

Introduction

Yogurt is one of the most popular fermented dairy products which has a wide acceptance worldwide whereas its nutritional and health benefits are well known for centuries. When the Neolithic people in the Central Asia transformed from a status of a food gatherer to a food producer where they began the practice of milking their animals. It is generally accepted that the fermented milk products including yogurt have been discovered accidentally when they used to store milk in sheep skin bags and has been evolved over centuries into commercial yogurt making, which paved the pavement for different commercially available varieties with a range of flavors, forms and textures (Mena *et al.* 2012).

Soured milk with thermophilic lactic acid bacteria became the preservation method of milk, and other communities learnt this technique. As a result the product “yoghurt”, coming from the Turkish name “Yogurt”, has been widely accepted (Erkus, 2007).

Plain yogurt has been referred to as a therapeutic product and has been used to treat fungal infections . Probiotic bacteria in plain yogurt namely of *Lactobacillus ssp.* have been reported to treat thrush, diarrhea, athlete’s foot, jock itch and vaginal yeast infections . Consumers are becoming more demanding when considering a food product purchase. Probiotics are normally consumed in the form of yogurt, kefir, koumiss, cheese and other fermented dairy and food products. Most probiotics fall into the group of organisms known as lactic acid-producing bacteria (Mena *et al.*2012).

Lactic acid bacteria are widely distributed in nature as indigenous micro flora. Lactic acid bacteria play multifunctional role in the production of fermented foods like curd, yoghurt, idli and dosa batter, alcoholic beverages, vegetables, fish and meat products. Lactic Acid Bacteria (LAB) have been utilized for thousands of years in fermentation of foods due to their ability to produce changes in the taste, flavour and texture as well as inhibit pathogenic and spoilage microorganisms. Since they are prevalent in raw and fermented foods, it is assumed that most representatives of this group do not pose any health risk to man, and are designed as Generally Recognized as Safe (GRAS) organisms. LAB have the ability to

produce a number of antimicrobial substances, such as organic acids (lactic acid, acetic acid), free fatty acids, ammonia, diacetyl, H₂O₂ and bacteriocin which have the capacity to inhibit growth of variety of food borne spoilage and pathogenic organism . These bacteria producing different antimicrobials can inhibit pathogenic and spoilage causing microorganisms, extending the shelf life and enhancing the safety of food products. (Samuel *et al.*2015).

Lactic acid bacteria (*LAB*) comprise a wide range of genera and include a considerable number of species. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal microflora. During the last fifteen years, the *Lactobacillus* genus has evolved and contains to date more than 80 species. They are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks. *Lactobacilli* comprise a large and diverse group of gram positive, nonspore forming, catalase negative rod bacteria, able to produce lactic acid as the main end product of the fermentation of carbohydrates (Sieladie *et al.*2011).

Probiotic lactic acid bacteria with a variety of applications have a great potential to control many infectious diseases of human . These bacteria are well known as having many properties which make them beneficial to control pathogenic microorganisms. These include, the ability to adhere to cell, reduce pathogenic bacteria adherents, co– aggregate, produce organic acids, hydrogen peroxide, bacteriocin and etc., be safe and non–pathogenic, which antagonize pathogenic microorganisms (Gulbe *et al.*2015).

Yavuzdurmaz (2007) reported that The term ‘probiotic’ firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. After this year the word ‘probiotic’ was used in different meaning according to its mechanism and the affects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined ‘probiotic’ as ‘substances and organisms which contribute to intestinal microbial balance’. In 1989, the meaning used today was improved by Fuller. Thus, probiotic is a live microbial supplement which affects host’s health positively by improving its intestinal microbial balance. Then this definition was broadened by Havenaar

and Huisin't Veld in 1992 including mono or mixed culture of live microorganisms which applied for animal and man (Yavuzdurmaz, 2007).

Probiotic bacteria are often belong to the LAB genera and are classified on the base of their morphology and an ability to glucose fermentation. They are commonly applied in the food industry due to their ability to convert fermentable sugars into lactic acid, ethanol and some other metabolites, which are responsible for lowering the pH value and preventing the potential growth of pathogenic microorganisms in food products as well as in the human intestines. These bacteria are classified as homofermentative producing lactic acid as main metabolite, and heterofermentative producing ethanol and carbon dioxide. It is known that some LAB strains may be used as probiotics because they tolerat ethecondition s present in the host gastrointestinal track and they are able to prevent the growth of undesirable pathogenic bacteria (Jakubczak *et al.*2012).

The functions of intestinal probiotic organism may include diverse actions in the gastrointestinal tract including production of metabolites, nutritional fermentation and participation in the host's immune defense system. One role of human probiotic organism may involve maintaining nutritional homeostasis in the intestine. Several compounds produced by probiotic co-metabolise nutrients with the host enzymes such as cytochrome P450 and conjugating enzymes in the liver. Ultimately these digested nutrients are absorbed by intestinal epithelial cells. The probiotic organism in the gastrointestinal tract may also produce or enrich metabolites such as glycans, amino acids, xenobiotics, vitamin K, folate and short-chain fatty acids (SCFA) . Starches are not easily digested by the human digestive system; however, the process is assisted by microbial fermentation (Thirabunyanon, 2011).

Objectives

General Objective

To isolate and identify the *lactobacillus spp* and specify their probiotic properties

Specific Objective

To determine the antimicrobial activity of *Lactobacillus spp* against the gram negative bacteria *E.coli*, *Klebsiella pneumonia*, *Pseudomonas. aeruginosa* and *salmonella spp* and gram positive bacteria *S.aureus* and *Bacillus spp*.

