rao, 1983). Moreover, there
are reports that fermented dairy products have anti-tumor properties (Friend and Shahani, 1984).

Lactic acid bacteria - which produce mainly lactic acid as a metabolic end-product - are responsible for mediating the souring of milk. At present there is considerable interest in these microorganisms and their products in fermented foods. The interest stems from the proven inhibitory effects of LAB on pathogenic microorganisms (Adams and Nicolides, 1995). Making use of the probiotic properties of LAB is of particular importance in the production of wholesome weaning foods. Products of lactic fermentation have traditionally been used by Africans for treating some human ailments and for other ends (Dirar, 1997). However, these foods are now believed to pose great danger to children of the Third World as a major vehicle of diarrhoeal diseases of children below five years of age (Motarjemi et al., 1993).

Dirar, (1993), divided the fermented dairy products of the Sudan into two major groups:
1- The truly indigenous group which includes: Rob, Gariss, Biruni and Mish; and
2- The quasi- indigenous products which include Zabady and Jibna- beida.

**Gariss:** is a product made from camel's milk. The camel boys tending these camels prepare Gariss, a fermented milk product on which they live for months as the sole source of nourishment. The word Gariss means pinching or stinging, denoting a high degree of sourness.
**Biruni and Mish:** Biruni is the name given to a fermented milk product that seems to be indigenous to the Nuba Mountains from where it spread into the low lands inhabited by pastoralist Arab tribes who gave it the name "laban-gadim" (aged milk); the product is aged for at least one year, but the aging period may extend to ten years or even more. The basic purpose behind making Biruni is one of long-term food security as it is sometimes consumed years after it has fermented (Dirar, 1993).

Mish is similar to Biruni in many ways. The starting material for the preparation of the product is Rob and fresh milk. Rob is stored in a large earthenware pot and fresh milk is added to it each morning for days. Whole cumin seeds and sometimes garlic are added to the fermenting milk. Mish is also made by nomadic tribes in the northern parts of the country around the town of Dongla and by nomads as far away as the Dinder region near the borders with Ethiopia. It is a recent introduction from Egypt.

**Zabady:** is not a truly indigenous fermented food of the Sudan. The product is only known to urban populations and is completely unknown to the older generations of rural communities or to those of the pastoral sector. Until the 1950s, only the inhabitations of Khartoum and a few relatively large towns knew zabady.

**Kush Kush cheese:** This type of cheese is prepared in Northern Sudan and is also known as "Rout" by the Nuer of the south (Dirar, 1993).

**Jibna-beida:** (white cheese) is the only cheese available to the general public in the Sudanese market. The art of making cheese came to Sudan from Egypt or through Greece.
2.1.1. Rob:

Rob is the major fermented milk product in the Sudan. It is mainly produced from surplus milk of the rainy season by nomadic tribes. About 80% of the milk produced during the rainy season is turned into Rob by the household. The milk turned into dairy products in the Sudan as reported by the Arab Organization for Agricultural Development (AOAD, 1983), amounts up to 65% of the total annual production. Dirar (1993) gave an estimate of 50-60%. Rob makes about 90% of all fermented milk products.

The aim behind the souring of milk into rob is not to obtain fermented milk for consumption. Milk souring here is only an expedient to facilitate the extraction of butter from the milk. Therefore the soured milk is considered as a by-product of butter production. Pastoralists commonly waste Rob away by spillage on the ground or give it to dogs or young animals and wild birds. Nonetheless, they daily consume part of it in one form or another.

Every few days the accumulated butter is heated to produce ghee or butteroil, called samin. The unboiled butter or "furssah" finds no other use except that part of it is fed to babies. On the other hand, all other fermented dairy products of the Sudan are fermented to be consumed without removing the butter in advance.

2.1.1.1. Methods of Rob preparation:

Methods of Rob preparation differ only slightly from one area to another. Most of the Rob is made from cow's milk but milk from sheep and goats is
likewise processed. All milk is obtained by hand milking and is grossly contaminated (EL-Rufa'i, 1990).

The containers used for fermentation and churning are the "si‘in" and "bukhssa". The former is a leather bag made from the whole skin of a goat or sheep, usually called “girba” in the rest of the Arab world. The "bukhssa" is a large gourd with a lidded narrow mouth. The "si‘in" as a rule is not washed and the traces of Rob from the previous batch serve as a starter when fresh milk is added in the course of making a new batch of Rob. The "bukhssa" on the other hand, is often washed with water, dried and smoked using wood of selected trees. When Rob is to be prepared in such a container, a starter in the form of a small quantity of Rob from a previous batch has to be added to the new batch of milk.

The fermentation process usually starts in the evening when the animals return from grazing, and the sour product is churned in the morning when the herd leaves for grazing.

2.1.1.2. Uses of Rob:
Freshly prepared Rob, which is available early in the morning, is a pleasantly sour product with an unmistakable characteristic buttery flavour and taste, with a pH of about 4.5, very well liked by those who habitually consume it, and people enjoy it much as a drink without any additives. However, after 1 to 2 hours, Rob sours and its pH drops to about 4.0. This sour Rob is also used as a drink, but after dilution with water and the addition of sugar. It is also used for sauce making or it is given to young animals as a milk replacer. Little specks of tiny butter pieces are always afloat on its face. Rob in this state is drunk as it is, or used to top a local
porridge (aceda) for breakfast. It is this product that any modernization or preservation attempt of milk products should aim at. As the day wears on, the product loses its original pleasant flavour and turns more and more sour till at one point the whey "safwa" separates from the curd. The part which floats on top, being fully impregnated with gas, is referred to as "Khush-Khush". Such Rob is put to a number of uses.

Fresh milk is milked directly into the container containing Rob to give a product with a fluffy head of foam called "umgufufu", a treat for children. In hot weather, Rob is diluted with 2 or 3 volumes of water to give "ghubasha", a thirst quencher (Dirar,1997). Sometimes pastoralists make a salad of a mixture of Rob and the grated fruit of a plant called "faggus " (Cucumis melo ). Urban people make a similar salad,"salatat-rob", using cucumbers and usually "laben rayeb" or zabady. They also make"salatat-aswad" using fried egg-plant slices and soured milk. "Ajinat-um-jingir" is made in western Sudan by mixing Rob with raw ground pearl millet and consuming the mixture without cooking.

Rob could be cooked to give various sauces such as "tektoka","mulah-rayeb" and " niemiya" which receives other additives such as dry okra powder and minced meat to impart a little flavor, in addition to salt and spices. These sauces are used with aceda porridge (Dirar 1993). Women of some tribes such as Kenana of the Blue Nile and White Nile, skim-off the floating curd of the over–soured Rob, dry it out in the sun and keep it for future use in the dry summer months to make sauce for stiff porridge, aceda. In recent years some Rob has found its way to markets of nearby towns. The whey or safwa is given to people fasting the holy month of Ramadan at their evening meal.
The Hausa tribe in the Sudan (originally from West Africa) have a popular dish,"gadu-gadu-bi-Rob" which consists of balls of pearl millet flour steamed in Rob.

2.1.1.3. Prospects for industrial production of Rob in Sudan:
Despite the widespread production of Rob in rural Sudan, it is still mainly produced at the household level. The traditional method of Rob fermentation is unsuitable for larger–scale industrial production since the technology has not been standardized. The quality of the resulting fermented product is likely to be of variable nature.

The industrial use of defined starter cultures in the fermentation of traditional cereal-based beverages has resulted in products that are consistent and of high quality (Steinkraus, 1983; Marshall, 1987). By using starter cultures isolated from traditional fermented milk, it may be possible to produce a product of consistent good quality with the retention of the characteristics of the traditional product. The development of suitable starter cultures for the production of Rob would facilitate standardization of the process and may create avenues for larger-scale industrial production, thus increasing the varieties of industrially fermented milk and dairy products in the country.

2.2. Lactic acid bacteria (LAB):
2.2.1. General properties of LAB:
lactic acid bacteria (LAB) constitute a phylogenetically diverse group and are defined as Gram-positive, non-sporing, and non-respiring cocci or rods, catalase negative organisms, devoid of cytochromes , of anaerobic habit but
aerotolerant, fastidious, acid-tolerant, and strictly fermentative bacteria that secrete lactic acid as the major end-product of sugar fermentation (Axelsson, 1998). LAB have a wide distribution in nature and are frequently isolated from environments rich in organic nutrients, such as decaying plant material or the intestinal tracts of mammals. They have a long history in industrial use in the fermentation of milk, vegetables and meat, and as industrial microbes. The importance of LAB is next only to that of the yeast *Saccharomyces cerevisiae*. Interest in LAB as health-promoting bacteria was raised early last century. Since then, several probiotic products have been marketed for human or animal use. LAB are major members of the complex microbial flora in the mammalian intestine and are gaining growing interest in basic and applied research.

Two major pathways of hexose fermentation can be distinguished among LAB. Glycolysis (Embden-Meyerhoff pathway) results in almost exclusively lactic acid as the end-product (homofermentative pathway). On the other hand, breakdown by the 6-phosphogluconate/phosphoketolase pathway results in significant amounts of other end-products, such as ethanol, acetic acid and CO₂, in addition to lactic acid (heterofermentative pathway).

