ISOLATION, IDENTIFICATION AND ANALYSIS OF PROBIOTIC PROPERTIES OF *LACTOBACILLUS SPP.* FROM SELECTIVE REGIONAL YOGHURTS

A thesis

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Submitted by

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Examination Roll No. MS-070725
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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been dully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Khulna University or other institutions.

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Date: 05.10.2009

Dedication

Dedicated

To

My Beloved parents

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ABSTRACT

Yoghurt is a potential source of probiotic lactobacilli. In the present study, *Lactobacillus spp.* were isolated from two regional yoghurts of Bangladesh, which were identified on the basis of their colony morphologies and biochemical assays. It was observed that isolated *Lactobacillus spp.* were resistance to inhibitory substances like phenol (0.4%), NaCl (1-9%) and bile acid (0.05-0.3%). Additionally, their good growth was observed in the presence of 1% NaCl and 0.3% bile acid. The isolated *Lactobacillus spp.* did show good survival abilities in acidic (pH 2.5) and alkaline (pH 8.5) conditions, while, their maximum growth were observed at pH 5.0 incase of lactobacilli isolated from Bogra yoghurt and at pH 6.5 incase of lactobacilli isolated from yoghurt of Khulna region of Bangladesh. Isolated lactobacilli were able to produce organic acid in the skim milk which was determined by titrimetric method. The *Lactobacillus spp.* also did show good survival abilities in simulated gastric juice at pH 2.22 and pH 6.6 (control). Their susceptibility to selected nine antibiotics was determined in terms of minimum inhibition concentration (MIC). The MICs results showed that, *Lactobacillus spp.* isolated from Bogra yoghurt were sensitive to amoxicillin, moderately sensitive to gentamycin, clindamycin, azithromicin and resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracyclin. On the other hand, *Lactobacillus spp.* isolated from yoghurt of Khulna region of Bangladesh were sensitive to gentamicin, clindamicin and resistant to amoxicillin, tetracyclin, kanamycin, nalidixic acid, metronidazol, azithromycin and cefradine. Observations from the present communications show that, there were variations in probiotic properties of the isolated *Lactobacillus spp.* from regional yoghurts. Therefore, species level isolation, identification and probiotic properties of regional lactobacilli need to be studied.

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Introduction

INTRODUCTION

The *Lactobacillus* genus consists of a genetically and physiologically diverse group of rod-shaped, gram-positive, non-spore forming, nonpigmenetd (Hasan and Frank, 2001), catalase negative and microaerophilic to strictly anaerobic (Vernoux et al., 2003) lactic acid bacteria (LAB) that have widespread use in fermented food production (Azcarate-Peril and Raya, 2001) and are considered as GRAS (generally recognized as safe) organisms and can be safely used for medical and veterinary applications (Fuller, 1989). In the food industry, LAB are widely used as starter cultures (Hammes, 1986; Skytta and Harris *et al.*, 1989) and have been cited to be part of human microbiota (Fuller, 1991; Holzapfel et al., 2001; Reid, 2001; Schrezenmeir and de Vrese, 2001; Sghir et al., 2000) microbiota. In raw milk and dairy products such as cheese, yoghurt and fermented milk, lactobacilli are naturally present or added intentionally, for technological reasons or to generate a health benefit for the consumer (Vernoux et al., 2003) and yogurt is one of the best-known of the foods that contain probiotics (Oscar et al., 2004). The beneficial effects of yoghurt were put on a scientific basis in 1907 by Elie Metchnikoff, the work that is regarded as the birth of probiotics (Fuller, 1992). According to Dave and Shah (1997) yoghurt or yoghurt-like products have been used as the most popular vehicle for incorporation of probiotic organisms.

From health perspective, ingestion of live cells of certain species and strains, probiotic concept, of lactobacilli in adequate amounts is believed to confer several beneficial physiological effects on the host (Tannock, 2004) such as maintaining a healthy and equilibrated intestinal microbiota and reducing incidence of intestinal infection (Gardiner et al., 2002), assimilation (Noh *et al.*, 1997) and reduction of serum cholesterol (Fernandes et al., 1987; Fukushima et al., 1999; Mann and Spoerry, 1974), enhanced resistance to tumergenesis (Saito *et al.*, 1987), prevent antibiotic associated diarrhea (Siitonen, et al., 1990), to treat acute infantile diarrhea (Saavedra, et al. 1994) and recurrent *Clostridium difficile* disease (Biller, et al., 1995), lactose intolerance, boosting of immune system (Dairy Counoil of California, 2000) etc. However, the impacts of a given species on the health of an individual within a specific probiotic species appear to be strain/host-dependent (Sellars, 1991). For example, a strain of *Lactobacillus acidophilus* isolated in North America may well be genetically different from a species recovered in Europe or the Middle East, and this difference could well be reflected in the

reaction of a host ethnic population remains a controversial topic, for the health-promoting properties of lactic acid bacteria (Haddadin, et al., 2004).

The choosing criteria for the *in vitro* selection of lactobacilli to be used as health-promoting, probiotic ingredients, in food and pharmaceutical preparations should possess the abilities of antibiotic tolerance due to their possible use to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis (Kacem and Nour-Eddine, 2006), as well as the production of lactic acid that inhibits the growth of other microorganisms, allow them to be established in the intestinal tract (Catanzaro and Green, 1997). Bile tolerance (Walker and Gilliland, 1983) and gastric juice resistance (Kilara, 1982) are important characteristics of probiotic lactic acid bacteria used as adjuncts because they enable them to survive, to grow, and to perform their beneficial action in the gastrointestinal tract (GIT). Although the degree of tolerance required for maximum growth in the GIT is not known, it seems reasonable that the most bile and acid-resistant species should be selected (Graciela and María, 2001).

In Bangladesh, yoghurt is perhaps the oldest fermented milk product known and consumed by large segments of our population as a part of their daily diet. In most of the areas of Bangladesh, different types of traditional yogurts are found but their probiotic role is in limited extent. Although yoghurt is the potential source of beneficial lactobacillus bacteria, a small number of evidence has been found so far regarding extensive study of its probiotic properties. Incorporation of probiotic live microorganism (isolated from indigenous yoghurts) in market yogurts can positively enhance health status of larger segment of communities of Bangladesh, having with probiotic foods. Therefore, the present study was undertaken with the following objectives for:

1. isolation and identification of presumptive strains of lactobacillus from different regional yoghurts of Bangladesh
2. determination of optimal growth and pH of concerned lactobacillus strains
3. quantification of lactic acid production in milk
4. determination of antibiotic and bile tolerance and
5. determination of resistance to gastric juice

Chapter Two

Review of Literature

REVIEW OF LITERATURE

2.1 Probiotics

The concept of probiotics evolved at the turn of the 20th century from a hypothesis first proposed by Nobel Prize winning Russian scientist Elie Metchnikoff (Bibel, 1988), who suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. He believed that when consumed, the fermenting bacillus (*Lactobacillus*) positively influenced the microflora of the colon, decreasing toxic microbial activities (Sanders, 1999).

Probiotics are defined as "live microbial food supplements or components of bacteria which have been shown to have beneficial effects on human health" (Salminen et al., 1998). The most frequently used genera fulfilling these criteria are lactobacilli and bifidobacteria (Harish and Varghese, 2006).

2.2 Probiotics selection criteria

According to Harish and Varghese (2006), organisms to be considered as probiotics, the following criteria need to be fulfilled:

1. It should be isolated from the same species as its intended host
2. It should have a demonstrable beneficial effect on the host
3. It should be non-pathogenic
4. It should be able to survive transit through the gastrointestinal tract
5. On storage, large number of viable bacteria must be able to survive prolonged periods

According to WHO and FAO working group reports (2002), In recognition of the importance of assuring safety, even among a group of bacteria that is Generally Recognized as Safe (GRAS), the Working Group recommends that probiotic strains be characterized at a minimum with the following tests:

1. Determination of antibiotic resistance patterns
2. Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation)
3. Assessment of side-effects during human studies

4. Epidemiological surveillance of adverse incidents in consumers (post-market)
5. If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production. One possible scheme for testing toxin production has been recommended by the EU Scientific Committee on Animal Nutrition.
6. If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required

2.3 Probiotics mechanism of action

Mechanisms for the benefits of probiotics are incompletely understood ((Harish and Varghese, 2006). However, as a general rule, include (Sartor, 2004)

- Adherence and colonization of the gut
- Suppression of growth or epithelial binding/invasion by pathogenic bacteria and production of antimicrobial substances
- Improvement of intestinal barrier function
- Controlled transfer of dietary antigens
- Stimulation of mucosal and systemic host immunity

2.4 Potential health benefits of probiotics

Probiotic cultures have been associated historically with cultured of milks and dairy products, from which there is substantial evidence for positive effects on human health and general well-being (Kaenhammer, 2000 and Reuter, 2001). A number of studies have found probiotic consumption to be useful in the treatment of many types of diarrhea, including antibiotic-associated diarrhea in adults, travellers' diarrhea, and diarrheal diseases in young children caused by rotaviruses (Siitonen et al., 1990; Oksanen et al., 1990; Isolauri et al., 1991). Besides these, several investigations have unveiled enormous health benefit of probiotics which are described in following headings:

2.4.1 Boosting of immune system

Evidence from in vitro systems, animal models and humans suggests that probiotics can enhance both the specific and nonspecific immune response, possibly by activating

macrophages, increasing levels of cytokines, increasing natural killer cell activity, and/or increasing levels of immunoglobulins (Sanders, 1999).