Chapter one

1.1 LITERATURE REVIEW

1.1 History of yogurt and probiotics

Probiotics defined by the World Health Organization/ Food and Agricultural Organization (2001) as "Live microorganisms whose administration in adequate amount to the body is able to confer a health beneficial effect on the host".

Trachoo (2002) reported that Yogurt has been known to mankind for over 6,000 years. The word "yogurt" is possibly derived from the Turkish word "jugurt" which first appeared in the 8th century but today various names are used to refer to yogurt or similar products.. It is likely that yogurt originated in the Middle East where there was limited availability of milk due to the desert environment. Ancient Turks who lived as nomads possibly introduced yogurt to village people as a preserved milk product. Popularity of yogurt is greatly attributed to Professor Elie Metchnikoff of the Pasteur Institute in Paris, who shared the Nobel Prize in Physiology and Medicine in 1908 and authored the book, "The Prolongation of Life" in which he advocated the health benefits of yogurt. Yogurt was first introduced to the U.S. in the early 20th century and gained significant consumer popularity during the 1960's and 1970's. Sales in the U.S. increased from 141 million kg in 1974 to 616 million kg in 1995 .Yogurt has now become a popular subject for researchers nationwide as it has been claimed to be a healthy food. During the past decade, full fat yogurt consumption has declined due to changes in dietary habits of consumers, in particular, reduction in milkfat consumption. Many modifications in yogurt manufacturing have therefore been developed to reduce milkfat content in yogurt resulting in the availability of non fat and low fat yogurt (Trachoo, 2002).

The word 'probiotic' comes from Greek language 'pro bios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The history of probiotics began with the history of man by consuming fermented foods that is well known Greek and Romans consume very much. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria

(*Lactobacillus spp.*). Therefore, these bacteria affect the gut microflora positively and decrease the microbial toxic activity (Kavitha and Devasena, 2013).

1.2 Yoghurt and starter culture

1.2.1 Definition of starter culture

Dairy starter cultures are carefully selected microorganisms, which are deliberately added to milk to initiate and carry out desired fermentation under controlled conditions in the production of fermented milk products. Most of them belong to lactic acid bacteria (*Lactococcus*, *Lactobacillus*, *Streptococcus* and *Leuconostocs*). In some cases, few non-lactic starters (bacteria, yeast and mold) are also used along with lactic acid bacteria during manufacturing of specific fermented milk products, such as kefir, kumiss and mold ripened cheeses(Gandhi, 2006).

1.2.2 Function of starter cultures

The major roles of starter culture during fermentation of milk are:

- a) Production of primarily lactic acid and few other organic acids, such as formic acid and acetic acid.
- b) Coagulation of milk and changes in body and texture in final products.
- c) Production of flavouring compounds, e.g., diacetyl, acetoin and acetaldehyde.
- d) Help in ripening of cheeses by their enzymatic activities.
- e) Produce antibacterial substances in the finished product.
- f) In addition, they may possess functional properties.

Thus, an ideal starter culture should be selected for the preparation of various fermented milks with the following characteristics.

1. It should be quick and steady in acid production.

2. It should produce product with fine and clean lactic flavour.
3. It should not produce any pigments, gas, off-flavour and bitterness in the finished products.
4. Should be associative in nature in product development.

Starter cultures can be used as single strain, mixed strain and multiple strains depending upon the type of products to be prepared. The ability of starter culture to perform its functions efficiently during manufacture of fermented dairy foods depends primarily on purity and activity of starter cultures (Gandhi, 2006).

1.3 The CONCEPT OF PROBIOTICS

A probiotic is ‘a live microbial food ingredient that is beneficial to health’ . Probiotics have recently received special attention on their application as an alternative approach to prevention of and therapy for several human gastrointestinal diseases . Most of these potential probiotics are of human origin and are isolated from microbiota in the human gastrointestinal tract . Other sources are several human food products which were also reported in our previous study of natural bacteria isolated from fermented milk products . Recently, probiotic bacterial formulations have been developed for consumers in the forms of dietary supplements, yogurts, drinks and capsules. Two genera, *Lactobacillus* and *Bifidobacterium*, have been found to be excellent potential sources of bacterial probiotics. In addition, some species of *Enterococcus*, *Streptococcus* and *Bacillus* have also been suggested to have probiotic properties. Many criteria must be met to establish that a new bacteria strain is probiotic. These include non pathogenicity, ability to inhibit the growth of pathogenic strains, tolerance for acid and bile salt conditions of the gastrointestinal tract, and ability to adhere to intestinal epithelial cells . In vivo testing must be conducted in order to evaluate the probiotic activity in the body. If both in vitro and in vivo studies are successful, the probiotic bacteria can be used as a biotherapeutic agent in humans (Thirabunyanon, 2011).

1.3.1 Therapeutic Properties of Probiotic Yoghurt

The efficacy of probiotics to exert a positive influence on host health or physiology was not confirmed for long, as evidence for their efficacy was low and information on the stability of the strains in the products and their survival in the gastrointestinal track was often lacking. However, a pharmacological approach has now been used to assess the effects and it is now known that the pharmacokinetics of probiotics varies between strains and that many are indeed effective. The most interesting studies are related to the immunomodulatory effects of probiotics with regards to treatment of allergic disease or inflammatory bowel diseases (IBD) . In recent times the proven benefits of probiotics in treatment and prevention of several diseases of GIT have been reviewed extensively. Interesting results have been published regarding food allergies and atopic eczema in children. Prevention of several infections and post surgical infections has also been reported. Promising results are being reported in patients with IBD and irritable bowel syndrome (IBS). It has also been suggested that probiotics could help treatment against *Helicobacter pylori* infection, but further studies are needed. Further, the role of probiotics in the process of carcinogenesis, as immune modulators in autoimmune disorders and even in conditions of the liver and skin are also being pursued. Some relevant studies reported and analyzed during the past decade are highlighted in this section of the review (Ramchandran, 2009).