Based on the fermentative characteristics, lactic acid bacteria can be divided into three groups: obligatory homofermentative, obligatory heterofermentative and facultatively heterofermentative (Holt *et al*. 1994). Obligatory homofermentative lactobacilli degrade hexoses exclusively to lactic acid and do not ferment pentoses or gluconate. Obligately heterofermentative lactobacilli degrade hexoses to lactic acid and additional products such as acetic acid, ethanol and CO₂, and degrade
pentoses to lactic and acetic acid. Facultatively heterofermentative lactobacilli ferment hexoses to lactic acid and may produce CO₂ from gluconate but not from glucose. They also ferment pentoses to produce lactic and acetic acids.

**Homolactic fermentation:**
The fermentation of 1 mole of glucose yields two moles of lactic acid:
\[ C_6H_{12}O_6 \rightarrow 2\text{CH}_3\text{CHOHCOOH} \]
Glucose → Lactic acid

**Heterolactic fermentation:**
The fermentation of 1 mole of glucose yields 1 mole of each of lactic acid, ethanol and carbon dioxide.
\[ C_6H_{12}O_6 \rightarrow \text{CH}_3\text{CHOHCOOH} + \text{C}_2\text{H}_5\text{OH} + \text{CO}_2 \]
Glucose → lactic acid + ethanol + carbon dioxide

In fermentation, the raw materials are converted by microorganisms (bacteria, yeasts, and molds) to products that have acceptable qualities of food. In common fermented products such as yogurt, lactic acid is produced by the starter bacterial culture to prevent the growth of undesirable microorganisms (Ray and Daeschel 1992). Food fermentations have a great economic value and it has been accepted that these products contribute to improving human health. Lactic acid bacteria have contributed to the increased volume of fermented foods worldwide, especially foods containing probiotics or health promoting bacteria. Microorganisms of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pediococcus* are involved in these fermentations. In addition, *Lactobacillus* spp. and species of *Bifidobacterium* (which is not
LAB in nature) are part of the normal human intestinal microflora and they have a positive effect on human health (Daly and Davis, 1998).

The lactic acid bacteria of importance in foods belong to the genera of *Cornebacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Paralactobacillus, Streptococcus* and *Weissella* (Sharpe, 1979; Kandler and Weiss, 1986; Vandamme et al. 1996; Stiles and Holzapfel, 1997; Leisner et al. 2000).

**2.2.2 Economic importance of lactic acid bacteria:**
Lactic acid bacteria are widely used in the production of fermented dairy products due to their specific metabolic activities. The production of lactic acid is essential for the production of fermented dairy products and the development of their typical flavor. Acidification (production of organic acid), and other antimicrobial substances, such as bacteriocins, contribute greatly to the preservation of fermented dairy products by inhibiting pathogens and other contaminations, and the transformation of lactose by lactic cultures improves the digestibility of fermented dairy products.

Various metabolic and enzymatic activities of lactic acid bacteria lead to production of volatile substances, which contribute to flavor, aroma and texture developments. Certain lactic acid bacteria produce exopolysaccharides which play a major role in texture development in many fermented dairy products.

Lactic acid bacteria are used extensively in the food industry. In particular, *Lactobacillus* species are essential to the dairy industry in the production of cheese, yoghurt, and other fluid milk products (Kosikowski 1982).
Species of lactic acid bacteria are widely used in the manufacture of dairy products, fermented vegetables and sourdough breads. Dainty and Mackey (1992), Borch et al. (1996), and Nattress and Jeremiah (2000) isolated lactic acid bacteria from stored vacuum-packaged meat and have reported them to be the major spoilage bacteria in these meats after extended periods of storage. Although lactic acid bacteria can cause meat spoilage, they have also been implicated in controlling the growth of meat-borne pathogens (Vold et al. 2000; Nissen et al. 2001).

Olsen et al. (1995) isolated Lactobacillus species from fermented maize dough in order to assess their technological properties such as production of antimicrobial substances and formation of acid in the maize dough. Lactobacillus plantarum is frequently used as a starter culture in food and feed fermentations (McKay and Bladwin 1990), and has been isolated from most of the traditional habitats of lactic acid bacteria (Kandler and Weiss 1986; Molin et al. 1993).

In spite of their wide distribution in nature, and frequent use in industry, the taxonomic data on genomic strain variation within the species is lacking. The need for new and rapid techniques for strain typing of Lactobacillus species has recently been pointed out (Dykes and von Holy 1994).

### 2.2.3. Developments in the biotechnology of LAB:

One area where genetic engineering would be of particular benefit to the dairy industry is the genetic modification of lactic acid bacteria which are commonly used as starter cultures in the production of fermented dairy foods. During the past 20 years much of the research on lactic acid bacteria
focused on dairy lactococci. Present investigations include different lactic acid bacteria involved in a wide variety of fermentation processes, and various lactobacilli and bifidobacteria belonging to the human microbiota. However, significant developments in bacteriophage biology and resistance mechanisms, pyruvate metabolism and production of bacteriocins have also been made (Fitzgerald and Hill, 1996; von Wright and Sibakov, 1998). Moreover, research on chromosomal genetics of LAB is also progressing rapidly. Physical and genetic maps of many of these strains have been constructed (Davidson et al. 1996).

New techniques, such as the ability to sequence large tracts of 16s and 23s rRNA genes using polymerase chain reaction (RAPD-PCR), and the use of pulsed field gel electrophoresis (PFGE) to fingerprint genomic restriction patterns, have contributed enormously to strain identification and classification (Axelsson 1998).

2.2.4 Identification of lactic acid bacteria:  
2.2.4.1 Identification of LAB by conventional methods:  
Traditionally, lactic acid bacteria have been identified by metabolic and other phenotypic characteristics; however this process is labor-intensive, time-consuming and difficult. Recent attempts to develop rapid methods to differentiate and identify strains of lactic acid bacteria in food have proven successful.

The identification with traditional methods is mainly based on physiological characters like the capability to ferment certain sugars, to produce gas or to exhibit certain enzyme activities. These are sufficient for a rough characterization but not for unequivocal identification purposes.
Furthermore, these procedures are time-consuming and ambiguous (Pot et al., 1993). Phenotypic responses can also be affected by environmental conditions (Schleifer et al., 1995), e.g. during the investigation of sourdough lactobacilli, certain wild-type strains fermented more carbon sources than the corresponding type strain (Lonner et al., 1990). Furthermore, it may be impossible for conventional methods to allow a differentiation between phylogenetically distinct species as has been shown by Hayford et al. (1999) for Lactobacillus reuteri and Lactobacillus fermentum, and hence the need for applying genotypic methods. For further phenotypic properties like cell or colony morphology similar observations can be made.

On the other hand, traditional laboratory culture techniques may not result in accurate identification of bacteria, and may take extremely long times before results can be obtained. An analysis using traditional techniques may take anywhere from days to weeks before getting results. For this reason, new techniques are being produced for the rapid, sensitive, and specific identification of bacteria. These are mainly molecular techniques, among which the use of genetic probes and utilization of the polymerase chain reaction are prominent (Josephson, et al. 1991).

To satisfy the need for a simplified means of identification of lactic acid bacteria, Microsystems - both in kit form and as part of automated identification systems- have been introduced to the microbiology community (Appelbaum et al. 1984; Kempfer and Dott, 1989). These systems offer an attractive alternative to conventional methodologies through such features as rapid (24-48 h) computer-assisted identification, convenience, ease of use and overall cost effectiveness.
2.2.4.2 Molecular identification of LAB:
During the last few years various approaches that use molecular methods based on genotype characterization, including PCR-based typing and identification methods, have been described for LAB strains (Klijn et al. 1991; Le-Bourgeois et al. 1992; 1993). The advance in molecular biology during the last decade has resulted in a large number of methods for the analysis and characterization of nucleic acids. In particular, since the introduction of the PCR (Saiki et al., 1988), most of the nucleic acid-based methods rely on the amplification of target sequences. In contrast to the described methods for identification by probes, typing methods are based on the generation of fingerprints through the electrophoretic separation of DNA fragments. Today the most important method to distinguish bacteria at the (sub) specific level is the Randomly Amplified Polymorphic DNA (RAPD), which is commonly used for bacterial identification.

2.3. RAPD-PCR
A few years ago, a new genetic assay was developed independently by two different laboratories (Welsh and McClelland, 1990; Williams et al. 1990). This assay is called Random Amplified Polymorphic DNA (RAPD) and is entirely dependent on the PCR protocol. The RAPD assay detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. In this reaction, a piece of primer binds to the genomic DNA at two sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocyclic amplification. The presence of each amplification product identifies complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer at each end of
the amplified product. Generally, each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphisms between individuals (Tingey and del Tufo, 1993).

In the RAPD assay, referred to as arbitrary-primed PCR, patterns are generated by the amplification of random DNA segments with single short (typically 10 bp) primers of arbitrary nucleotide sequences. The primer is not targeted to amplify any specific bacterial sequences and will hybridize at multiple random chromosome locations and initiate DNA synthesis. After separation of the amplified DNA fragments by agarose gel electrophoresis, a pattern of bands results, which is characteristic of the particular bacterial strain (Williams et al. 1990; Welsh and McClelland, 1992; Maunier and Grimient, 1993).

RAPD analysis has been used to compare inter- and intra-specific differences in bacteria (Welsh et al. 1992; Welsh and McClelland, 1990; Mazurier and Wernars, 1992; Mazurier et al. 1992). By reducing the stringency of the primer annealing step in the PCR reaction, a single primer that has no known homology to a genome can anneal at sites for which the match is imperfect, and allow certain regions of the genome to be amplified.