2.4.2 Lactose intolerance

Several lines of evidence show that the appropriate strains of lactic acid bacteria, such as *S. thermophilus*, *L. bulgaricus* and other lactobacilli in fermented milk products, can alleviate symptoms of lactose intolerance by providing bacterial lactase to the intestine and stomach. Because lactose intolerance affects almost 70% of the population worldwide, consumption of these products may be a good way to incorporate dairy products and their accompanying nutrients into the diets of lactose intolerant individuals (Dairy Council of California, 2000).

2.4.3 Allergy

Probiotics may exert a beneficial effect on allergic reaction by improving mucosal barrier function. In addition, probiotic consumption by young children may beneficially affect immune system development. Probiotics such as *Lactobacillus GG* may be helpful in alleviating some of the symptoms of food allergies such as those associated with milk protein (Majamaa and Isolauri, 1997).

2.4.4 Cancer

Studies of the effect of probiotic consumption on cancer appear promising (Dairy Council of California, 2000). Animal and *in vitro* studies indicate that probiotic bacteria may reduce colon cancer risk by reducing the incidence and number of tumors. One clinical study showed an increased recurrence-free period in subjects with bladder cancer (Aso and Akazan, 1992). Results, however, are too preliminary to develop specific recommendations on probiotic consumption for preventing cancer in humans.

2.4.5 Constipation

Constipation is common especially in elderly people. An increase in the number of bowel movements or a decrease in transit time has been reported in controlled studies that employed probiotics for treating constipation (Koebnick et al., 2003 and Ouwehand et al., 2002).

2.5 *Lactobacillus spp.* as probiotics

From early times until now many Lactic Acid Bacteria (LAB) were used in functional foods manufacture, since they contribute to a healthy nutrition (Holzapfel et al., 1998; Marteau and Rambaud, 1993). Among the LAB, species within the genera *Lactobacillus* and *Bifidobacterium* are the best known and the most frequently used in food industry (Harish and Varghese, 2006; Bayane et al., 2006). Yoghurt usually contains the probiotics *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and *Streptococcus thermophilus* which are used to start fermentation process that create yoghurt from milk (Ganesh, 2006 and Nyam news, 2005). In some countries less traditional microorganisms, such as *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *lactis*, are sometimes mixed with the starter culture (McKinley, 2005). According to a recent listing (www.bacterio.cict.fr, 2003) 88 species and 15 subspecies of lactobacillus have been identified but all of the species are not using as a probiotic bacteria. Most commonly used of probiotics *lactobacillus* in the dairy industries is given in Table 1.

Table 1. Probiotic *Lactobacilli* used in dairy products

Lactobacillus acidophilus	***Lactobacillus gasseri***
Lactobacillus casei	***Lactobacillus johnsonii***
Lactobacillus crispatus	***Lactobacillus paracasei* ssp.**
***Lactobacillus delbrueckii* ssp.**	***paracasei***
delbrueckii	***Lactobacillus plantarum***
Lactobacillus farciminis	***Lactobacillus reuteri***
	Lactobacillus rhamnosus

Source: www.bacterio.cict.fr, 2003

2.6 Sources of *Lactobacillus spp.*

Lactic acid bacteria are widely distributed in the nature. In this group are included representatives of the genus *Lactobacillus, Lactococcus, Pediococcus* and *Leuconostoc*. They could be isolated from dairy products, soils, waters, plants, silages, waste products, and also from the intestinal tract of animals and humans (Tserovska et al., 2002 and Kandler and

Weiss, 1986). Among them milk products specially yoghurts are good source of *lactobacillus spp* (Vernoux, et al., 2003).

2.7 Isolation of *Lactobacillus spp.*

Given the great potential economic value of lactobacilli, one of the main objectives of microbiologists is to develop a clear picture of the microflora present in the various dairy products, and the way in which it changes during processing. Quality assurance programmes associated with research, development, production and validation of the health or technological benefits of these bacteria require the relevant isolation, counting and identification of bacteria (Vernoux, et al., 2003). Several protocol and guidelines have been published for the extraction of lactobacilli from milk or dairy products (IDF, 1997).

2.8 Isolation media

Several elective and selective media have been developed for the isolation and counting of *Lactobacillus* species and for the differential counting of mixed populations of lactic acid bacteria. Oxygen tolerance, nutritional requirements, antibiotic susceptibility, colony morphology and colour are used to differentiate strains in these methods.

Lactobacilli are generally isolated on rich media such as MRS (De man, Rogosa and Sharpe, 1960) which is routinely used for the isolation and counting of lactobacilli from most (fermented) food products. The addition of a reducing agent such as cysteine 0.05% to MRS improves the specificity of this medium for *Lactobacillus* isolation (Hartemink et al., 1997; Lankaputhra et al., 1995; Shah, 2000). MRS and M17 are, respectively, the medium of choice for the differential counting of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in yoghurt (IDF, 1997). Birollo et al. (2000) proposed the use of a cheaper medium skim milk agar for counting and differentiating colonies of these two bacteria. When yogurt microflora was supplemented by other lactobacilli, specific methods were needed. Enumeration of *Lb. delbrueckii* ssp. *bulgaricus* in the presence of *Lb. acidophilus* was possible, on acidified MRS at pH 5.2 or on reinforced clostridial agar (RCA) at pH 5.3, at 45 °C for 72 h (Shah, 2000). The composition of MRS and M17 media are given below in Table 2. and Table 3:

Table 2. The compositions of MRS broth media for 1 L

a. Nitrogen source:	**1. polypeptone (10 g)**
	2. meat extract (10 g) and
	3. yeast extract (5 g)
b. Carbon source:	**1. glucose (20 g)**
c. Salts:	**1. K_2HPO_4 (2 g)**
	2. ammonium citrate (2 g)
	3. sodium acetate (5 g)
	4. $MgSO_4 \cdot 7H_20$ (0.2 g) and
	5. $MnS0_4 \cdot 4H_20$ (0.05 g).
d. Growth factor:	**1. Tween-80 (1 mL).**

The pH is adjusted to 6.4 ± 0.2 before autoclaving at 121°C for 15 min.

Source: De man, Rogosa and Sharpe, 1960

Table 3. The compositions of M17 media for *lactococci* for 1 L

a. Nitrogen source:	**1. Phytone peptone (5 g)**
	2. Polypeptone (5 g)
	3. Yeast extract (5 g) and
	4. Beef extract (2.5 g)
b. Carbon source:	**1. Lactose (5 g).**
c. Others	**1. Ascorbic acid (0.5 g)**
	2. β-disodium glycerophosphate (19 g)
	3. 1.0 *M MgSO4*·$7H_2O$ (1 mL).

The pH is adjusted to 7.1 before autoclaving at 121°C for 15 min.

Source: Raibaud, et al., 1961

2.9 Identification of *lactobacillus spp.*

Depending on the taxonomic level desired, several phenotypical or molecular methodologies (polyphasic analysis) can be used for isolation, characterisation and identification of lactobacilli. Recent methodologies, which are culture-independent such as single strand conformation polymorphism (SSCP), temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) have also been proposed for characterization of microbial diversity (Vernoux, et al., 2003).

Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species. Their common traits are: Gram-positive, usually catalase negative, growth under microaerophilic to strictly anaerobic conditions and lactic acid production (Vernoux, et al., 2003).

2.10 Probiotic properties of lactobacilli

Bile tolerance (Walker and Gilliland, 1983) and gastric juice resistance (Kilara, 1982) are important characteristics of probiotic lactic acid bacteria used as adjuncts because they enable them to survive, to grow, and to perform their beneficial action in the gastrointestinal tract (GIT). Although the degree of tolerance required for maximum growth in the GIT is not known, it seems reasonable that the most bile- and acid-resistant species should be selected.