1.3.2 Probiotics for diarrhea

Especially in developing countries with poor hygiene and sanitation. Acute diarrhea is one of the most common diagnoses in daily clinical practice. It is often a primary sign and symptom of gastrointestinal tract disorder which may cause serious problems both in children and adults. The symptom ranges from mild and self-limiting to severe, which may become a common cause of death in developing countries. The traveler's diarrhea in adults would be a special problem. Therefore, a prompt and effective treatment to manage acute diarrhea is critical in reducing the high morbidity and mortality rate. Furthermore, there are great concerns on the use of antibiotics in managing acute diarrhea, as most of infectious diarrhea cases are viral. Inappropriate use of antibiotics will disturb the gastrointestinal flora, which may cause a longer duration of diarrhea, greater side effect and lead to the

development of antibiotic resistance. Thus, oral rehydration therapy, ongoing fluid replacement and nutritional support, including prescribing probiotic supplementation to promote recovery from acute diarrhea are parts of the core foundation treatment for acute diarrhea. The concept of taking probiotic supplementation for better health and improving acute episodes of diarrhea is not new. Several studies have indicated that probiotic supplementation may normalize the gut microflora, which is an important protection of the host against gastrointestinal (GI) tract diseases (Simadibrata, 2012).

1.3.3 probiotic for infectious organism

Ingestion of LAB has also been found to be beneficial in people infected with the bacterium *Helicobacter pylori* which is responsible for gastritis and peptic ulcers . Various strains of LAB probiotics such as those isolated from yoghurt have been proven to reduce the growth of *H.pylori* in vitro .In animal studies and human clinical trials, presumably by producing selectively antibacterial substances known as bacteriocins and by inhibiting binding ability.*Lactobacillusjohnsonii* is probably the most successful species of probiotic shown to reduce *H.pylori* infection. Further investigations in Switzerland have found that *L. johnsonii* probiotics are capable of producing a favourable effect on *H.pylori* induced gastritis in human subjects including a trial which found that 2 weeks of *L. johnsonii* consumption suppressed *H.pylori*. infection regardless of whether it was combined with a standard medication used to treat*H. pylori* called omeprazole, or with a placebo (Tarun, 2009) .

Further more some strains of *Lactobacillus* and *Bifidobacterium* have been shown to exert bacteriostatic or bactericidal effects against *H. pylori* through the release of bacteriocins or organic acids in both in vitro and in vivo models . Probiotics also have a possible role in stabilization of the gastric barrier function and decreasing mucosal inflammation in the gastric mucosa . They recently conducted a systematic review of the clinical trials using probiotics in adults and children colonized with *H. pylori* and concluded that probiotics as a single therapy do not appear to eradicate *H. pylori* but may maintain lower levels of this pathogen. The probiotic strains *L. johnsonii* ,*L. gasseri*, *L. casei* and *Clostridiumbutyricum* appear to be most promising in reducing the density of colonization of *H. pylori*. When

probiotics are administered in combination with antibiotics, probiotics may increase eradication rate and decrease adverse effects associated with antibiotic therapy

1.3.4 probiotic for allergic

The epidemic rise in allergic disease over the last decades has coincided with progressive westernisation (increased hygiene, smaller family sizes, dietary change and excessive antibiotic use). As one of the leading candidates in the allergy epidemic, there has been long standing interest in the critical role of microbial for normal immune development and regulation. To explain this rise, Strachan introduced the hygiene hypothesis suggesting that microbial exposures in childhood are critical for normal immune development . This hypothesis was later revised by the “gut microbial deprivation hypothesis”, proposing that the observed changes in early intestinal colonisation patterns over the last decades in Western countries have resulted in failure to induce and maintain tolerance . The infant gut microbiota and its corresponding genes (the microbiome) undergo dynamic changes during development, resulting in an adult-like microbiome at about 3 years of age . This process is influenced by genetic, epigenetic and environmental factors such as country of origin, delivery mode, antibiotics and breast feeding (West *et al.* 2015).

1.4 lactic acid bacteria:

Lactic acid Bacteria (LAB) is an important bacteria in fermented products, it is functioned both in fermentation process or increasing nutrient value of fermented products (Yelnetty *et al.*2014).

Lactic acid bacteria (LAB) are groups of related bacteria that produce lactic acid as a result of carbohydrate fermentation . These microbes are broadly used by man in the production of fermented food products, such as yogurt (*Streptococcus spp.* and *Lactobacillus spp.*), cheeses (*Lactococcus spp.*), sauerkraut (*Leuconostoc spp.*), sausage, vegetables and meats. Most species have multiple requirements for amino acids and vitamins, thus LAB are generally abundant only in communities where these requirements can be provided. They are often associated with animal oral cavities and intestines (e.g. *Enterococcus faecalis*) plant leaves (*Lactobacillus*, *Leuconostoc*) as well as decaying plant or animal matter such as

rotting vegetables, fecal matter, compost, . LAB are used in the food industry for several reasons. Their growth lowers the carbohydrate content and the pH of foods they ferment due to lactic acid production, it is this acidification process which is one of the most desirable side effects of their growth. The pH may drop to 4, low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods prolong shelf life (Arimah *et al.*2014).