RAPD-PCR could be considered as a powerful tool to reveal extensive DNA polymorphisms between the genomes of different species under the same genus. Keinath et al. (1995) used the RAPD- technique to distinguish between Phoma cucurbitacearum, the causal agent of gummy stem blight
of cucurbits, and other *Phoma* spp., such as *P. exigua* which has been reported to cause symptoms of gummy stem blight.

RAPD-PCR analysis also plays an important role in studying genetic variation within fungal species differing in their geographic locations and virulence. Guthrie *et al.* (1992) used RAPD markers for comparison of sorghum isolates of *Colletotrichum graminicola* collected from Africa, United States, India and Puerto Rico. They found that RAPD marker analysis was a simple and fast technique for isolates differentiation.

Duncan *et al.* (1993) used the RAPD assay in analyzing genetic variation between isolates of *Rhizoctonia solani*, collected from different geographic locations and belonging to different anastomosis and pectic zymogram groups. They found that all the anastomosis and pectic zymogram groups could be distinguished by using RAPD assay. In some groups, there was considerable variation in the fingerprint patterns between isolates, and this variation was more marked between isolates from different locations. They concluded that RAPD-PCR analysis was a very useful alternative to anastomosis grouping for identification of *R. solani* isolates.

Recently RAPD techniques specific for lactic acid bacteria and enterococci isolated from dairy products, vegetable fermentations and gastrointestinal microflora have been developed and successfully applied (Cocconcelli *et al* 1995, 1997; Drake *et al.* 1996; Roushdy *et al.* 1998; Tailliez *et al.* 1996)

The application of molecular techniques, such as RAPD analysis has allowed the characterization of the bacterial associations of natural starter culture for fermented milk production. Since these days the RAPD-PCR
techniques has been applied to many problems in bacterial microbiology mainly in the characterization of complex habitats or the differentiation of isolates. Furthermore, the RAPD analysis demonstrated that variation. Sohier et al. (1999) showed that RAPD-PCR was more efficient than phenotypic or biochemical approaches for the identification of *Lactobacillus hilgardii* and *Lactobacillus brevis* at the species level. Torriani et al. (1996) used the discriminatory power of RAPD-PCR to classify lactic acid bacteria strains isolated from wines.

The RAPD assay has been shown to be suitable for different bacteria (Mazurier and Wernars, 1992; Stephan et al., 1994) including *Lactobacillus* spp. (Duplesia and Dicks, 1995). It allows discrimination at species level (van der Vossen and Hofstra, 1996). However, the use of empirically designed primers makes standardization of the method difficult and the discriminatory power depends largely on the primers used (Welsh and McClelland, 1992; Maunier and Grimont, 1993; Kerr, 1994).

Rodtong and Tannock (1993) suggested that restriction fragment length polymorphism (RFLP) of the 16S rRNA genes was a suitable method for strain typing of lactobacilli. However, chromosomal DNA cleaved by the restriction enzymes *EcoR*I and *Hind*III gave identical patterns for most of the tested strains of *Lactobacillus plantarum* (Johansson et al. 1995) which indicates that RAPD might be more appropriate in this case.

Abdelgadir et al. (2001) isolated the predominant microorganisms in the Sudanese traditional fermented dairy product "Rob" from cows. One hundred and eighty nine representative strains of lactic acid bacteria and 109 representative isolates of yeasts were identified using morphological,
physiological and molecular typing techniques. Through use of the internal transcribed spacer- polymerase chain reaction, they divided the LAB isolates into nine groups, according to the size of the PCR product formed. Representative members of all groups were identified by the API 50 CH system for lactic acid bacteria. Confirmation of identification of lactic acid bacteria species was achieved by sequencing the 16S rRNA genes. The predominant lactic acid bacteria in Rob fermentation were found to be *Lactobacillus fermentum*, *Lactobacillus acidophilus*, and *Streptococcus salivarius*.

Roushdy (1999) used RAPD-PCR with an OPL-O1 primer to investigate the genetic diversity of 35 strains of LAB isolated from “Laban Rayeb” and divided the strains into seven clusters based on numerical analysis of RAPD-PCR profiles. The representative strain of each cluster was identified by the API 20 STREP identification kit. These isolates were identified as *Enterococcus faecalis* (4 strains), *Enterococcus faecium* (14 strains), *Lactococcus lactis* subsp. *lactis* (13 strains), and *Streptococcus agalactiae* (4 strains).

Johansson *et al.* (1995) used RAPD-PCR for the rapid typing of *Lactobacillus plantarum* strains isolated from traditional habitats. RAPD may be used with either purified chromosomal DNA serving as template in the polymerase chain reaction, or with crude cell extracts, using a 9-mer primer with 80% G+C content. They found that the two sources of template DNA gave the same clusters and sub-clusters of the strains at the similarity level of 50%. About 50% of the strains could be individually separated from all the other tested strains.
Booysen et al. (2001) used numerical analysis of total soluble cell protein patterns and RAPD-PCR banding patterns to identify a number of lactic acid bacteria to species level which they isolated during the malting of two different barley cultivars, and they found that Leuconostoc argentinum, Leuconostoc lactis, and Weissella confuse were most predominant in both cultivars. A few strains were identified as Weissella paramesnteroides (four strains), Lactobacillus casei (five strains), Lactococcus lactis (five strains) and Lactobacillus rhamnosus (two strains).

Alice et al (1999) used RAPD for the characterization of 172 dominant Lactobacillus isolates from present and previous studies of Ghanaian maize fermentation. They found that heterofermentative lactobacilli dominate the fermentation flora since approximately 85% of the isolates belong to this group; and the cluster analysis of the RAPD profiles obtained showed the presence of two main clusters: cluster 1 included Lactobacillus fermentum, whereas cluster 2 comprised the remaining Lactobacillus spp.

2.3.1 Polymerase chain reaction

Polymerase Chain Reaction (PCR) technology has led to the development of several novel genetic assays based on selective DNA amplification (Innis et al. 1990; Saiki et al., 1988). Since then, a great revolution in the different aspects of biological sciences has occurred.

Polymerase chain reaction (PCR) amplification of DNA and RNA (Saiki et al. 1985) has become a key protocol in many biological laboratories. The polymerase chain reaction technique can be used to identify very low quantities of a specific nucleotide sequence contained within the genome of an organism by a process of directed DNA synthesis.
As described by Mullis and Faloona (1987) a typical cycle of polymerase chain reaction (PCR) involves denaturing the double-stranded DNA into single strands, annealing short oligonucleotide primers to the single strands and extending the primer sequence using a DNA polymerase to complete the synthesis of strands complimentary to the original single strands. The cycling is repeated to obtain an exponential increase in the number of copies of the original DNA strand (Kureishi et al. 1994; Victor et al., 1991).

Only a decade ago, the prospect of producing billions of amplified copies of a specific nucleic acid sequence by performing successive rounds of \textit{in vitro} nucleic acid replication would have been considered science fiction. Interestingly, the basic ingredients for an \textit{in vitro} nucleic acid amplification method were described by Kleppe et al. (1971). In 1983, K. Mullis envisioned a process of an \textit{in vitro} nucleic acid amplification that eventually became known as the Polymerase Chain Reaction (PCR) (Mullis, 1990). Buoyed by several technologies that matured in the 1980s, including automated oligonucleotide synthesis, PCR became a reality in a relatively short time. (Saiki et al. 1985). There are three steps in a PCR amplification cycle; these are:

\subsection*{2.3.1.1 Template denaturation:}
Template denaturation occurs at temperatures greater than the melting temperature of the DNA. Denaturation separates template DNA into single strands allowing subsequent primer annealing by heating (95\textdegree{}-100\textdegree{}C), thereby breaking the hydrogen bonds which bind the two strands of DNA together.
2.3.1.2 Primer annealing:
Primer annealing occurs at a lower temperature which is typically 40-70°C. The higher temperature of annealing, and the extent of annealing of mismatched primer to template is reduced. However, as primer annealing temperature increases, there is an associated decrease in sensitivity (Yamane et al. 1989).

2.3.1.3 Primer extension:
Extension, which involves the synthesis of a DNA strand complementary to the template, extends from the primer and proceeds from the 3' end of each primer and results in a double stranded copy of target DNA from each original single strand (Tokue et al. 1991).

Primer extension occurs at 72°C and is catalyzed by the Taq DNA polymerase. All the reaction components are placed in a microfuge tube and cycles of PCR amplification carried out in the temperature cycles. The cycling process, which involves alternately heating the reaction mixture to 95°C and then rapidly cooling it to 50°C, continues efficiently as a result of the use of heat-stable DNA polymerase from the bacterium Thermus aquaticus (The Taq polymerase). This bacterium usually lives in hot springs (Olive, 1989). The optimum temperature for the work of Taq polymerase is 72°C, at which the enzyme will start constructing the complementary strands using the four deoxynucleotides [deoxyadenosine 5-triphosphate (dATP), deoxycytidine 5-triphosphate (dCTP), deoxyguanosine 5-triphosphate (dGTP), and deoxythymidine 5 triphosphate (dTTP)] (Pepper, et al. 1995).
At each cycle of denaturation, binding and extension is performed, and the number of DNA copies increases by a factor of $2^n$, where $n$ is the number of cycles. Thus, after 30 cycles, approximately $2^{30}$ fold amplification of a specific DNA segment can be achieved.