The techniques used to selecting LAB for probiotic purposes will be considered under the following headings (Graciela and María, 2001):

1. Bile tolerance, using differents bile salts concentrations.
2. Resistance to gastric juice.
3. In vitro cholesterol reduction on the presence of bile salts.
4. Bile salt hydrolase activity, determined by three complementary methods: plate assay, colorimetric method, and high-performance liquid chromatography (HPLC).

2.11 Organic acid accumulation

Lactobacilli have been found to produce metabolic products that play important role in controlling undesirable microflora in the gut (Itoh et al., 1995). They were able to prevent an increase of pathogenic bacteria by production of antimicrobials such as organic acids, hydrogen peroxide, and bacteriocins (Jin et al., 1996). Hence, they can be used as biopreservative agent.

2.12 Influence of antibiotics on lactobacilli

Lactic acid bacteria (LAB) are common inhabitants of the gastrointestinal (GI) tract and have important role in maintaining the equilibrium of GI flora, which can be influenced by various factors like diets, antimicrobials and stress (Andreja and Matijaši, 2001).

Medical treatment with antibiotics causes non-selective elimination of part of the microbial population from the gastrointestinal tract, which in this way are weakened and present an easy target for microorganisms dangerous to health. To prevent such a threatening situation and to restore the normal state of intestinal microflora, consumption of biologically active fermented foods containing probiotic bacteria during and/or after antibiotic intake is recommended (Andreja and Matijaši, 2001). Bacteria of the genus *Lactobacillus* have been proposed as probiotic microorganisms to restore the ecological equilibrium of the intestinal, respiratory, and urogenital tracts (Hammes et al., 1995). Therefore, probiotic LAB present an appropriate adjunct in antibiotic therapy, especially if they posses intrinsic resistance to antibiotics.

Currently, there is concern over the possible spread of resistance determinants (from the food chain) to antimicrobials (Teuber et al. 1999). Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes that could be transferred to pathogens, either in the food matrix or, more importantly, in the gastrointestinal tract. The production of fermented dairy products from raw milk in antibiotic challenged environments may select antibiotic-resistant LAB harbouring transmissible resistance genes (Perreten et al. 1997). In fact, horizontal gene transfer is essential for bacteria to survive and adapt to new environments (Kurland et al. 2003). Strains intended for the use in food systems as starters or

probiotics should therefore be carefully examined for antimicrobial susceptibility, especially those isolated during the so called "post-antibiotic era" (Teuber et al. 1999).

However, there is still a lack of agreement on the resistance susceptibility breakpoints for most antimicrobials in LAB (Felten et al. 1999; Charteris et al. 2001; Katla et al. 2001; Danielsen and Wind 2003). Distinguishing between intrinsic, nonspecific, and acquired resistance is difficult and requires that the antimicrobial-resistance patterns of many LAB species from different sources be compared (Teuber et al. 1999). This is a very important task since genes conferring resistance to several antimicrobials (i.e., chloramphenicol, erythromycin, streptomycin, tetracycline, and vancomycin) located on transferable genetic elements (plasmids or transposons) have already been characterized in lactococci (Perreten et al. 1997), lactobacilli (Axelsson et al. 1988; Danielsen 2002; Gevers et al. 2003; Gfeller et al. 2003), and enterococci (Eaton and Gasson 2001; Ozawa et al. 2002 and Huys et al. 2004) from foods. Furthermore, under certain circumstances, LAB strains themselves have been reported to cause infections in humans (Gasser 1994; Husni et al. 1997). The most common way to analyse the susceptibility of a bacterium to antibacterial substance is via detecting the minimal inhibitory concentration (MIC) (Andreja and Matijaši, 2001).

Chapter Three

Materials and Methods

MATERIALS AND METHODS

3.1 Collection of samples

Yoghurt samples were collected from Grameen Dannone yoghurt, Bogra and Khulna Districts of Bangladesh. Immediately after collection, the samples were stored aseptically in low temperature (-4 ^{0}C) refrigerator to protect from contamination and deterioration. In preliminary investigations the yoghurt sample of Grameen Dannone and Barisal region were discarded due to lack of sufficient probiotic properties.

3.2 Media

The bacteria *Lactobacillus spp.* was isolated from yoghurt samples by using modified MRS broth and MRS agar media (De Man, Rogosa and Sharpe, 1960). The composition of the media were: universal peptone (10 gm/liter), meat extract (5 gm/liter), yeast extract (5 gm/liter), dextrose (20 gm/liter), dipotassium hydrogen phosphate (2 gm/liter), diammonium hydrogen citrate (2 gm/liter), sodium acetate (5 gm/liter), magnesium sulfate (0.1 gm/liter), manganous sulfate (0.05 gm/liter), tween 80 (1 ml/liter), bacteriological agar (12 gm/liter for semisolid media). Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of lactobacillus (Hartemink et al., 1997; Lankaputhra et al., 1995; Shah, 2000). The pH of the media was adjusted to 6.5.

3.3 Isolation of bacteria

Lactobacillus was isolated from regional yoghurts by using MRS medium. One gram of each sample was directly dissolved into 100 ml of MRS broth at pH 6.5. After dissolving into MRS broth they were shaken homogeneously. After that, they were incubated at 37^{0}C for 24 h in aerobic condition as a result many fastidious organisms removed in the presence of acetate, citrate, tween-80 etc. and growth of lactic acid bacteria was ensured. The cultures were subjected to five subculture at 37^{0}C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO_2 to remove unwanted bacteria. After seven subcultures, the bacterial culture was streak onto MRS agar media at pH 4.8. Finally, the single colony of lactobacillus was isolated by observing their colony morphology and some biochemical tests and the culture were maintained in MRS broth at pH 5.5.

3.4 Identification

The isolated bacteria were identified as *Lactobacillus spp.* by observing their morphological characteristics and by means of some biochemical tests, such as gram stain, motility test, catalase test, endospore test, milk coagulation activities, 0.4% bacteriostactic phenol tolerance test and 1-10% NaCl tolerance test. MRS broth containing inhibitory substances such as 0.4% phenol and 1-10% NaCl were inoculated with 1% (v/v) 24 h active culture of lactobacillus and incubated (anaerobically) for 24 h at 37^0C in the presence of 10% CO_2.

3.5 Determination of optimal growth and P^H

For the determination of optimal growth and P^H of lactobacillus, 1% (v/v) fresh over night culture of lactobacillus were inoculated into MRS broth with varying pH ranging from 2.5-8.5. The pH were adjusted with concentrated acetic acid (99%) and 5 N NaOH. The inoculated broths were incubated in anaerobic condition 24 h at 37^0C in the presence of 10% CO_2. After 24 h of incubation growth of the bacteria were measured using a spectrophometer, reading the optical density at 560 nm (OD_{560}) against the uninoculated broth.

3.6 Assay for NaCl tolerance

For the determination of NaCl tolerance of isolated lactobacillus 10 test tube containing MRS broth were adjusted with different concentration (1-10%) of NaCl. After sterilization, each test tube was inoculated with 1% (v/v) fresh over night culture of lactobacillus and incubated at 37^0C for 24 h. After 24 h of incubation their growth were determined by observing their turbidity. Maximum growth were indicated as double positive sign (+ +), normal growth as single positive sign (+) and no growth were indicated as negative sign (-).

3.7 Quantification of organic acid and determination of pH value

One percent (v/v) 24 h active culture of lactobacillus was used to inoculate 10% sterilized skim milk obtained from Milk Vita Co-operative Bangladesh Ltd. and initial P^H (6.68) was determined by a digital electrode P^H meter. The inoculated skim milk was incubated at 37^0C for 72 h and samples were collected in every 24 h, 48 h and 72 h and liquids of coagulated milk were separated by filtration. P^H of the separated liquid was recorded using a digital electrode P^H meter and quantification of organic acid was performed through titration with 0.1 N NaOH.

3.8 Assay for determination of bile tolerance

Bile tolerances of isolated lactobacillus from regional yoghurts were determined using protocol described by Graciela and Maria (2001).

3.8.1 Materials

3.8.1.1 Growth media

MRS broth was used for culturing lactobacilli. Final P^H of the medium was adjusted to 6.5 and was sterilized at 121^0C for 20 minutes.

3.8.1.2 Active culture

An active culture was grown in MRS broth for about 16 h at 37°C (overnight culture).

3.8.1.3 Phosphate buffer

Solution A (0.2 M NaH_2PO_4): 27.6 g $NaH_2PO_4 \cdot H_2O$ was taken and made up to 100 ml with distilled water.

Solution B (0.2 M Na_2HPO_4): 53.05 g $Na_2HPO_4 \cdot 7H_2O$ was taken and made up to 100 ml with distilled water.