The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures and configuration of the lactic acid produced ability to grow at high salt concentrations and acid or alkaline tolerance. Lactic acid bacteria are among the best studied microorganisms for human health advantageous effects and fermentation. Significant novel developments have been made in the research of lactic acid bacteria in the area of multidrug resistance, bacteriocins, osmoregulation, autolysins and bacteriophages. Advancement has also been made in the production of food grade genetically modified lactic acid bacteria (Nikita and Hemangi, 2012).

Lactic acid bacteria consist of bacterial genera within the Firmicutes comprised of about 20 genera. The main members of the Lactic acid bacteria are genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, *Aerococcus*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella*. *Lactobacillus* is the largest genus of this group, comprising around 80 recognized species. The lactic acid bacteria (LAB) comprise a clade of Gram positive, acid tolerant, non-sporulation, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria are usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end product of carbohydrate fermentation. This trait has historically linked LAB with food fermentation as acidification inhibits the growth of spoilage agents (Saranraj *et al.*2013).

1.4.1 genera of lactic Acid bacteria

1.4.1.1 *Lactobacillus*

The characteristics of *Lactobacillus* are rods, usually long and slender, that forms chains in most species. They are microaerophilic, but some are strict anaerobe is knowing, catalase-negative and Gram positive, and they ferments sugars to yield lactic acid as the main product. They ferment sugars chiefly to lactic acid if they are homofermentative, with small amount of acetic acid, carbon-di-oxide and trace products, if they are heterofermentative, they produce appreciable amounts of volatile products, including alcohol, in addition to lactic acid. Most species of this non spore forming bacterium ferment glucose into lactate hence the name *Lactobacillus* is industrial production of fermented food production of fermented food products (Saranraj *et al.*2013).

1.4.1.2 *Streptococcus*

The characteristics of cocci occur in pairs, short chains or in long chain, depending upon the species and the conditions of growth and all are homofermentative. *Streptococcus.lactic* grows well in milk and ferment the lactose to 0.8 to 1.0 percent acid of which L(+) lactic acid constitutes nearly all of the acid formed, although traces of acetic and propionic acid may be present. Optimum temperature of 30°C and a temperature range of 10°C to 40°C were required (Saranraj *et al.*2013).

1.4.1. *Pediococcus*

The characteristics of cocci occur in single, in pairs or in short chains or in tetrads and are Gram positive, catalase negative and microaerophilic. They are homofermentative, fermenting sugars to yield 0.5 to 0.9 percent acid, mostly lactic and they grow fairly well in salt brines upto 5.5 percent and poorly in concentrations of salt upto about ten percent. They grow in the temperature 45°C but the best in 32°C. *Pediococcus* have been found growing, during the fermentation of brined vegetables (Saranraj *et al.*2013).

1.4.2 LAB as probiotic organism

“Lactic acid bacteria” (LAB) refers to a large group of bacteria, rather than a single species or strain, that produce lactic acid as a by-product of digesting their food source (usually carbohydrates). The lactic acid accumulates to ferment or “pickle” the food, and LAB are capable of surviving in acidic (low-pH) environments. LAB are widespread in nature and are beneficial probiotics in our digestive systems. They are among the most important groups of microorganisms used in food fermentation, contributing to the taste and texture of fermented products and inhibiting food spoilage caused by other microorganisms. LAB are responsible for production of yogurt, cheese cultured butter, sour cream, sausage, kimchee, olives, and sauerkraut (Ikeda *et al.*2013).

1.4.3 The role of the LAB on health:

The relationship between certain food and health benefits has been investigated for many years. In recent years, there has been a lot of active research in the field of probiotics due to the growing commercial interest in the probiotic food. The research work has also resulted in the understanding and ability to characterize specific probiotic organisms and their health benefits (Pyar and Peh , 2014).

Some probiotics have also a documented high total antioxidative activity (TAA) and total antioxidative status (TAS) of their intact cells and lysates, and are characterized by a complete glutathione system; thus, they can help in limiting the negative effects of the so called Reactive Oxygen Species (ROS), whose high levels can cause damage to DNA, lipids, proteins or carbohydrates, and contribute to age-related disorders, such as cancer, atherosclerosis and hypertension (Nazzaro, 2012).

Among several therapeutic applications of the probiotics can be cited the prevention of urogenital diseases, alleviation of constipation, protection against traveller's diarrhoea, reduction of hypercholesterolaemia, protection against colon and bladder cancer, prevention of osteoporosis and food allergy. Ingestion of LAB has been suggested to confer a range of

health benefits including immune system modulation, increased resistance to malignancy and infectious illness. Host immune modulation is one of the suggested benefits of the consumption of probiotic functional food. However, comparative studies on the immunological properties that support the selection of strains of the same species for specific health benefits are limited.. Infectious diseases are still the biggest human health problem for the world to solve. Intestinal infection caused by the intake of pathogenic microorganisms with the contaminated water and food are the main causes of death. Under this circumstance, probiotics can assist in part the food borne problematic situation, LAB can reduce the severity of infection caused by the enterohemolytic pathogen *Escherichia.coli* O157: H7 and suggested that this reduction may be associated with enhanced immune protection conferred by the probiotic. LAB also demonstrated the ability to provide a significant degree of protection against *Salmonella* infection. (Soccol , *et al.* 2010)

1.5. Mechanism of the probiotic action against the pathogenic bacteria

1.5.1. Competitive exclusion of pathogenic microorganisms

Probiotic bacteria compete with invading pathogens for binding sites to epithelial cells and the overlying mucus layer in a strain specific manner. Surface layer proteins purified from *L. helveticus* R0052 inhibited enterohemorrhagic *E.coli*O157:H7 adherence and the subsequent rise in permeability, without altering the growth of the pathogen. *S. boulardii* secretes heat labile factor which has shown to be responsible for the decreased bacterial adherence (Gogineni *et al.*2013).