The majority of the DNA produced consists of short products representing only the area between the oligonucleotide primers (Tokue et al. 1991). The amplified DNA is detected via electrophoresis and subsequent ethidium bromide staining. When viewed under a UV transilluminator, the DNA appears pink (Mecca, et al. 1995; Fratamico et al., 1995; Tornieporth et al. 1995).
CHAPTER THREE
MATERIALS AND METHODS

3.1. Sampling:
Ten samples of traditional Sudanese fermented cow milk (Rob) were collected from ten different production sites in the Sudan, viz. Khartoum, Madani, Nyala, El-Fasher, Kasala, ElObied, Elgadarif, Dongola, Khartoum North and Omdurman. The Rob samples were collected in 250-ml sterile screw-cap bottles kept in a refrigerator (5°C) until the time of sampling.

3.2. Isolation of strains of lactic acid bacteria:
Form each sample, 10 ml of Rob were diluted with 90 ml of sterile 1.5% peptone water (Difco, USA, pH 7.0) and homogenized. Serial dilutions were prepared and 100µl from the 10⁻⁷ dilution of the samples were inoculated on plated of MRS Agar medium (de Man, Rogosa and Sharp 1960). The medium consisted of: Casein peptone tryptic digest 10g, meat extract 10g, yeast extract 5.0g, glucose 20.0g, K₂HPO₄ 2.0g, sodium acetate 5.0g, di-ammonuim citrate 2.0g, MgSO₄.7H₂O 0.2g, MnSO₄.H₂O 0.05g., and distilled water 1000 ml. The pH was adjusted to 6.2-6.5 according to Hitchner et al. (1982). All plates were incubated at 37°C for 3 days under anaerobic conditions using anaerobic jars with a gas generating kit (Oxoid, BR 38).

3.3. Purification and preservation of LAB isolates:
Bacterial colonies from the plates of the appropriate dilution were picked at random for characterization, based on differences in morphology (size, form and shape). The different isolates were tested for their Gram reaction and catalase and oxidase activity. Only Gram- positive, catalase and
oxidase negative isolates were purified by subculturing and successive streaking onto the same agar medium before being subjected to preliminary identification, and then pure cultures were maintained in MRS broth with glycerol at 4°C.

3.4. Preliminary identification and biochemical tests:
The preliminary identification tests included Gram staining, microscopic examination, catalase and oxidase activity, carried out according to the methods described by Marshall (1993).

3.5. Gram stain:
A discrete colony was carefully picked with sterile wire loop. The colony was emulsified in a drop of saline, placed on a clean slide and evenly spread to make a thin film. The slide was allowed to dry in air and the smear was stained with crystal violet solution for 1 minute and rinsed rapidly with water. Gram iodine solution was added and left for 1 minute, then poured off and the preparation was left to dry, and then the slide was washed with 95% ethanol. The slide was rinsed under tap water and stained with safranin for 20 seconds, and then washed well and blot dry. The smear was then examined microscopically by the oil immersion lens.

3.6. Biochemical tests:
3.6.1. Oxidase test:
A piece of filter paper was impregnated with oxidase test solution (1% aqueous solution of tetramethyle –p-phenylenediamine hydrochloride). A loopfull from a 24 hours culture was streaked onto the filter paper. A positive reaction was indicated by development of purple colour within 10-15 seconds.
3.6.2. Catalase activity:
One drop of 3% hydrogen peroxide solution was placed on a clean slide. A loopfull from 24 hour culture was added. The release of bubbles of gas (oxygen) indicated the presence of catalase enzyme.

3.7. Growth temperatures:
Growth at 10º C and 45º C as reported in Holt et al. (1994) was assayed using two sets of tubes, each of which contained 9 ml MRS broth inoculated with 1 ml of a 24 hours-old broth culture of each LAB isolate. The first set was incubated at 10º C, and the second at 45º C. Incubated tubes were examined for growth (turbidity) after 48 hours at 45º C, and after 5 days at 10º C.

3.8. Growth in 6.5% NaCl:
Growth in the presence of 6.5% NaCl in MRS broth was determined according to the method described in Holt et al. (1994), in which 9 ml of the MRS broth were inoculated with 1 ml of a 24 hours-old broth culture of each LAB isolate, and incubated at 37º C for 48 hours. Growth was assayed by visual assessment of turbidity.

3.9. Production of acid and gas (CO₂) from glucose anaerobically:
Homo- and hetero-fermentative LAB isolates were differentiated according to Hitchner et al. (1982) using 1.5% peptone water + 1% glucose + 1.0 ml of fresh Andrade indicator solution per 100 ml distilled water, distributed in test tubes, and the medium tubes contained inverted Durham tubes. The medium was autoclaved at 110ºC for 10 minutes then inoculated and anaerobically incubated at 37ºC. All tubes were daily examined for acid
and/or gas production for 7 days. The tubes were examined for change in colour and gas accumulation in the Durham tubes.

3.10. **Action on litmus milk:**
A few drops of Andrade indicator were added to Litmus Milk medium (10.0 ml of litmus milk solution in 1.0 liter of defatted milk) till a purple colour developed, then the medium was distributed in test tubes and sterilized at 121°C for 20 minutes, inoculated by the isolates and incubated at 37°C for 24 hours. Results were expressed as positive (curdling) or negative (no curdling).

3.11. **Production of exopolysaccharides (EPS):**
For the selected 42 isolated strains EPS production was observed on MRS agar in which glucose was replaced by 5% sucrose. Plates were incubated for 24 hours at 30°C and growth was compared to that of the same isolates grown in media without sucrose. Polysaccharide producing colonies were recognized by their larger size and mucoid appearance in the glucose-supplemented medium. Confirmation of the phenotype was done by microscopic examination of negatively stained cell preparations (Hitchener *et al.* 1982).

3.12. **Molecular techniques:**
The method used in the molecular characterization of the isolates in this study is Random Amplified Polymorphic DNA-polymerase chain reaction (RAPD-PCR).
3.12.1. Sample preparation:
Selected lactic acid bacteria (42 isolates) were grown overnight in MRS agar, a loopful of growth was transferred to 0.1 ml H₂O and then transferred to boiling water for 10 min to lyse the cells. The resulting cell lysate was centrifuged for 10 seconds at 10000 rpm (Eppendorf model 4515C centrifuge), and 5µl of supernatant was used as a DNA sample in the PCR, as described by Carozzi et al. (1991).

3.12.2. Preparation of random amplified polymorphic DNA-PCR:
The amplification reaction was carried out in a volume of 50 µl. Each reaction mixture contained 10x PCR reaction buffer (Tris 108.0g +boric acid 55.0g +EDTA 7.44g + H₂O(dd) up to 1 liter), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2mM MgCl₂ (0.01% W/V), 250µl of each of the four deoxynucleotide triphosphates (dNTPs) (dGTP, dATP, dCTP and dTTP), 2.5 units of *Taq* polymerase (Promega Corp., Madison, USA), 50 pmoles of each primer and 5µl of the template DNA. A master mix was prepared in a 5ml microfuge tube, according to the number of PCR reactions to be performed, with an extra reaction included to compensate for the loss of part of the solution due to frequent pipetting. An aliquot of 45µl master mix solution was dispensed in each PCR tube (0.2ml), containing 5 µl of the appropriate template DNA, so that each reaction contained:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per one PCR reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs mix</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Primer</td>
<td>2.0 µl</td>
</tr>
<tr>
<td><em>Taq</em> polymerase</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>10x PCR buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>H₂O (dd)</td>
<td>up to 50 µl</td>
</tr>
</tbody>
</table>

The PCR contents were mixed and overlaid with a few drops of mineral oil.

### 3.12.3. Primers used in RAPD analysis:

The oligonucleotide sequences of the four ten-mer random primers used in this study were: O-10 (5′TCAGAGCGCC3′), O-16 (5′TCGGCGGTTTC3′), A-11 (5′CAATCGCCGT3′) and C-04 (5′CCGCATCTAC3′). These primers were synthesized at the Agricultural Genetic Engineering Research Institute (AGERI), Giza, Egypt.

### 3.12.4. PCR program and temperature profile:

DNA amplification was carried out in a DNA thermal cycler programmed for 40 cycles after initial denaturation for 3 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min. The primer extension phase was extended to 7 min at 72°C in the final cycle. The amplified product was cooled at 4°C until samples were retrieved.

### 3.12.5. Gel electrophoresis of PCR products:

The PCR amplification products were analyzed by electrophoresis separation. Twenty µl of each PCR product were mixed with 3µl loading dye and loaded into the wells of the 1.5% agarose gel. The gel was stained with 43µl ethidium bromide (10 mg/ml). One Kb ladder DNA (Gibco BRI) was used as molecular marker. The gel was run for about 2 hours at 100 V in 1X TBE electrophoresis buffer (1x buffer: 10g Tris –base, 5.5g boric acid and 4 ml 0.5M Na-EDTA, pH 8.0 in 1.0 liter).
3.12.6. Visualization and photography:
After electrophoresis, the RAPD patterns were visualized with a UV Trans-illuminator and the gels were photographed using a Polaroid camera (MP4 camera) and Polaroid films type S7 (ASA 3000).