To prepare 1 L of 0.1 *M* sodium phosphate buffer (pH 7.0), 195 ml of solution A and 305 ml of solution B were mixed and made up to 1000 ml with distilled water. Sterilization was done at 121°C for 20 min.

3.8.1.4 Media for Determination of Bile Tolerance (for 1 L)

3.8.1.4.1 MRSO broth: MRS broth were supplemented with 0.5, 1, 1.5, and 3 g bile oxgall (Sigma Laboratories, UK) to obtain a final concentration of 0.05%, 0.1%, 0.15%, and 0.3 %, respectively and were sterilized at 121°C for 20 min.

3.8.2 Methods

The lactobacilli under study were grown in MRS broth at 37°C for 16 h and the cells were harvested by centrifugation at 5000*g* for 10 mins. The pellets were washed twice, obtained with 0.1 M phosphate buffer at pH 7.0. The cells were resuspended to the original volume with the buffer by vortexing. MRS and MRSO broth were inoculated with 0.5% bacterial suspension. The broths were incubated at 37^0C in a CO_2 incubator in the presence of 10% CO_2 gas. The optical density was read at 560 nm (OD_{560}) against the blank (uninoculated broth) every hour for the first 8 h and after 24 h of incubation. The optical density values were plotted against incubation time and results have shown in table and graphs.

3.9 Assay for determination of gastric juice resistance

Gastric juice resistances of isolated lactobacillus from regional yoghurts were assayed using protocol described by Graciela and Maria (2001).

3.9.1 Media for Determination of Acid Resistance (for 1 L)

3.9.1.1 Artificial gastric juice: NaCl (2 g), pepsine (3.2 g) (Sigma, UK), were adjusted at a final pH 2.22 with HCl without dilution, and taken to 1 L with distilled water. As a control, artificial gastric juice was adjusted at a final pH 6.6 with 5 *N* NaOH. Sterilization was done by filtration (filter membrane 0.22 μm).

3.9.2 Materials for Gastric Juice Resistance

1. An overnight culture in MRS broth.
2. 0.1 *M* phosphate buffer, pH 7.0, sterile.
3. Artificial gastric juice, pH 2.22 and pH 6.6
4. 0.1% peptone water: To prepare 1 L, 1 g peptone were weigh and brought it to 1000 ml with distilled water and Sterilization was done at 121°C for 20 min.
5. MRS agar.

3.9.3 Methods

The lactobacilli under study were grown in MRS broth at 37°C for 16 h and the cells were harvested by centrifugation at 5000*g* for 10 min. The pellets were washed twice, obtained with 0.1 M phosphate buffer at pH 7.0. The cells were resuspended to the original volume with the buffer by vortexing. The artificial gastric juice having pH 2.22 and pH 6.6 were inoculated with the 2% (v/v) bacterial suspension. Both the media were incubated at 37°C and samples were taken at 0, 1, 2, 3, and 4 h and after 24 h for cell viability measurement. The samples were plated in MRS agar (in mass). Proper dilutions from 10-fold serial dilutions were prepared in 0.1% peptone water. The plates were incubated at 37°C for 24 h, and counted the resulting colonies after that time. Results were expressed as colony-forming units (CFU) per milliliter (CFU/ml).

3.10 Assay for determination of sensitivity to antibiotic

Sensitivity of lactobacillus to different antibiotics was determined by minimum inhibitory concentration (MIC). MIC was done using the method described by Andrews (2001). According to this method stock solutions 128 μg/ml of 9 different selected antibiotics such as amoxicillin, gentamicin, tetracyclines, clindamicins, kanamycin, nalidixic acid,

metronidazole, azithromicin and cefradine were prepared. All antibiotics except Kanamycin were obtained from Square Pharmaceuticals Ltd. Bangladesh. Stock solutions of selected antibiotics were two-fold diluted in the ranges 0.25 μg/ml to 1024 μg/ml in MRS broths. For preparation of the test inoculums the 16 h active culture of lactobacillus were adjusted to 0.5 McFarland standards (10^7 to 10^8 cfu/ml of bacteria) by adding sterile distilled water. 50 μl of test inoculums were inoculated into each dilution and incubation were done 24 h at 37^0C in the presence of 10% CO_2 in a CO_2 incubator. After 24 h incubation, MIC were observed with no visible growth of bacteria.

Chapter Four

Results

RESULTS

4.1 Identification

Bacteria isolated from regional yoghurts were identified as *Lactobacillus spp.* by observing their colony morphology, physiological and as well as some biochemical characteristics. The isolates grows on MRS agar media with 0.05% cysteine showed their colonies morphology small circular, white-creamy in colors. Microscopically they were Gram-positive (Figure 7), rod shaped (Figure 8), non-motile, catalase negative and absence of Endospore (Figure 9). The isolates have the abilities to coagulate milk and were able to tolerate inhibitory substances such as 0.4% bacteriostatic phenol and showed growth in MRS broth containing 1-9% NaCl. Their characteristics have shown in Table 4. According to their characteristics the isolates were identified as *Lactobacillus delbrueckii* subspecies *Bulgaricus, L. acidophilus, L. casei* and *L. rhamnosus.*

Table 4. Morphological, physiological and biochemical characteristics of isolated *Lactobacilli.*

Biochemical and physiological characteristics	*Lactobacillus delbrueckii ssp. Bulgaricus*	*L. acidophilus*	*L. casei*	*L. rhamnosus.*
Colonies morphology	1.0 mm, white, rough, irregular and round	small 0.1-0.5 mm, rough, dull and brownish	white, shiny, smooth and 1.0 mm diameter	Small circular, white-creamy in colors and disc like.
Gram stain	+	+	+	+
Motility test	Non motile	Non motile	Non motile	Non motile
Catalase test	-	-	-	-
Endospore test	-	-	-	-
0.4% phenol	+	+	+	+
Milk coagulation	+	+	+	+
NaCl tolerance	+	+	+	+

+: positive, -: negative.

4.2 Optimal growth and pH

Maximum growths (OD= 2.054 A) of isolated lactobacilli from Bogra yoghurts were observed at pH 5.0 and maximum growth of lactobacilli isolated from Khulna yoghurt (OD= 1.93 A) were observed at pH 6.5. The OD reading was taken by average value of two readings, where control OD was 0.152 A. The results have shown in Figure 1 and Figure 2.

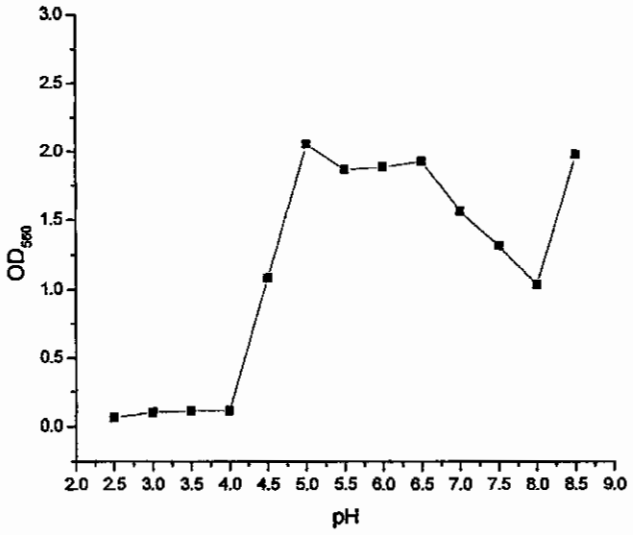

Figure 1. Optimal growth and pH of isolated *Lactobacillus spp.* from Bogra yoghurt sample

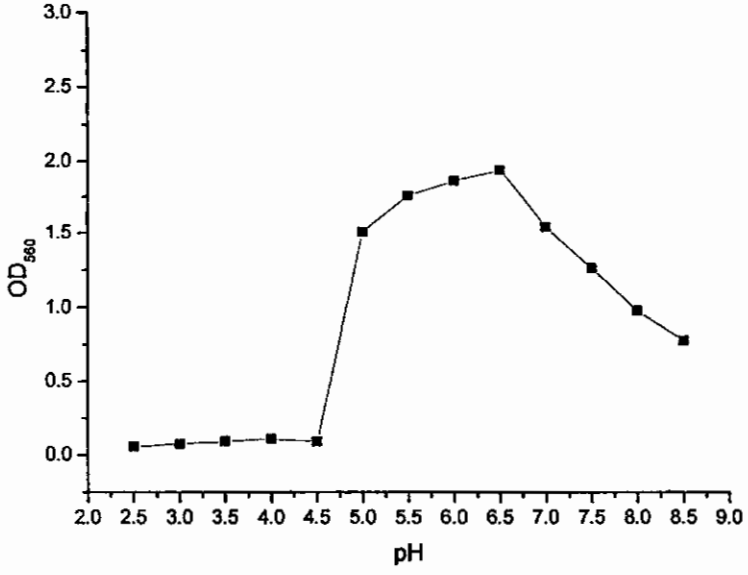

Figure 2. Optimal growth and pH of isolated *Lactobacillus spp.* from Khulna yoghurt sample

4.3 Tolerance to NaCl

The isolated lactobacilli from regional yoghurts were able to tolerate 1-9% NaCl. Good growths were observed in the presence of 1% NaCl, where no growths were observed at 10% NaCl. This result indicates that isolated lactobacilli are able to tolerate high concentration of NaCl like inhibitory substance. The results have shown in Table 5.