Nevertheless *L. acidophilus* and *L. delbrueckii* are able to bind to ferric hydroxide at their cell surface rendering it unavailable to microorganism in contract to *lactobacillus* the probiotic relies on iron and express iron uptake systems to transport it into the bacterial cell. this property is also common to pathogenic bacteria. An important example for limited substance in the host is iron but for most all bacteria, iron is an essential element with exception of *Lactobacilli*. They do not need iron in their natural habitat. They might be crucial advantage in competition with other microorganism which depend on uptake

systems transport into the bacteria cell. This property is also common to pathogenic bacteria however is able to compete very effectively for this limited resource because it encodes at least even different iron uptake systems (Oelschlaeger, 2009).

1.5.2 Antagonistic effect on pathogenic microorganisms:

Gastrointestinal environment contains a wide range of contents ranging from harmless beneficial production of antimicrobial substances (bacteriocins), in situ in the intestine can be improved by increasing the ability of probiotic bacteria to adhere to the intestinal mucosa. Bovine colostrum contains substances that can triple the capacity of *Lactobacillus casei* species to adhere to intestinal cell line. However, in situ production of microbial substances adversely affect intestinal microflora beneficial to the host organism. Ruminal bacteria can also produce such bacteriocins which by their presence are able to modify the ruminal ecosystem. dietary and microbial flora to harmful pathogenic bacteria. The mammalian organism fights against these pathogenic bacteria through various ways: blocking pathogenic bacteria effects by producing bactericidal substances and competing with pathogens and toxins for adherence to the intestinal epithelium, regulation of the immune responses by enhancing the innate immunity. Anti-pathogenic action of probiotics consists in production of lactic acid which decreases the pH, interacts with the toxins produced by pathogens, with the production of hydrogen peroxide and synthesis bacteriocine. dietary and microbial flora to harmful pathogenic bacteria (Corcionivoschi, *et al.*2010).

1.5.3. Stimulation of immune response

There is considerable evidence from the animal studies that probiotic organisms can modulate the mucosal and systemic immune systems. feeding 16 volunteers with fermented milk supplemented with *L. acidophilus*, *Bifidobacterium* and *Streptococcus thermophilus* for three weeks during when they injected attenuated Salmonella typhi Ty21a vaccine. Results showed that the specific serum IgA titre rise was significantly higher in the controls, denoting an improvement in the humoral immune response. The 39 children with acute rotavirus diarrhoea were randomly assigned to receive Lactobacillus casei GG or a placebo

milk product. They found an increased IgA specific antibody secreting cell response to rotavirus in the probiotic group, associated with a reduction in diarrhoea. When they repeated this study with heat inactivated *Lactobacillus.casei* GG, specific IgA levels were unaltered but the duration of diarrhoea was reduced. Finally, they concluded that probiotics must have interacted beneficially with the mucosal immune system. Different species of lactobacilli exert different activation patterns on the dendritic cells. Further, *L. reuteri* DSM12246 inhibited the activities of other species. Clearly, not all the probiotics share the same immunomodulating properties and can even have opposite effects on some parameters (Hemaiswarya, 2013).

The immunomodulatory effects of probiotics are related to important parts of their beneficial effects. Initially, ingested probiotic bacteria interact with gut epithelial cells. In studies using cell lines . probiotic *Lactobacillus* stimulated the production of pro- and anti-inflammatory cytokines by these cell lines in a strain-dependent manner . Because intestinal epithelial cells regulate the intestinal immune response probiotic *Lactobacillus* may modulate the intestinal immune response through the stimulation of certain cytokine secretion by epithelial cells. Immune activation by probiotic *Lactobacillus* has been demonstrated in in vitro studies . Probiotic *Lactobacillus* induces the production of interferon-gamma and interleukin-12 from antigen-presenting cells through activation of the NF-kB and STAT signaling pathway. These cytokines inhibit the production of interleukin-4 but stimulate the production of interferon-gamma by helper T(thymus) cells to alter the T helper1/T helper2 equilibrium toward T helper1. On the other hand, it has been suggested that probiotics have anti-inflammatory effects on the host and relieve inflammatory bowel disease (Ohashi and Ushida, 2009).

CHAPTER TWO

MATERIALS AND METHODS

2.1 materials

2.1.1 Sample collection

One hundred Samples of curd were collected from local market Then all these samples were stored at 4°C. Afterwards all these samples were taken to Microbiology lab.

2.1.2 Preparation of media

2.1.3 de Man, Rogosa, Shrap(MRS)medium broth/Agar

The medium was prepared by dissolving 13g of the powder into100 ml distilled water and sterilized by autoclaving at 121°C for 15 minutes as selective medium to isolation of *Lactobacillus*.

2.1.4 Tryptone water

1.5g of Tryptone Water medium was suspended in the 100 ml distilled water. Dispensed into tubes and sterilized by autoclaving at 15 lbs pressure121°C for 15minutes as enrichment medium.

2.1.5 Muller Hinton Agar

The medium was prepared by dissolving 13g of the powder into100 ml distilled water and sterilized by autoclaving at 121°C f for 15 minutes .the MRS Agar was prepared by the same and addition of agar agar.