3.12.7. Reading of band patterns:
Amplified products were visually examined and the presence or absence of each band was scored as 0 or 1, respectively.

3.12.8. RAPD analysis:
The banding patterns generated by the RAPD-PCR analysis were compared to determine the genetic relatedness of LAB isolates. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical. The similarities between RAPD-PCR profiles were calculated using the Dice coefficient, and then a dendrogram was derived from the distance by the unweighted paired-group method, arithmetic mean (UPGMA) algorithm contained in a computer program: Diversity Data Base™ Fingerprinting Software (Bio-Rad).

3.13. Carbohydrate fermentation:
Ability of the isolates to ferment carbohydrates was studied using the API 50 CHL STREP system for the identification of lactobacilli and API 20 STREP for the identification of lactococci (La Balme les Grottes, 38390, Montalieu Vercieu, France). The results were recorded after 24 and 48 h. as recommended by the manufacture. The selected strains were subcultured in MRS broth before tests were performed.
3.14. Computer analysis

Patterns of fermented carbohydrates were used in a preliminary attempt of identification, using the computer-aided identification programme for lactic acid bacteria as developed by Cox and Thomsen (1990).
CHAPTER FOUR
RESULTS

4.1. Phenotypic characterization of LAB strains:

The lactic acid bacteria that are considered as natives in milk and milk products include *Lactobacillus delbreuckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. acidophilus*, *Lb. plantarum*, *Lb. fermentum*, *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecium*, *En. faecalis*, *Sterptococcus thermophilus* and *Leuconotoc* spp.

One hundred and sixty LAB isolates were obtained from 10 samples of traditional Sudanese fermented cow's milk (Rob) collected from different locations in the Sudan. Microscopic examination revealed that some isolates were rod-shaped while others were cocci. Among the 160 isolates, 32 isolates (20%) were rods which occurred either singly or in chains, produced lactic acid but no gas (CO₂) from glucose anaerobically. These were considered as obligatory homofermentative lactobacilli. The other 128 isolates (80%) were cocci which occurred either in pairs or in chains, and produced lactic acid from glucose anaerobically. Among these, 15 isolates (11.72%) produced gas (CO₂) from glucose anaerobically, and they were presumptively identified as *Leuconostoc* spp. The other 113 coccal isolates (88.18%) produced no gas from glucose anaerobically, and they were presumptively identified as aerococci, enterococci, lactococci and streptococci. The various groups of the isolated strains are presented in Table 1.
Table 1. Major groups of the 160 LAB isolates obtained from 10 Rob samples:

<table>
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<tr>
<th>Shape of isolates</th>
<th>No. of isolates</th>
<th>Gas from glucose</th>
<th>Percentage of total</th>
</tr>
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<tbody>
<tr>
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<td>32</td>
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<tr>
<td>Cocci</td>
<td>123</td>
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<tr>
<td>Cocci</td>
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<td>Total</td>
<td>160</td>
<td></td>
<td>100</td>
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</table>
Morphological examination and physiological tests were carried out. These tests included: Gram stain reaction, catalase and oxidase activity, growth in presence of 6.5% NaCl, growth at 10° and 45°C, gas production from glucose anaerobically to differentiate heterofermentative from homofermentative strains, action on litmus milk and production of exopolysaccharides from sucrose. All isolates were found to be Gram-positive, oxidase and catalase negative.

On the basis of a number of preliminary tests which are commonly used for the identification of lactic acid bacteria, 42 homo- and heterofermentative isolates were selected for further investigations and characterization. Fourteen of these were rods, five were gas-producing cocci and 23 were cocci that produced no gas from glucose anaerobically as shown in Table 2.

4.2 Molecular fingerprinting:
4.2.1 RAPD-PCR profiles:
The RAPD technique was applied to the 42 selected isolates of lactic acid bacteria in an attempt to detect specific DNA profiles of species, strains or biotypes in order to elucidate the genetic diversity of the microflora, with the aim of confirming the phenotypic identification and for further characterization.

The 42 isolates included homo- and heterofermentative isolates of the lactic acid bacteria (Table 2), and four primers were used for the RAPD-PCR fingerprinting. The primers: O-10 (sequence 5′TCAGAGCGCC3′, Fig. 1), O-16 (sequence 5′TCGGCGGTTC3′, Fig. 2) and A-11 (sequence 5′CAATCGCGGT3′, Fig. 3) gave the largest number of bands. The 4th primer C-4 (sequence 5′CCGCATCAC3′ Fig. 4) resulted in fewer bands.
The RAPD-PCR fingerprinting patterns of the selected strains obtained with the four above primers were analyzed together as a single set of data to obtain single dendrograms as shown in Fig. 5 (coci) and Fig. 6 (rods). Numerical analyses of RAPD-PCR profiles were conducted. The similarities between RAPD-PCR profiles were calculated using the Dice coefficient. Cluster analysis was performed by the unweighted pair-group method using arithmetic averages (UGGMA). The computer program: Diversity Data Base™ Fingerprinting Software (Bio-Rad) was used for fingerprinting.

The profiling of the 14 rod-shaped lactic acid bacterial isolates resulted in five clusters (Table 3). These clusters were observed at the similarity level of about 80% (Fig. 6). On the other hand, the 28 coccal strains were clustered in nine different clusters as shown in Table 4.
Table 2. The isolates of lactic acid bacteria that were subjected to morphological and physiological tests.

<table>
<thead>
<tr>
<th>Serial NO.</th>
<th>Gram reaction and shape</th>
<th>Catalase activity</th>
<th>Oxidase activity</th>
<th>Growth in 6.5% NaCl</th>
<th>Growth at 10°C</th>
<th>Growth at 45°C</th>
<th>Milk curdling</th>
<th>Acid from glucose anaerobically</th>
<th>Gas from glucose anaerobically</th>
<th>Sugar fermentation</th>
<th>Polysaccharide from sucrose</th>
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<td>Oxidase activity</td>
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<td>Growth at 45(^{\circ})C</td>
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<td>Acid from glucose anaerobically</td>
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<td>Polysaccharide from sucrose</td>
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<td>Catalase activity</td>
<td>Oxidase activity</td>
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Fig 1. RAPD profiles of the 42 selected LAB isolates generated with primer O10 (70% G+C). Lanes bear the strain numbers shown in Table 2. Lanes "M" are molecular weight markers (1Kb ladder, Gibco BRL).
Fig2. RAPD profiles of the 42 selected LAB isolates generated with primer O16 (70%G+C). Lanes bear the strain numbers shown in Table 2. Lanes "M" are molecular weight markers (1Kb Ladder, Gibco BRL).
Fig 3. RAPD profiles of the 42 selected LAB isolates generated with the primer A11 (60%G+C). Lanes bear the strain numbers shown in Table 2. Lanes "M" are molecular weight markers (1Kb Ladder, Gibco BRL).
Fig 4. RAPD profiles of the 42 selected LAB isolates generated with the primer C-4 (60% G+C). Lanes bear the strain numbers shown in the Table 2. Lanes "M" are molecular weight markers (1Kb ladder, Gibco BRL).
Figure 5. Dendrogram showing the clustering of the 28 coccal-shaped LAB isolates (Nos 1-28) from Rob
Obtained by numerical analysis of RAPD-PCR profiles. Clustering was obtained by UPGMA programme.
Figure 6. Dendrogram showing the clustering of the 14 rod-shaped LAB isolated from Rob (Nos 29-42). Obtained by numerical Analysis of RAPD-PCR profiles. Clustering was obtained by UPGMA program.
4.2.2. Clustering and biochemical tests of the selected rode-shaped isolates:

Representative strains of each RAPD-PCR cluster were further identified using the API 50 CHL STREP system for the bacilli (Table 5), and the API 20 STREP system for the cocci (Table 6). A computer program (Cox and Thomsen, 1990) was utilized for analysis of the API STREP identification system which resulted in assignment of the isolates to genera and species (Table 7). Results of the biochemical tests confirmed the clustering scheme obtained through the RAPD-PCR profiling system (Figs 5 and 6).

Cluster one (rods) comprised only one strain (No 40) which was identified as *Lactobacillus pentosus* (Fig 5). This strain was not able to grow at 10°C, 45°C or in the presence of 6.5% NaCl, did not curdle litmus milk, and was unable to produce polysaccharides from sucrose (Table 2). It was able to ferment amygdaline, galactose, glucose, lactose, and mannose. All strains failed to ferment raffinose, rhamnose or maltose (Table 7).