Table 5. Tolerance to NaCl of isolated *lactobacilli*

Concentration of NaCl (%)	*Lactobacilli* isolated from yoghurt of Khulna region	*Lactobacilli* isolated from yoghurt of Bogra Region
1	++	++
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	–	–

4.4 Quantification of organic acid and determination of P^H value

Lactobacillus spp. isolated from regional yoghurt was lactic acid bacteria (LAB) as they produced organic acids in the sterilized skim milks detected by titrimetric methods. After 24 h, 48 h and 72 h incubations, the quantities of organic acids were 2.29%, 6.615% and 6.53% and the changed pH were 5.09, 3.73 and 3.65 respectively (incase of lactobacillus spp. isolated from Bogra yoghurt). In case of *Lactobacillus spp.* isolated from the yoghurt of Khulna region, after 24 h, 48 h and 72 h incubations, the quantities of organic acids were 2.13%, 3.92% and 6.26% and the changed pH were 5.13, 4.5 and 3.88 respectively. The results have shown in Table 6.

Table 6. Organic acids (%) and P^H in skim milk produced by isolated *Lactobacillus spp.*

Sources of Bacteria	Name of the bacteria	Incubation time (Hour)	Incubation temp. (^{0}C)	Organic acid (%)	P^H
Bogra yoghurt	*Lactobacillus spp.*	24	37	2.29	5.09
		48	37	6.615	3.73
		72	37	6.53	3.65
Khulna yoghurt	*Lactobacillus spp.*	24	37	2.13	5.13
		48	37	3.92	4.5
		72	37	6.26	3.88

4.5 Bile tolerance

Isolated *Lactobacillus Spp.* was able to survive in 0.05%, 0.1%, 0.15% and 0.3% inhibitory substance, bile acid. The isolated *Lactobacillus spp.* was also able to multiply in above mentioned concentrations of bile acid. In the figure 3 and figure 4 the optical densities values against incubation time has shown.

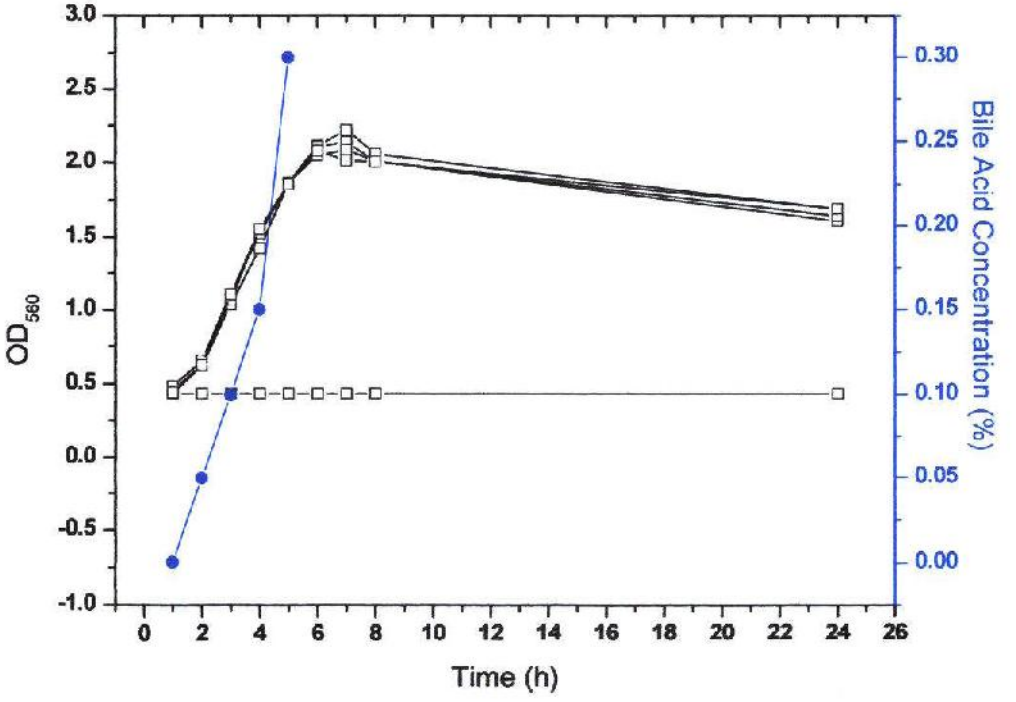

Figure 3. Bile acid tolerance of *Lactobacillus spp.* isolated from Bogra yoghurt

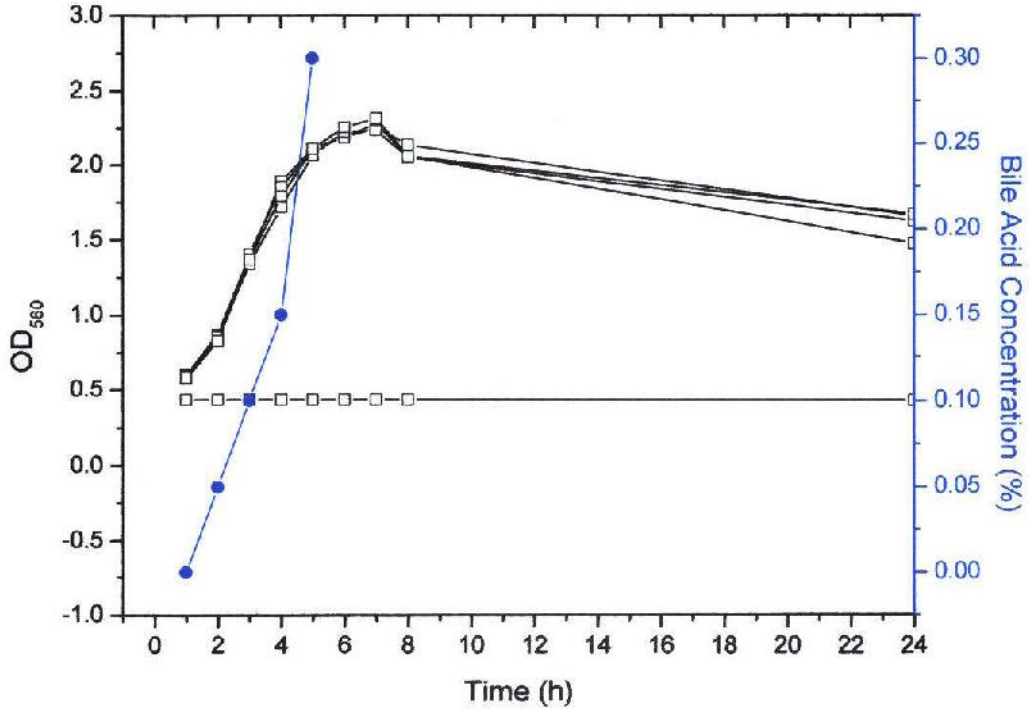

Figure 4. Bile acid tolerance of *Lactobacillus spp.* isolated from Khulna yoghurt.

4.6 Gastric juice resistance

Isolated *Lactobacillus spp.* was able to survive in gastric environment at low pH (2.22) but they were not able to multiply. In per ml artificial gastric juice (pH 2.22) the colony forming unit at 0 h, 1 h, 2 h, 3 h, 4 h and 24 h were 1.5×10^4 (CFU/ml), 1.6×10^4 (CFU/ml), 1.5×10^4 (CFU/ml), 1.36×10^4 (CFU/ml), 1.4×10^4 (CFU/ml) and 1.59×10^4 (CFU/ml) respectively. On the other hand, they were able to survive and had partial multiplication abilities at pH 6.60. In per ml gastric juice (pH 6.60) the colony forming unit at 0 h, 1 h, 2 h, 3 h, 4 h and 24 h were 1.9×10^4 (CFU/ml), 1.93×10^4 (CFU/ml), 1.83×10^4 (CFU/ml), 1.89×10^4 (CFU/ml), 1.94×10^4 (CFU/ml) and 1.99×10^4 (CFU/ml) respectively. Results have shown in figure 5 and figure 6 as colony forming unit (CFU/ml) after 0,1,2,3, 4 h and after 24 h incubations.