2.1.6 chemical reagents

1. 3% H_2O_2
2. Bromothymol blue
3. Gram stain reagents
4. Normal saline
5. Bile salt
6. NaCl

2.1.7 Tools

1. Test tubes
2. Clean slide
3. Petri dish
4. Flask
5. Cotton wool swab
6. Cutter
7. Anaerobic Jar
8. Centrifuge
9. Filter paper
10. Incubator
11. Autoclave
12. Water bath
13. Wire loop
14. Cotton
15. Microscope

2.2 Methods: (according to cowan and steel for the identification of bacteria)

2.2.1. isolation and selection of *Lactobacillus*

Samples obtained from local markets were serially diluted in Sterile distilled water and 1ml of appropriately diluted sample was poured plated on MRS agar and incubated at 37°C anaerobically for 48 hours for enumeration of *Lactobacillus* in MRS agar. based on colony characteristic like colour subculture to get pure colonies. The pure isolates were subjected to identification. These colonies were streaked on MRS slants for further use and stored at 4 °C and subcultured every 2 weeks.

2.2.2. Identification of *Lactobacillus* spp

2.2.2.1. gram stain

The gram reaction of the isolates was determined by light microscopy after gram staining. LAB are known to be gram positive. It means that they give blue-purple color by gram staining. Cultures were grown in MRS media at 37 °C for 24 h under anaerobic conditions. Cells from fresh cultures were used for gram staining. Gram staining procedure was applied. Then, under light microscopy gram Positives and purified isolates were determined.

2.2.2.2. Catalase Test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme. $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, two methods were applied, Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h 3% hydrogen peroxide solution was dropped onto randomly chosen colony. Also fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution into 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were chosen. Since, LAB is known as catalase negative.

2.2.2.3 Motility test

The motility of the isolates were done by hanging drop method using hanging drop slide.

2.2.2.4 carbohydrate fermentation

Bromothymolblue base medium was used as a medium for this test. Different sugar substrates namely, Glucose, xylose, sucrose, galactose, lactose, maltose, trehalose, ribose, mannitol and dextrose, arabinose, sucrose, maltose, sorbitol and were used. 1 g of each sugar substrate was added to 100 ml of the medium. 5 ml of each mixture was transferred into each tube. For gas detection, Durham tube was inserted into the test tube containing glucose. All the tubes were sterilized for 15 min at 121 °C. The tubes were inoculated with a single colony of the bacteria under study. The positive reaction of the bacteria was indicated by the changes in the colour of the medium .

2.2.3 Probiotic Properties of Isolates

2.2.3.1 Resistance to Low pH

For determination optimum culture pH growth of the isolates, 100 µl overnight culture of the isolates were inoculated into MRS agar with varying pH ranging from 4.5, 5.5, 6.5, 7.5 and . The inoculated MRS agar were then incubated in anaerobic condition for 48 h at 37°C. Viable colonies were enumerated using colony counter.

2.2.3.2 Growth at Different NaCl Concentrations

Isolates were tested for their tolerance against different NaCl concentrations. For this purpose 100 µl overnight culture of the isolates were inoculated into MRS agar plates with varying NaCl concentrations 4.5, 5.5, 6.5, 7.5 2.5%,4.5% and 6.5% NaCl concentrations. these plates were incubated at 37 °C for 48. Viable colonies were enumerated using colony counter.

2.2.3.3 Tolerance against Bile

Concentration of bile salt 0.1, 0.3 ,0.5 ,0.7, were used by adjust MRS at this concentrations of bile salt. MRS medium containing these concentrations (incubated for 48h). viable colonies were enumerated using colony counter.

2.2.4 Antimicrobial Activity by a Well Diffusion Assay

A culture of *L. acidophilus* inoculated in 100 mL MRS broth for 48 h at 37°C was centrifuged at 10,000 rpm for 30 min. The supernatant was filtered by passage through a 0.25 µm filter . Inhibitory activity of the test suspensions was investigated by a well diffusion method. From the nutrient agar plates, colonies of *P.aeruginosa*, *Ecoli*, *K. pneumonia*. *S.typhi* , *S.aureu* and *bacillus spp* .were transferred in 5 mL buffered Tryptone water and bacterial cultures was inoculated on Muller Hinton agar , by streaking the swab over the entire Muller Hinton agar surface. Wells sized 6 mm were cut with a sterile metal cylinder into the agar plate. On each Muller Hinton agar plate 3 wells were cut. Then, of each test suspension was placed into each well. The plates were incubated for 24 h at 37 °C and inhibition was examined by growth free inhibition zones surrounding each well. Inhibition zones were measured in millimetres (mm) by the diameter of the wells. As the controls, sterile peptone water were used. This experiment was carried out in duplicate.

Chapter Three

RESULTS

From the tested samples, lactic acid producing were isolated from different yohurt samples. Colonies were observed on the surface of MRS agar Petri plates. More than one type of colony was observed on surface of MRS agar Petri plates. The cultural and morphological characteristics were further resolved on the basis of microscopic examination. All isolated were Gram positive rods shaped bacteria. After characterization, some of them were determined as representative of genus *Lactobacillus*. The isolates were phenotypically characterized on the basis of their morphological, cultural, and biochemical characteristics. Catalase test showed isolates were not able to produce bubbling when mixed with 3% H₂O₂ This showed that there was absence of catalase enzyme. The absence of catalase enzyme showed that identified bacteria were from *Lactobacillus* spp.the morphological characteristics ,type of colony, colour, Gram reaction and motility the results are shown in (Table1). The three isolates showed high growth on MRS agar after 48h of incubation. Some of the isolate colonies appeared small in their shape. The colour of colonies ranged from white to creamy white. Further the colour pigments were absent in all pure colonies of isolates and appeared white to creamish in colour.