Cluster two consisted of one isolate (No 42) which was identified as *Lactobacillus delbreukii* subsp.*bulgaricus*. It was able to produce lactic acid, but did not produce gas from glucose anaerobically. These results suggested that this strain was obligatory homofermentative. The strain was able to grow at 45°C but not at 10°C or in the presence of 6.5% NaCl. It was able to produce polysaccharides when grown in a medium with 5% sucrose. It curdled litmus milk very well (Table 2). It showed good fermentative ability with glucose, fructose, lactose and ribose, but was unable to ferment amygdaline, arabinose, mannose, melibiose, mannitol, maltose, rhamnose, raffinose or D-xylose (Table 7).
Table 3. Clustering of the 14 rod-shaped LAB isolates obtained from Rob (based on the results of cluster analysis by RAPD-PCR)

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<th>% of the 42 selected isolates</th>
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<td>2.38</td>
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<td>3</td>
<td>2 (No. 33 and 41)</td>
<td>4.76</td>
</tr>
<tr>
<td>4</td>
<td>1 (No. 39)</td>
<td>2.38</td>
</tr>
<tr>
<td>5</td>
<td>9 (No 29,30,31,32,34,35,36,37 and 38)</td>
<td>21.42</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
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</tr>
</tbody>
</table>
Table 4. Clustering of the 28 coccal-shaped LAB isolates obtained from Rob (based on the results of cluster analysis by RAPD-PCR)

<table>
<thead>
<tr>
<th>Cluster No.</th>
<th>Isolates</th>
<th>% of the selected isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1 (No. 26)</td>
<td>2.38</td>
</tr>
<tr>
<td>7</td>
<td>2 (No. 8 and 18)</td>
<td>4.76</td>
</tr>
<tr>
<td>8</td>
<td>2 (No. 1 and 2)</td>
<td>4.76</td>
</tr>
<tr>
<td>9</td>
<td>2 (No. 15 and 17)</td>
<td>4.76</td>
</tr>
<tr>
<td>10</td>
<td>3 (No. 5, 6 and 10)</td>
<td>7.14</td>
</tr>
<tr>
<td>11</td>
<td>2 (No. 13 and 16)</td>
<td>4.76</td>
</tr>
<tr>
<td>12</td>
<td>7 (No. 3, 4, 7, 22, 23, 24 and 27)</td>
<td>16</td>
</tr>
<tr>
<td>13</td>
<td>4 (No. 9, 11, 12 and 14)</td>
<td>66</td>
</tr>
<tr>
<td>14</td>
<td>5 (No. 19, 20, 21, 25 and 28)</td>
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<tr>
<td>Total</td>
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</tr>
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</table>
Table 5. Identification of representatives of the 5 clusters of rod-shaped isolates using the API 50 CHL STREP identification system.

<table>
<thead>
<tr>
<th>Cluster No.</th>
<th>Serial No of representative</th>
<th>API identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td><em>Lactobacillus pentosus</em></td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td><em>Lactobacillus delbreuckii ss. bulgaricus</em></td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td><em>Lactobacillus plantarum</em></td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td><em>Lactobacillus casei</em></td>
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<tr>
<td>5</td>
<td>29</td>
<td><em>Lactobacillus rhamnosus</em></td>
</tr>
</tbody>
</table>
Table 6. Identification of representatives of the 9 clusters of coccal-shaped isolates using the API 20 STREP identification system.

<table>
<thead>
<tr>
<th>Cluster No.</th>
<th>Serial No of representative</th>
<th>API identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>26</td>
<td><em>Streptococcus acidominimus</em></td>
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<tr>
<td>7</td>
<td>8</td>
<td><em>Enteococcus faecium</em></td>
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<tr>
<td>8</td>
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<td><em>Enteococcus gallinarum</em></td>
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<tr>
<td>9</td>
<td>15</td>
<td><em>Streptococcus bovis</em></td>
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<tr>
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<td>10</td>
<td><em>Leuconostoc sp.</em></td>
</tr>
<tr>
<td>11</td>
<td>13</td>
<td><em>Leuconostoc sp.</em></td>
</tr>
<tr>
<td>12</td>
<td>23</td>
<td><em>Streptococcus acidominimus</em></td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td><em>Aerococcus viridans</em></td>
</tr>
<tr>
<td>14</td>
<td>25</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
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</table>
Table 7: Biochemical characterization of representative strains of each cluster of the selected LAB isolates from Rob.

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<th>Isolate No.</th>
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<th>Ara</th>
<th>Celle</th>
<th>Esc</th>
<th>Fru</th>
<th>Gal</th>
<th>Glu</th>
<th>Lac</th>
<th>Mal</th>
<th>Mani</th>
<th>Mann</th>
<th>Meli</th>
<th>Raf</th>
<th>Rham</th>
<th>Rib</th>
<th>Sor</th>
<th>Suc</th>
<th>Tre</th>
<th>D-xy</th>
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<td>-</td>
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<td>Lb. delbruekii ss bulgaricus</td>
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<td>d</td>
<td>+</td>
<td>Lactococcus lactis ss.lactis</td>
<td></td>
</tr>
</tbody>
</table>

Positive reaction (+), negative reaction (-), most strains are positive (d), not determined (ND).

Amyg= Amygdaline, Arab= Arabinose, Celle= Cellebiose, Esc= Esuclin, Fru= Fructose, Gala= Galatose, Glu= Glucose, Lac= Lactose, Mal= Maltose, Mani= Mannitol, Mann=Mannose, Meli= Melibiose, Raff= Raffinose, Rham= Rhamnose, Rib= Ribose, Sor= Sorbitol, Suc= Sucrose, Tre=Trehalose, D-xy= D-xylose.
Cluster three included two isolates (Nos 33 and 41) the representative of which (No 33) was identified as *Lactobacillus plantarum*. They grew at 10°C, but not at 45°C or in presence of 6.5% NaCl, produced polysaccharides from sucrose, and curdled litmus milk (Table 2). It was able to ferment amygdaline, arabinose, fructose, lactose, melibiose, raffinose, sucrose, trehalose, but not D-xylose or rhamnose (Table 7).

Cluster four consisted of one isolate (No 39) which was identified as *Lactobacillus casei*. It was able to grow at 10°C but not at 45°C. It could grow in the presence of 6.5% NaCl and curdled litmus milk, but was not able to produce polysaccharides from sucrose (Table 2). The representative strain of this cluster (No 39) was able to ferment amygdaline, fructose, galactose, lactose, maltose, mannose and rhamnose but failed to ferment arabinose or raffinose (Table 7).

Nine isolates formed cluster 5 (Nos 29, 30,31,32,34,35,36,37 and38). Their representative (No 29) was identified as *Lactobacillus rhamnosus*. It was able to grow at 45°C but not at 10°C. It grew in the presence of 6.5% NaCl, failed to produce dextran from sucrose and also failed to curdle litmus milk (Table 2). Most strains in this group fermented amygdaline, arabinose, galactose, glucose, fructose, lactose and rhamnose, but failed to ferment melibiose, raffinose or D-xylose (Table 7).

4.2.3. Clustering and biochemical tests of the selected coccal-shaped isolates:

At the 80% level of cut-off similarity, the coccoid strains were grouped into nine distinct clusters designated 6-14 (Fig 5). Representative strains of each RAPD-PCR cluster were identified by the API 20 STREP identification system as presented in Table 6.
Cluster six included only one isolate (No 26) which was identified as *Streptococcus acidominimus*. It was able to grow at 10°C but not at 45°C or in the presence of 6.5% NaCl, was not able to curdle litmus milk and also did not produce polysaccharides from sucrose (Table 2). This isolate fermented glucose, lactose, mannitol, rhamnose, and trehalose but was not able to ferment raffinose or ribose (Table 7).

Cluster seven included two isolates (Nos 8 and 18) the representative of which (No 8) was identified as *Enterococcus faecium*. Most strains in this cluster grew at 10°C, 45°C and in the presence of 6.5% NaCl, produced polysaccharides from sucrose and curdled litmus milk (Table 2). Their representative was able to ferment fructose, glucose, lactose, mannitol, maltose, melibiose, ribose and sucrose, but failed to ferment amygdalin, cellobiose, sorbitol, raffinose, rhamnose or D-xylose (Table 7).

Cluster eight comprised two strains (Nos 1 and 2) the representative of which (No 2) was identified as *Enterococcus gallinarum*. Both strains were able to grow at 10°C, 45°C and in the presence of 6.5% NaCl, were not able to produce polysaccharides form sucrose, and curdled litmus milk as shown in Table 2. It was noticed that the selected strains of cluster seven (No 8) and cluster eight (No 2) were phenotypically identical, but the representative strain in cluster eight failed to ferment raffinose or D-xylose (Table 7).

Cluster nine consisted of 2 isolates (Nos 15 and 17) the representative of which (No 15) was identified as *Streptococcus bovis*. It was able to grow at 45°C but not at 10°C or in the presence of 6.5% NaCl, was unable to curdle litmus milk or to produce polysaccharides from sucrose (Table 2). They were able to ferment...
lactose, sucrose, raffinose, salicine, mannitol and trehalose but were not able to ferment arabinose, ribose or sorbitol (Table 7).

Cluster ten included three strains (Nos 5, 6 and 10) the representative of which (No 10) was identified as *Leuconostoc* sp. These strains were able to grow at 10°C but not at 45°C or in the presence of 6.5% NaCl, curdled litmus milk, produced gas from glucose anaerobically, and produced polysaccharides from sucrose (Table 2). These were considered to be obligatory heterofermentative. The strains of this group were able to ferment galactose, glucose, lactose, raffinose, fructose, salicine and trehalose, but failed to ferment arabinose, maltose, mannose or rhamnose (Table 7).

Cluster eleven included two isolates (Nos 13 and 16) the representative of which (No 13) was identified as *Leuconostoc* sp. Both strains were able to grow at 10°C but not at 45°C or in the presence of 6.5% NaCl, curdled litmus milk, and were unable to produce polysaccharides from sucrose (Table 2). The phenotypic and physiological characteristics of this cluster were similar to those of cluster ten; the strains in both clusters were morphologically and physiologically similar, and produced mainly lactic acid and gas from glucose anaerobically. These results suggested that these strains were obligatory heterofermentative and were presumptively classified as members of the genus *Leuconostoc*. Their carbohydrate fermentation profiles were also identical, with the exception of fructose and trehalose fermentation and the production of exopolysaccharides (Table 7).