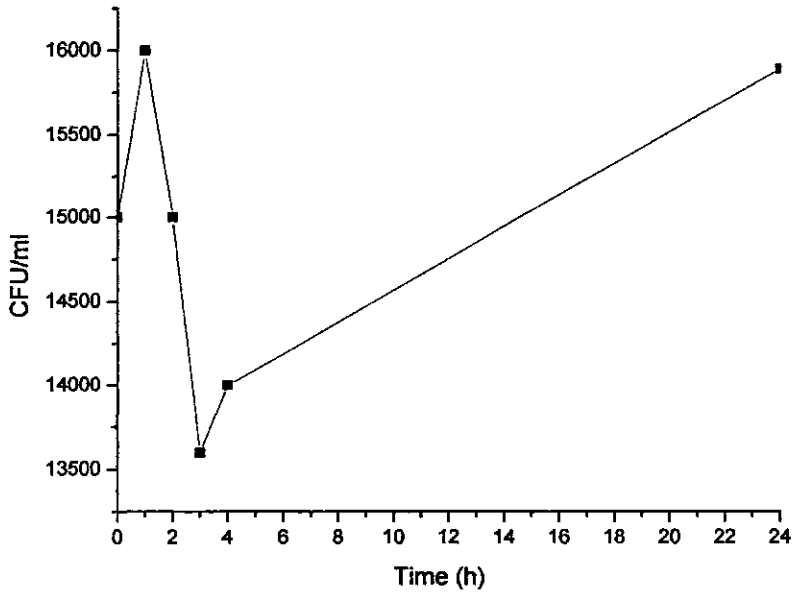

Figure 5. Survival abilities of *Lactobacillus spp.* in simulated gastric juice at pH 2.22.

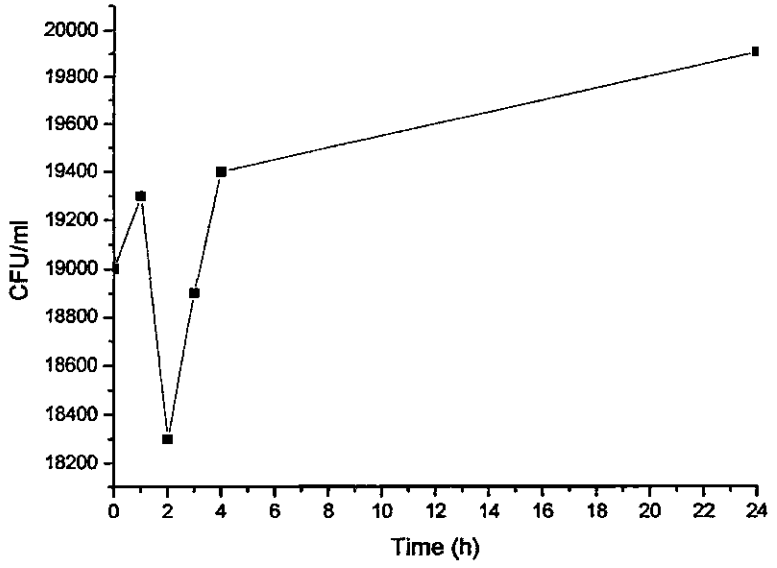

Figure 6. Survival and partial multiplication abilities of isolated *Lactobacillus spp.* in simulated gastric juice at pH 6.6.

4.7 Sensitivity to antibiotic

Sensitivity of *Lactobacillus spp.* and to different antibiotics was determined in term of minimum inhibitory concentration (MIC). The MIC value for *Lactobacillus spp.* isolated

from Bogra yoghurt were 2µg/ml (amoxicillin), 8 µg/ml (gentamycin), 32 µg/ml (tetracyclin), 8 µg/ml (clindamycin), 256 µg/ml (kanamycin), >1024 µg/ml (nalidixic acid), >1024 µg/ml (metronidazol), 8 µg/ml (azithromicin) and 128 µg/ml (cefradine) respectively. On the other hand, isolated *Lactobacillus spp.* from yoghurt of Khulna region of Bangladesh had variation in their MIC value among twelve antibiotics. Their MIC value were 256 µg/ml (amoxicillin), 16 µg/ml (gentamycin), 128 µg/ml (tetracyclin), 8 µg/ml (clindamycin), >1024 µg/ml (kanamycin), >1024 µg/ml (nalidixic acid), >1024 µg/ml (metronidazol), >1024 µg/ml (azithromicin) and >1024 µg/ml (cefradine) respectively. The results have shown in the Table 7.

Table 7. Minimal Inhibitory Concentrations (MIC) of selected antibiotics for isolated *Lactobacillus spp.* from regional yoghurts

Sources of bacteria	Species	MIC (µg/ml)								
		Amoxi	Genta	Tetra.	Clinda	Kana	Nalid	Metro	Azit	Cefra
Bogra Yoghurt	*Lactobacillus spp.*	2	8	32	8	256	>1024	>1024	8	128
Yoghurt Khulna	*Lactobacillus spp.*	256	16	128	8	>1024	>1024	>1024	>1024	>1024

Legend:
Amoxi – amoxicillin, Genta – gentamycin, Tetra – tetracyclin, Clinda – clindamycin, Kana – kanamycin, Nalid – nalidixic acid, Metro – metronidazol, Azit – Azithromicin, Cefra – Cefradine.