Table 1, macroscopic and microscopic characteristic of the isolates

isolates	Type of colony	Colony colour	Gram stain	Catalase test	Morpho type	Motility
Isolates	Small	Creamy white, shiny	+	-	Long rod	-

Identification of *Lactobacillus* spp. isolates in food and dairy samples in (Table 2) that were used for sugar fermentation, showed that the isolates were homofermentative , The microorganism fermented

glucose to acid which was evident by changing colour of medium from red to yellow. Glucose , Lactose , Sucrose , Maltose , Trehalose , Galactose , Sorbitol Dextrose a and Arabnose fermented by isolated 1 and isolated 2 and not able to ferment Manose and Xylose . Were isolated 3 ferment the glucose, lactose, Xylose but not Maltose ,T rehalose ,M anose , Galactose ,Dextrose, Sorbitol, Arabnose.

Sugars	Isolate1	Isolate2	Isolate3
Glucose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
lactose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁺ G ⁻
sucrose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻
Maltose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻
Trehalose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻
Manose	A ⁻ G ⁻	A ⁻ G ⁻	A ⁻ G ⁻
Galactose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻
Xylose	A ⁻ G ⁻	A ⁻ G ⁻	A ⁺ G ⁻
Dextrose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻
sorbitol	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻
Arabnose	A ⁺ G ⁻	A ⁺ G ⁻	A ⁻ G ⁻

A⁺ = Acid Positive,

A⁻ = Acid Negative,

G⁺ = Gas Positive

G⁻ = Gas Negative

Identification of isolated lactic acid bacteria

The isolates were identified on the basis of cultural ,morphological and biochemical characteristics as given in the Bergey's Manual of Systematic Bacteriology vol. 2 (Holt *et al.*, 1984) . Isolates 1 and 2 were identified as *Lactobacillus acidophilus* but they may have different strains or subspecies due to clear difference in Fig.1 and Fig.2. And isolate 3 was identified as *Lactobacillus delbrueckii sub sp. Bulgaricus*.

Characteristics of *Lactobacillus acidophilus* :

Growth at different pH

As can be seen (Table 3) below showed the results of the growth of *L. acidophilus* at different pH values range of 4.5 to 7.5 adjust in the MRS medium and colonies were counted by colony counter. The growth of *L. acidophilus* indicating that the bacteria preferred to grow in acidic and neutral environment. The growth was 25×10^6 and increase with increase of pH values towards the neutral but decrease after that 7.5.

Bile salt tolerance

the effect of different concentrations of bile salts on the growth of *L. acidophilus* bacteria in MRS agar was investigated and the results are presented in (Table 3). The total viable count of *L. acidophilus* decreased with an increase in the bile salt concentration showing pattern.

NaCl

Growth of *L. acidophilus* at different NaCl concentrations was observed. The *Lactobacillus acidophilus* have ability to grow at 2.5% NaCl concentration but does not show the ability to grow at 7% NaCl concentration. That mean the growth decrease with increase with NaCl.

Table 3, show the colony count of *Lactobacillus acidophilus* the growing on MRS medium with different concentration of NaCl ,bile salt and different pH values.

Parameter and count of bacterial growth				
PH	4.5	5.5	6.5	7.5
Colonies count	25x10 ⁶ CFU	120x10 ⁶ CFU	136x10 ⁶ CFU	56x10 ⁶ CFU
Bile salt	0.1	0.3	0.5	0.7
Colonies count	17x10 ⁶ CFU	25x10 ⁶ CFU	110x10 ⁶ CFU	82x10 ⁶ CFU
NaCl	2.5%	4.5%	6.5%	7%
Colonies count	65x10 ⁶ CFU	27x10 ⁶ CFU	15x10 ⁶ CFU	0

(table 4)

Table Showed the antagonistic zone (mm)

	Antagonistic zone (mm)	Min.	Max.
<i>E.coli</i> (n=21)	14.33±3.73 ^a	3	19
<i>Salmonella</i> (n=19)	10.32±3.43 ^b	5	15
<i>Klebsiella</i> (n=20)	14.15±5.37 ^a	6	25
<i>Pesudomonas</i> (n=17)	12.35±3.30 ^{ab}	5	17
<i>Proteus</i> (n=5)	14.4±02.88 ^{ab}	11	18
<i>Shigella</i> (n=15)	14.6±5.10 ^a	6	29
Significant	*		

*= Significant differences at P<0.05

Different superscript letters means significant differences at P<0.05.

The higher antagonistic zone show against *E.coli*, and following against *Klebsiella*, *protus* then against *Pesudomonas*, and the smallest against *Pesudomonas* and *Salmonella*.

Chapter Four

Discussion

The goal of this research work was to isolate and characterize potential probiotic bacteria from bioyoghurt samples and to assess their antibacterial activity against some common pathogenic bacteria. Result showed that isolated bacteria is gram positive, non sporeforming and catalase negative which is similar to that reported by Guessas and Kihal, (2004) that isolated lactic acid bacteria from goats milk in Algerian arid zone and reported that all the isolates were gram positive, catalase negative and non- spore forming.

In the current study, some of the isolates showed the absence of catalase enzyme and these were from the genus *Lactobacillus* this agree with Zourari , *et al.* (1992) who reported that lactic acid bacteria are facultative anaerobes with a preference of anaerobic conditions. They cannot synthesis eporphyrins and consequently they do not synthesise cytochromes or catalase. Oxygen is sometimes used for formation of hydrogen peroxide, which is toxic for lactic acid bacteria and do not contain catalase to break it down; Aerobic organisms that have the enzyme catalase break down hydrogen peroxide in following reaction. This study showed the isolates were non motile when examined under microscopic and this is similar to Ahmed and Kanwal , (2004) finding who isolated different strains of lactic acid bacteria from camel milk. and reported that all the strains were non-motile.