Cluster twelve is a large assemblage of isolates of lactic acid bacteria which included seven isolates (Nos 3, 4, 7, 22, 23, 24 and 27). The representative of this cluster (No 7) was identified as *Sterptococcus acidominimus*. They were able to
grow at 10°C but not at 45°C or in the presence of 6.5% NaCl. They did not curdle litmus milk nor did they produce polysaccharides from sucrose (Table 2). The representative strains of cluster six (No 26) and cluster twelve (No23) were phenotypically identical, with exceptions in the rhamnose fermentation. Most strains in cluster twelve fermented glucose, lactose, mannitol and trehalose but were not able to ferment raffinose or ribose (Table 7).

Cluster thirteen consisted of 4 isolates (Nos 9, 11, 12 and 14). The representative of this cluster (No 12) was identified as *Aerococcus viridans*. Most strains of this cluster were able to grow at 10°C but not at 45°C, and were able to grow in the presence of 6.5% NaCl. They did not curdle litmus milk and were unable to produce polysaccharides from sucrose (Table 2). The representative strain in this group fermented glucose, lactose, raffinose, ribose, sorbitol, sucrose and trehalose, but not arabinose (Table 7).

Cluster fourteen consisted of 5 isolates (Nos 19,20,21,25 and 28) the representative of which (No 25) was identified as *Lactococcus lactis* subsp. *lactis*. All strains were able to grow at 10°C but not at 45°C or in the presence of 6.5% NaCl. All strains were able to produce polysaccharides from sucrose and curdled litmus milk (Table 2). The representative strain in this group was found to ferment glucose, lactose, mannose, ribose and trehalose, but all failed to ferment arabinose, mannitol, raffinose or sorbitol (Table 7).
CHAPTER FIVE
DISCUSSION

Lactic acid bacteria (LAB) have been used for thousands of years to produce a
variety of fermented food products, including milk products. Fermented milk
products contain a variety of microbial strains belonging to different genera and
species each with the major common characteristic of producing lactic acid. The
metabolism of lactic acid bacteria leads to products with improved shelf-life and
different flavors and textures from the original food, and may contribute to possible nutritional and health benefits as well.

Fermentation of milk with lactic acid bacteria leads to specific organoleptic characteristics (taste, aroma) of the final products. Fermentation by LAB adds nutritional benefits to milk, particularly in its ability to reduce symptoms of lactose intolerance (Danone World Newsletter, issue 2) and may also provide various health benefits, ranging from the preservation of the balance of the normal intestinal flora to the modulation of the immune system.

In the present investigations, the identification of lactic acid bacteria isolated from Rob, and the differentiation between the isolates has been carried out using the RAPD-PCR fingerprinting technique and biochemical characteristics. Arbitrarily selected 10-mer oligonucleotide primers - when used under well defined and optimized conditions- were capable of generating reproducible amplification of random fragments of DNA from the isolates of lactic acid bacteria to differentiate between the various isolates of LAB isolated from traditional fermented milk (Rob).

One hundred and sixty isolates of lactic acid bacteria were obtained from ten locally- obtained Rob samples, and different clusters of lactic acid bacteria were observed at the 80% similarity-cut off level. With ten-mer primers, the use of the RAPD-PCR technique allowed the delineation of 14 different clusters of selected local LAB isolates. Analysis of the representatives of these clusters (42 isolates) showed that 33.33 % belonged to the genus Lactobacillus, 23.81% to the genus Streptococcus, 11.91% to the genus Lactococcus, 11.91% to Leucocostoc, 9.52% to Enterococcus and 9.52% to Aerococcus, as can be calculated from the numbers of isolates in different clusters.
The predominant genus isolated from Rob samples was *Lactobacillus*, representing 33.33% of the 42 selected representative isolates. Predominance of lactobacilli in Rob samples in the Sudan has been reported by El-Mardi (1988), and also by Sulieman *et al.* (2001) who found that *Lactobacillus* and *Leuconostoc* were the dominant genera of lactic acid bacteria isolated from different Rob samples collected from Gezira. Abdelgadir *et al.* (2001) found that *Lactobacillus* and *Streptococcus* were the dominant lactic acid bacteria in Rob samples collected from seven production sites in Khartoum.

Dominance by these species was accompanied by high lactic acid production. Similar results were obtained in many African fermented milk and other food products. Hounhouigan *et al.* (1993) found that *Lactobacillus* spp were predominant (94 %) in the fermentation of Mawe (a fermented maize dough) in Benin. Savadogo *et al.* (2004) reported that the predominant spp. in fermented milk products in Burkina Faso belonged mainly to the genus *Lactobacillus*.

Based on the RAPD-PCR analysis, cluster five (*Lactobacillus rhamnosus*) was the predominant species of lactic acid bacteria representing 21.42 % of the selected 42 representative isolates. This was followed by cluster 12 (*Streptococcus acidominimus*) representing 16.66 %, then cluster 14 (*Lactococcus lactis* subsp. *lactis*) representing 11.90 %.

Identification by the API kit system showed complete agreement with the RAPD-PCR clustering scheme as far as the rod-shaped isolates were concerned, and it generally confirmed the delineation of the coccal forms into clusters by the RAPD-PCR technique. However the API 20 STREP identification system failed to separate between clusters 6 and 12 (both identified as *Streptococcus acidominimus*) and between cluster 10 and 11 (both identified as *Leuconostoc*
Nevertheless, the predominance of *Lactobacillus rhamnosus* (21.42%) and *Lactococcus lactis* subsp. *lactis* (11.9%) remains the same in both systems of identification, but according to the STREP identification system the percentage of *Streptococcus acidominimus* increases to 19.04%.

*Lactobacillus* is one of the most important genera of lactic acid bacteria because members of this genus play a major role in human and animal gastrointestinal tracts, as well as in the production of many foods, feeds and beverages. The majority of the lactobacilli in this investigation were traced to the species *Lactobacillus rhamnosus* (21.42%) which constituted cluster 5 in the RAPD-PCR clustering system. The isolates in this cluster showed very similar phenotypic characteristics.

The second most prevalent sp. in the lactobacilli was *Lactobacillus plantarum*, representing 6.76% of the 42 selected representative isolates. This species has been identified as the predominant species in most vegetable fermentations. It belongs to the group of the mesophilic lactobacilli which are commonly met in the later phases of the maturation of cheese, together with *Lb. casei*, *Lb. brevis*, and *Lb. buchneri* (Hammes et al. 1999). Ollrata et al. (2000) noted that the presence of *Lb. plantarum* in the production of cheese from goat's milk resulted in decreasing the numbers of enterobacteria and fecal coliforms in the final product. It has been identified as the terminal organism in many natural lactic acid fermentations and this is due to its high acid tolerance (Fleming and McFeeters 1981).

The presence of *Lactobacillus plantarum* was also reported in many African dairy products. Of, 21 isolates from naturally fermented milk in Zimbabwe, three were identified as *Lactobacillus plantarum*. Isono et al. (1994) identified
four out of 100 isolates as *Lactobacillus plantarum* from fermented milk in Northern Tanzania, and Jiwana and Millier (1990) identified 47 out of 426 isolates as *Lactobacillus plantarum* from cultured milk, in Cameroon. *Lactobacillus plantarum* is frequently used as starter culture in food and feed fermentations (Mckay and Baldwin, 1990) and has been isolated from most of the traditional habitats of lactic acid bacteria (Kandler and Weiss 1986; Molin *et al.* 1993).

*Lactobacillus delbrueckii* subsp. *bulgaricus* represents one of the required microflora in milk fermentation, as it plays an important role in the production and conservation of food-stuffs, especially in the dairy industry. Mixed starter cultures containing selected strains of *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* are generally used in the elaboration of yoghurt and soft cheese. They two species represent an example species present a protocooperative relationship (Beal and Corrieu 1994; Higashio *et al.* 1977). Proteolytic enzymes produced by *Lb. delbreuckii* subsp. *bulgaricus* degrade casein, releasing low molecular weight peptides and amino acids. These low-molecular weight compounds were identified as growth factors for *Streptococcus thermophilus*.

*Lactobacillus casei* is usually included in the starter cultures and it has ability to increase the nutritional and/or physiological value of the food, thus resulting in health promoting properties and improving the vitamin profiles (FIL/IDF 1992; Salmnen and von Wright 1993; Friend *et al.* 1983; Marshall 1993). *Lb. pentosus* is the dominant homofermentative *Lactobacillus* species associated with rice meals, fish fillets and plant materials.
The second predominant genus isolated from Rob samples was *Streptococcus*, representing 23.81% of the 42 selected isolates in this investigation. The RAPD-PCR profiles showed 3 different clusters of streptococci (6, 9 and 12), identified by the API 20 STREP identification system as *Streptococcus acidominimus* (clusters 6 and 12) and *Streptococcus bovis* (cluster 9). Many research workers reported the widespread occurrence a third species in this genus, viz. *Streptococcus thermophilus* (El-Mardi 1988; Savadogo et al., 2004). However, this species was not detecting in the present study, probably due to the requirement for high growth temperatures. The species comprising this genus include the pathogenic and the oral streptococci (Stiles and Holzapfel 1997).