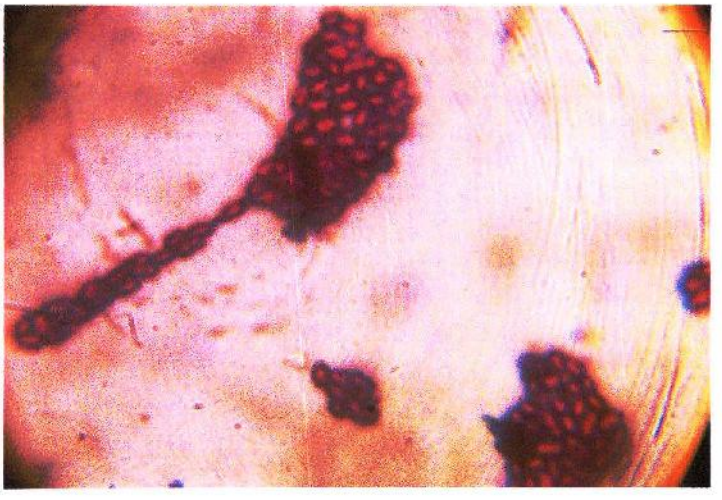

Figure 7. Gram-positive *Lactobacillus spp.*

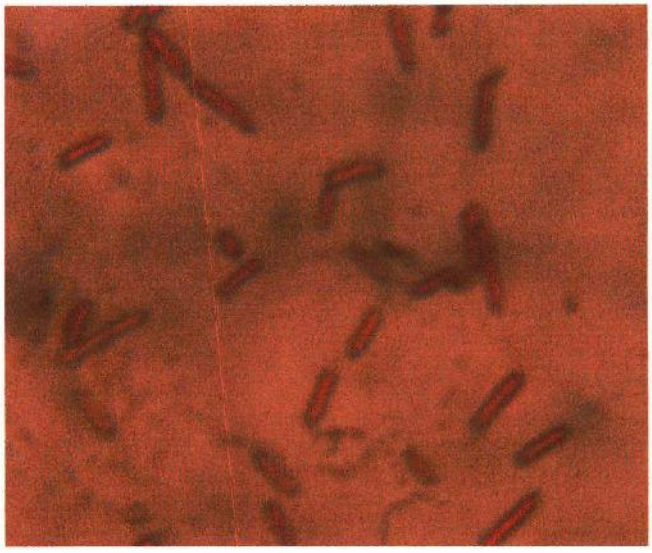

Figure 8. Isolated rod-shaped *Lactobacillus spp.*

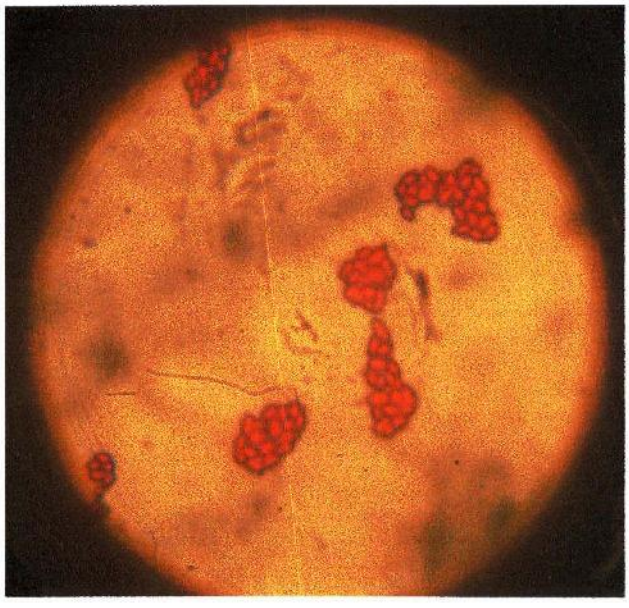

Figure 9. Non-spore forming *Lactobacillus spp.*

Chapter Five

Discussion

DISCUSSION

5.1 Isolation and identification

Lactobacilli were isolated from regional yoghurts (Bogra and Khulna) using modified MRS media (MRS media with additional 0.05% cysteine). Some of the compositions of MRS culture media such as Tween 80, acetate, magnesium and manganese which are known to act as special growth factors for lactobaciili as well as a rich nutrient sources. Among them, Tween 80 is a surfactant, facilitate uptake of nutrients and acetate, ammonium citrate act as selective agents (de Man et al., 1960). On the other hand, addition of 0.05% cysteine in MRS medium increases the specificity for isolation of lactobacilli (Hartemink et al., 1997; Lankaputhra et al., 1995; Shah, 2000). From the literature and experiments it has shown that lactobacilli can survive in extreme low pH and can grow in strictly anaerobic to aerobic conditions. In the present study, the cultures were subjected to seven subcultures at pH 4.5 and incubated in the presence of 10% CO_2. As a result many fastidious organisms were removed and growth of lactobacilli favoured. From their colonies morphologies and biochemical characteristics (gram positive, catalase negative, endospore absence, non-motile, tolerance to inhibitory substances e.g., 0.4% phenol, 1-9% NaCl, and milk coagulation activities) we were able to identified them as *Lactobacillus delbrueckii ssp. Bulgaricus* (1.0 mm, white, rough, irregular, round and white colony), *L. acidophilus* (small 0.1-0.5 mm, rough, dull and brownish colony), *L. casei* (white, shiny, smooth and 1.0 mm diameter) and *L. rhamnosus* (2.0-2.5 mm, white, shiny, smooth and disc like colony) which can be correlated with the study of Tharmaraj and Shah (2003), Kimoto et al. (2004), Siddlingiaya et al. (2007), Vamanu et al. (2005), Emanuel et al. (2005), Lilia et al. (2002), Oyetayo (2004) and Eduardo et al. (2003), who have found the similar aforementioned characteristics in their isolated *lactobacilli.*

Elezete and Carlos (2005) stated that, tolerance to bile and phenol (phenols can be formed in the intestines by bacteria that desaminate some aromatic amino acids delivered by the diet or produced by endogenous proteins) is an important characteristic for a better survival ability, not necessarily of multiplication, in the intestines.

Xanthopoulos et. al. (2000) stated that a 0.4% concentration of phenol causes a bacteriostatic action in some microorganisms

5.2 Optimal growth and P^H

pH is an important factor which can dramatically affect bacterial growth. The pH affects the activity of enzymes—especially those that are involved in biosynthesis and growth (Prescott et al., 2001). Each microbial species possesses a definite pH growth range and a distinct pH growth optimum. Acidophiles have a growth optimum between pH 0.0 and 5.5; neutrophiles between 5.5 and 8.0; and alkalophiles 8.5 to 11.5 (Prescott et al., 2001). In general, different microbial groups have characteristic pH optima. The majority of bacteria and protozoa are neutrophiles. In our experimental design we have observed the growth of our isolated *Lactobacillus spp.* in various pH values ranges from 2.5 to 8.5. The reason for chosen this ranges of pH to determine whether LAB species can grow in acidic and alkaline conditions and also to predict the optimum pH value for good growth. From the experimental results we have found that the isolated *Lactobacillus spp.* from yoghurts was able to survive in extreme acidic pH (pH 2.5 to 3.5) and basic pH (pH 7.5 to 8.5). Their growth was not observed in acidic condition but they had survival abilities, in the contrary they were able to survive and multiplication in alkaline conditions. Maximum growths (OD= 2.054 A) of isolated lactobacilli from Bogra yoghurts were observed at pH 5.0 and incase of lactobacilli isolated from Khulna yoghurt maximum growths (OD= 1.93 A) were observed at pH 6.5. From our investigated results we also found a variation of optimum growth pH between the isolated Lactobacilli from mentioned two regions. It was possibly due to the geographical and ecological niches variations (Haddadin et al., 2004).

Shah (2000), have enumerated *Lb. delbrueckii* ssp. *Bulgaricus* and *Lb. acidophilus* on acidified MRS media at pH 5.3. De Man et al. (1960), Vinderola and Reinheimer (1999), Kandler and Weiss (1984) and MacFaddin (1985) in their study isolated *Lactobacillus casei* ATCC393, *Lactobacillus fermentum* ATCC9338, *Lactobacillus acidophilus* ATCC4356 and *Lactobacillus plantarum* ATCC8014 by using MRS medium at P^H 6.5. Therefore, our study results recommend that *Lactobacillus spp.* could be isolated (from yoghurt) and maintained their culture at pH 5.0 or pH 6.5 for better performances. Our performed study also addressed that rigorous study need to unveil the mechanism behind the optimum growth pH variation among isolated *Lactobacillus spp.* from region to region.

5.3 Tolerance to NaCl

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. NaCl may present in our diet as well as intestinal environment. In our study we have tested isolated *Lactobacillus spp.* with 1-10% of NaCl, to determine whether they can tolerate NaCl or not and also to determine in which concentration of NaCl LAB species show good growth. Our experimental results show that *Lactobacillus spp.* isolated from regional yoghurts is able to tolerate 1-9% of NaCl and good growth was observed at 1% NaCl. From the performed investigation we can certify that lactobacilli isolated from yoghurts are able to survive NaCl riches environment and also able to performed good growth in the presence of 1% NaCl. Therefore, isolated lactobacilli can be selected as potential probiotic organisms. Our experimental results have similarities with previous research findings. Elezete and Carlos (2005) did isolate lactobacilli from gastrointestinal tract of swine that were tolerable to 4-8% NaCl. Schillinger and Lucke (1987) did grow lactobacilli in the presence of 7.5% NaCl isolated from meat and meat products and the result is almost similar to the findings of present study.

5.4 Organic acid and P^H value

Lactic acid bacteria are either homo-fermentative (producing more than 85% of fermentative products as lactic acid) or heterofermentative (producing lactic acid, carbon dioxide, ethanol and/or acetic acid) (Tannock, 2004). Our isolated *Lactobacillus spp.* may possibly heterofermentative due to the production of mixed acids in the reconstituted skim milk and they were also able to coagulate the milk into curd. The present experiment indicates that organic acid production was increased with the incubation time. On the other hand, pH of the media was decreased with the increasing acid production. Highest acidity (6.53%) and lowest pH (3.65) was observed after 72 h incubation at 37^0C incase of probiotic LAB isolated from Bogra yoghurt. On the other hand, probiotic bacteria isolated from yoghurt of Khulna region of Bangladesh had highest acid (6.26%) and lowest P^H (3.88) value after 72 h incubation. This investigation clearly indicates that, there is a minor variation in organic acid production due to their regional variation. This finding has the similarity with opinion of Haddadin et al. (2004) who stated that, a strain of *Lactobacillus acidophilus* isolated in North America may well be genetically different from a species recovered in Europe or the Middle East.

Lactobacillus delbrueckii ssp. Bulgaricus M3 40-3 isolated from traditional fermented milk "Dahi' by Rashid et al. (2007) were able to produce highest 2.13% acid in reconstituted skim milk at 37^0C for 72 h incubation period. These properties of the isolated *Lactobacillus spp.* will help to withstand in gastric environment as well as inhibition of many pathogenic microorganisms in the intestine due to their production of lactic acid with other organic acids. Therefore, isolated *Lactobacillus spp.* could be use as a good probiotic candidate.