Lactobacillus when inoculated in the MRS with different concentration of the bile salt showed the growing below the 0.1 see (table 3) that is similar to the result obtained by Sieladie, *et al.* (2011) who concluded that, all isolates demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C.

Results of the well diffusion assay method showed that *Lactobacillus* isolated from yoghurt were able to inhibit the growth of the selected pathogens. The spectrum of their antibacterial effects varied. Probiotics of yoghurts inhibited the growth of *E. coli*, *S. typhi* , *K. pneumonia*, *P.aeruginosa* , *bacillus* spp, proteus, shigella .see (Table2) the antibacterial effects of the

Lactobacillus isolated from yoghurts this may be due to the production of acetic and lactic acid that lowered the pH of the media (Bezkorovainy, 2001).

Quwehand and Vesterlund . (2004) reported that till today there are some researches showing that different species produce different antimicrobial substances, eg. *Lactobacillus reuterii* produce a low molecular weight antimicrobial substance called reuterin, subspecies of *Lactococcus lactis* produce a class I bacteriocin known as nisin *Enterococcus faecalis* produces a class I bacteriocin cytolysin ,*Lactobacillus plantarum* produces classII bacteriocin plantaricin and *Lactobacillus acidophilus* produces a class III bacteriocin acidophilucin. Production of bacteriocins is highly affected by the factors of the species of microorganisms, ingredients and pH of medium, incubation temperature and time.

Ray, (1996) reported that Other than bacteriocins, some are also capable of reuterine production that is known to act as an antibacterial compound .

CHAPTER FIVE

Conclusion and Recommendation

Conclusion

Although bioyoghurt is very nutritive and essential for human health it is also important in protection human health, due to the fact that whatever the starter may be lactic acid bacteria will appear soon After production. From materials and methods all bioyoghurt samples isolates were characterized on the basis of their colony morphology and other biochemical aspects. Colonies were circular, small and cream-white after incubation on MRS agar plate. . All the isolates studied were non-motile, Gram-positive, rod-shaped that produce lactic acid. Also all isolates were not producing catalase enzyme. Antimicrobial sensitivity were done by well diffusion method. Results showed the presence of antibacterial effects among the probiotics that were isolated from bioyoghurts.

Recommendations

Lactobacillus species can be use in the production of other functional foods.

A wareness of the beneficial health promoting effects ,when food treated by probiotics is consumed.

More research works should be performed on this area.

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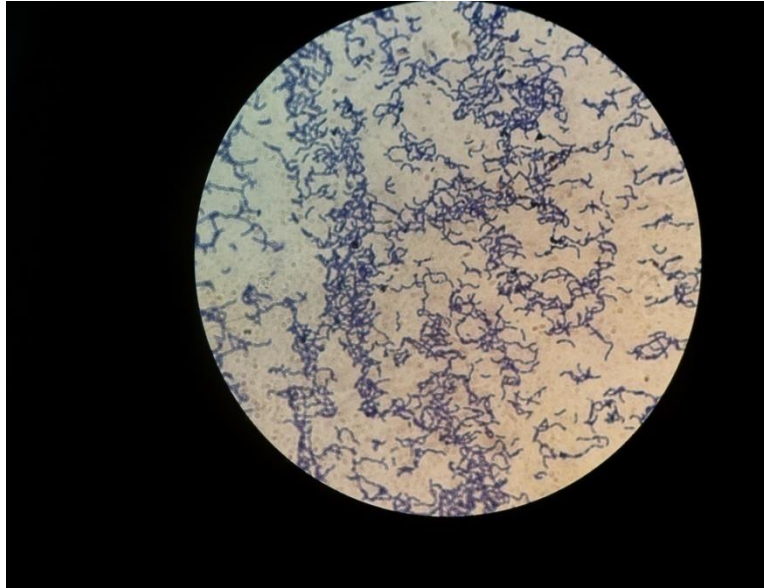
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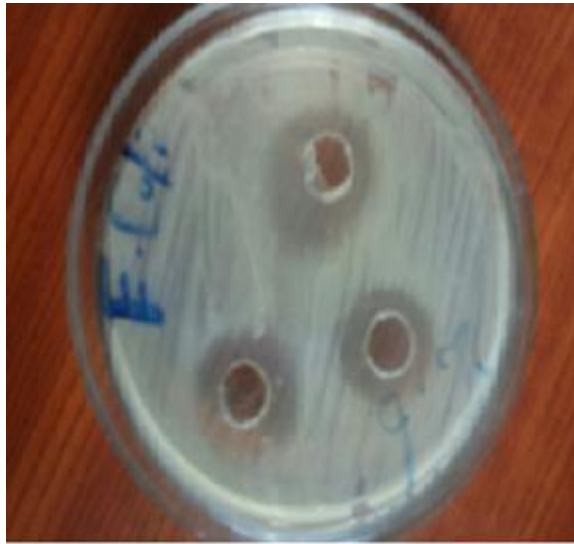
Appendices



microscopic characteristics showing gram positive long rods grown in MRS medium at 37°C after 48 hrs.



showing the zone of the inhibition of *L. acidophilus* was more of *P. aeruginosamm*



showing the zone of the inhibition of *L. acidophilus* of *E. coli*,



showing the zone of the inhibition of *L. acidophilus* of *K. pneumoniae*