The API identification system has been widely used in the identification of lactic acid bacteria found in milk and fermented milk products (Doliezil and Kirsop 1977; Laban et al. 1978; Abdelgadir 2001). These authors found that this system gave results which agreed with those obtained using more traditional methods. In the present study the API 50 CHL and the API 20 STREP identification systems proved very satisfactory, being simple to use and having good reproducibility. However, adoption of the RAPD-PCR profiling system seems to give more subtle differentiation. For instance the RAPD-PCR showed two different clusters (6 and 12), but the API system could not differentiate between representatives of these clusters. It thus appears that RAPD-PCR fingerprinting has more discriminative power and sensitivity as compared to the API system.

Roushdy (1999) isolated 35 strains of lactic acid bacteria and found that use of the RAPD-PCR fingerprinting was a very sensitive and reliable technique in the differentiation of LAB species. However, as RAPD-PCR profiling does not
provide genetic or specific labeling, the two systems have to be used in conjunction with each other till exhaustive fingerprint libraries are established.

The genera *Lactococcus* and *Leuconostoc* represented the third most predominant genera (11.91%). The only representative of the genus *Lactococcus* that was found in the present study was *Lactococcus lactis* subsp *lactis* which constituted cluster 14. This bacterium is of great economic importance and has been extensively studied for its biochemical and physiological characteristics, and its effect on foods (Teuber *et al.* 1991). Mutukumira (1996) isolated *Lactococcus lactis* subsp. *lactis* and *Lactobacillus acidophilus* from Amazi, a Zimbabwean naturally fermented milk. The sub-species of *Lactococcus lactis* are the most important of the commercially used lactic acid bacteria; and the most recognized habitat for lactococci is dairy products (Stiles and Holzapfel 1997).

Of the other genera found in the present study is genus *Enterococcus* which represented 9.52 % constituting clusters 7 and 8, and identified by the API 20 STREP identification system as *En. faecium* (cluster 7) and *En. gallinarum* (cluster 8). Although the cluster analysis by the RAPD-PCR profiling and biochemical identification by the API kit showed two distinct clusters (7 and 8) the isolates of both clusters showed very similar phenotypic and physiological characteristics. This again indicates the stronger discriminative power of RAPD analysis as supported by biochemical characterization than phenotypic characterization alone.

Enterococci have been frequently isolated from milk and dairy products (Wessels *et al.* 1988, Roushdy *et al.* 1998). Species most frequently detected are *En. faecalis, En. faecium,* and *En. durnas*. Moreover enterococci are used as
starter cultures in the preparation of some foods and they are commercially available as probiotics for preservation and treatment of intestinal disorders of humans (Lewenstien et al. 1979) and animals (Ushe and Nagy, 1985). In particular, *En. faecium* is associated with the fermentation of a number of southern European cheeses and is often applied in their processing.

Five of the 42 representative isolates (11.9%) were identified as *Leuconostoc* spp. (three in cluster 10, and two in cluster 11). The isolates in both clusters showed very similar phenotypic and physiological characteristics. They were differentiated by the RAPD-PCR analysis but could not be differentiated by phenotypic or biochemical means and this again confirms the higher differentiating power of the RAPD-PCR analysis as compared to phenotypic and biochemical characterization. Similar results were obtained by Cocconcelli et al. (1996) and by Quiberoni et al. (1998), who found that RAPD-PCR was able to group *Leuconostoc* spp. in accordance with their genetic diversity. Drinon et al. (1976) reported that *Leucocnostoc* grew slowly in milk and is normally not considered as important for acidification, but is useful for the formation of flavor components (diacetyl) in acidified milk. *Lactobacillus plantarum* can also produce diacetyl when sufficient quantities of citrate are present in the medium as reported by El-Gendy et al. (1983). *Leuconostoc* spp. are important in the production of taste and aroma in the fermented milk and other food products. They are usually used as starters in the preparation of fermented milk products due to their capacity for polysaccharide production and their production of aromatic (flavor) compounds. Exopolysaccharides modify the texture of the product by increasing its viscosity, or "ropy" texture (Roissart and Luquet 1994; Salminen and von Wright 1993; Hartley and Denariaz 1993; Zourari et al. 1992; Nakazawa and Hosono 1992; Cerning 1990; Marshall 1993).
*Leuconostoc mesenteroides* is usually found as the initiating flora in many fermentation processes (Pederson, 1979; Gashe, 1985). *Leuconostoc lactis*, on the other hand, was isolated from many fermented dairy products. It was one of the main species recovered from "Laban", a traditional soft cheese from Morocco (Hamama, 1992). Two strains of *Leuconostoc lactis* were isolated from South African traditional fermented milk by Beukes *et al.* (2000), and from 72 *Leuconostoc* isolates obtained from Aruza cheese (Spain cheese), only one was identified as *Leuconostoc lactis* (Centeno *et al.* 1996).

Four of the 42 selected representative isolates were identified as *Aerococcus viridans* (cluster 13) which represented 9.52% of the selected representative isolates. Presence of strains of the genus *Aerococcus* was not surprising since untreated raw milk was used for preparation of Rob, and strains of these genera of lactic acid bacteria are known to contaminate raw milk during milking. They enter milk from various sources such as air, dust, and dairy utensils. Therefore presence of such organisms in milk is not desirable due to their possible involvement in food-borne illnesses (Teuber and Geize, 1981).

In other traditional fermented milk products, such as "Maziwa Lala" from Kenya, Miyomoto *et al.* (1989), identified *Lb. plantarum, Lb. curvatus, Lactococcus lactis* subsp. *lactis*, and *Leuconostoc mesenteroides* subsp. *mesenteroides*. In Ethiopian "Ergo and Ltitu", Gonfa *et al.* (1996) isolated the genera *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus*, together with yeasts from fermented milk. In a "Laban" from Morocco, Tantaoui and El-Marrakchi (1987) reported the presence of *Lactococcus lactis* subsp *lactis* biovar *diacetylactis*, *Leuconostoc lactis*, *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuconostoc dextranum.*
*Lactobacillus rhamnosus* and *Streptococcus ascidominimus* were predominant in the Rob samples in the present study, but they are not usually reported in other traditional African fermented milks. However they are dominant in the dairy products in Finland (Tilsala, Timisjarvi and Alatossava 1998).

5.1. Exopolysaccharides (EPS) production:
Exopolysaccharides produced by lactic acid bacteria have gained increasing attention over the last few years because of their contribution to the rheology and texture of food products (Cerning and Marshall 1999). EPS in their natural environment are thought to play a role in the protection of the microbial cell against desiccation, phagocytosis, phage attack, antibiotics or toxic compounds, predation by protozoans, osmotic stress, adhesion to solid surfaces, and in cellular recognition. In the food industry, microbial exopolysaccharides are used as thickeners or for improving viscosities, stabilizing or emulsifying agents, and as gelling and water-binding agents or textures (Sutherland 1994).

In this study a few of the tested isolates of lactic acid bacteria produced EPS under the conditions used, including species of the genus *Leuconostoc* and *Lb. delbrueckii* subsp.*bulgaricus, Lb plantarum, and Lb. pentosus* which were able to synthesize EPS. It is reported that temperature and time of incubation and the composition of the growth medium (availability of nitrogen and carbon) affect both the sugar composition of the polymer and the amount produced. Similar results were obtained by (Cerning, (1990), Sikkema, and Oba (1998), De Vuyst and Degeest (1999), Ricciardi and, Clementi (2000). However, Roushdy, (1999) found that none of the strains he tested produced EPS under the conditions he used, probably due to excessive subculturing. This is not surprising since EPS production, at least in lactococci, is normally plasmid-associated and is therefore easily lost on subculturing. Schellhaass and Morris (1985), Teggatz
and Morris (1990), Mozzi et al. (1995) demonstrated that a single-strain culture of *Lb. delbureckii* subsp.*bulgaricus* and *Streptococcus thermophilus* produced more EPS at low temperature, leading to high viscosity.

RAPD-PCR together with kit system demonstrated that they were more definitive when they use for the characterization of LAB of species and subspecies with distinct zeologic, organoleptic, flavors, aroma for new products.

RAPD-PCR analysis demonstrated a higher differentiating power as compared to phenotypic and biochemical characterization.
REFERENCES


ABBREVIATIONS

AFLP, amplification fragment length polymorphism
AOAD, Arab Organization for Agricultural Development
ESP, exopolysaccharides
FAO, Food and Agricultural Organization
CONCLUSIONS

1- This study demonstrated the shortcomings of relying on phenotypic methods alone in the identification of lactic acid bacteria.

2- The results obtained demonstrated the diversity of lactic acid bacteria that can be isolated from traditional fermented milk "Rob" in the Sudan.
3- Fingerprinting of the isolated strains by the RAPD-PCR technique proved to be a sensitive tool for the fast evaluation of the genetic diversity of strains of lactic acid bacteria in fermented milk.

4- The predominant lactic acid bacteria strains present in Rob samples were enterococci, lactococci, streptococci, leuconostoc spp. and lactobacilli.

5- These isolates can be used as starter cultures with predictable characteristics that may contribute to the development of small-scale and commercial production of fermented milk with safe, stable and consistent quality.

6- Selection of promising strains with specific benefits can be put to use in tailor-making more healthful and appetizing fermented milk products.