5.5 Bile tolerance

Bile tolerance is one of the important characteristic of probiotic lactic acid bacteria used as adjuncts because they enable them to survive, to grow, and to perform their beneficial action in the gastrointestinal tract (GIT) (Walker and Gilliland, 1983). Although the degree of tolerance required for maximum growth in the GIT is not known, it seems reasonable that the most bile- and acid-resistant species should be selected (Graciela and María, 2001). In our experimental design, we used 0.05%-0.3% of bile concentration, this is, because, it is corresponded to that found in the human intestinal tract and 0.3% bile is the maximum concentration that is present in healthy men (Graciela and María, 2001). Therefore, before selection of probiotic bacteria for human purpose it must be endurable to 0.3% bile concentration (Gilliland et al., 1984; Pennacchia et al., 2004). Our isolated *Lactobacillus spp.* from both yoghurts (Bogra and Khulna regions of Bangladesh) is able to tolerate up to 0.3% of bile concentrations. The bile tolerances of *Lactobacillus spp.* from same yoghurt were similar; this possibly attributable to presence of bacteria in of the same ecological niche. In fact, it was possibly due to horizontal gene transfer between the bacterial species (Kurland et al., 2003). Elizete and Carlos (2005) have stated that, tolerance to bile is an important characteristic for better survival ability, not necessarily of multiplication, in the intestines. Our isolated *Lactobacillus spp.* are able to survive and multiplication in the presence of 0.3% bile concentration. Therefore, our isolated bacterial species could be used as potential probiotic organisms with better survival and multiplication abilities.

5.6 Gastric juice resistance

Kilara (1982) stated that gastric juice resistance is one of the important characteristic of probiotic lactic acid bacteria to survive, to grow, and to perform their beneficial action in the gastrointestinal tract (GIT) (Catanzaro and Green, 1997). In our experimental medium pepsin

were added to simulate the activity of gastric environment. The viable cell count of *Lactobacillus spp.* on MRS agar media were 1.5 × 10^4 (CFU/ml) at 0 h and 1.59× 10^4 (CFU/ml) at 24th h (cell plated from artificial gastric juice at pH 2.22). These results indicate that there were no losses of viability of cell in simulated gastrointestinal condition. On the other hand, cell plated from artificial gastric juice with pH 6.6 the viable cell counts were 1.9× 10^4 (CFU/ml) at 0 h and 1.99 × 10^4 (CFU/ml) at 24th h. These results indicate that the cells were able to survive and may have little multiplication abilities in artificial gastric juice at pH 6.6. From this experiment we are also able to apprehend that in GIT environment the gastric juice will have minor or possibly no adverse effect on our isolated probiotic bacteria. In our simulated gastric juices there were no nutrients for bacterial growth and multiplication. On the other hand, bacteria will be in touch of some minimal nutrients in our GI tract, therefore it will help to supply some nutrients for bacterial growth and multiplication. Gibson et al. (year anon) stated that, the typical transit time of a small food bolus in the stomach is approximately 20 minutes, where our isolated *Lactobacillus spp.* were able to survive in simulated gastric juices 24 hours. In our investigation we have counted the viability of bacteria on MRS agar media instead of counted by optical densities. This is because in spectrophotometric method dead cell may present as false enumeration. Bacterial culture plated on MRS agar media from both simulated gastric juices (pH 2.22 and 6.6) showed four morphologically different colonies at 0 h and 24th h. We able to identified them as *Lactobacillus delbrueckii ssp. Bulgaricus, Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus rhamnosus.* This result indicates that mentioned four species of *Lactobacilli* are able to survive in simulated GI tract.

Our experimental results have similarities with the results obtained by Gibson et al. (year anon), where seven Lactobacillus strains: *L. casei immunitass* (strain 46), *L. casei shirota* (strain 47*), L. plantarum* (WCH1*), L. pentosus (WCA2), L. reuterii* (strain 48*), L. acidophilus* subsp. *johnsonii (*WCG2) *and L. delbrueckii* subsp. *bulgaricus* (WCF1) did show strongest acid and bile tolerance in simulated gastric environments.

5.7 Sensitivity to antibiotic

Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes (Flórez et al., 2005). It is a controversial issue, that LAB may transfer antimicrobial resistance genes to different species of bacteria. Curragh and Collins (1992) stated that the antibiotic resistances of LAB are intrinsic and nontransmissible and these intrinsically antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of various antimicrobial agents (Salminen et al., 1998). Some researches have shown that antimicrobial resistant gene may transfer to pathogens present either in food matrix or GIT (Flórez et al., 2005; Kruse and Sorum, 1994). Therefore, it is important to determine the antibiotic susceptibility of LAB and the most common way to analyse the susceptibility of a bacterium to antimicrobial substances via detecting the Minimal Inhibitory Concentration (MIC) (Majhenic and Matijasic, 2001). In our experiment we have used 9 selected antibiotics which did belong to 3 groups: inhibitors to cell wall synthesis (e.g., amoxicillin, cefradine), inhibitors of protein synthesis (e.g., kanamycin, gentamycin, azithromicin, tetracyclines, clindamycin etc.) and inhibitors of nucleic acid synthesis (e.g., nalidixic acid and metronidazol). From the MIC values of 9 tested antibiotics it was found that, *Lactobacillus spp.* isolated from Bogra yoghurt of Bangladesh was sensitive to amoxicillin (MIC = 2 μg/ml), moderately sensitive to gentamycin (MIC = 8 μg/ml), clindamycin (MIC = 8 μg/ml), azithromicin (MIC = 8 μg/ml) and resistant to kanamycin (MIC = 256 μg/ml), nalidixic acid (MIC > 1024 μg/ml), metronidazol (MIC > 1024 μg/ml), cefradine (MIC = 128 μg/ml) and tetracyclin (MIC = 32 μg/ml). On the other hand, *Lactobacillus spp.* isolated from yoghurt of Khulna region of Bangladesh were sensitive to gentamicin (MIC = 16 μg/ml), clindamycin (MIC = 8 μg/ml) and resistant to amoxicillin (MIC = 256 μg/ml), tetracyclin (MIC = 128 μg/ml), kanamycin (MIC > 1024 μg/ml), nalidixic acid (MIC > 1024), metronidazol (MIC > 1024 μg/ml), azithromicin (MIC > 1024 μg/ml) and cefradine (MIC > 1024 μg/ml). This investigation revealed that, some antibiotics such as amoxicillin, gentamycin, clindamycin and azithromicin intake can drastically drop the *Lactobacillus spp.* from intestinal microflora, on the other hand five antibiotics viz. tetracyclin, kanamycin, nalidixic acid, metronidazol and cefradine will not influence the growth of lactobacilli population. Although exceptional result was observed from *Lactobacillus spp.* isolated from yoghurt of Khulna region. Where isolated lactobacilli were sensitive to only gentamycin and

clindamycin and were resistant to seven antibiotics including amoxicillin (higher MIC). There are several mechanisms underlying behind the LAB species resistant to wide ranges of antibiotics, one of the main mechanisms resistance to inhibitors of cell-wall synthesis, is due to cell-wall impermeability, since LAB species lack cytochrome-mediated electron transport (Condon 1983). One of the important reasons, LAB species resistance to protein synthesis inhibitors antibiotics, is position of potentially transferrable genes on their plasmid which conferring resistance to one or more protein synthesis inhibitors antimicrobial agents (Axelsson et al. 1988; Perreten et al. 1997; Danielsen 2002; Gevers et al. 2003; Gfeller et al. 2003). All the tested *Lactobacillus spp.* is resistant to metronidazol, since LAB have no hydrogenase activity (Church et al. 1996). From our experiments we did find that *Lactobacillus spp.* isolated from yoghurt of Khulna region have shown broad ranges of resistances to most of the antibiotics including amoxicillin. This was possibly due to wide use of antibiotics in veterinary medicine and agriculture which could be contributing to the dissemination of resistances into yoghurt related ecological niches, and is supported by the opinion of Flórez et al. (2005). Another important reason, production of dairy products such as yoghurts from raw milk in antibiotic challenged environments may select antibiotic resistant LAB harbouring transmissible resistance genes (Perreten et al. 1997). In Bangladesh a wide ranges of non-specific antibiotics treatment is a common practice, as a result many pathogenic bacteria in the geographical location of Bangladesh has gained resistance to several groups of antibiotics including amoxicillin. One important reasons of becoming our isolated LAB species resistant to aforementioned antibiotics, is due to a strong link between the use of a particular antibiotic in a locality and the incidence of resistant bacterial strains (Stuart, 2005). This is because of selective pressure favouring the resistant of bacterium. Other important reasons of bacterial species resistant to antibiotic are horizontal gene transfer (Kurland et al. 2003) and mutation, which occur spontaneously in bacteria (Stuart, 2005), it is also a common phenomenon among LAB species (Yanfen et al. 2004), which render them resistant to one antibiotic or another (Stuart, 2005). Whereas many other bacteria can pump the antibiotic back out of the cell before it has had a chance to act, by means of enzymes called *translocases*; this the fairly non specific, leading to multiple drug resistance (Stuart, 2005). Our experimental results have similarities with many previous research findings. The LAB species isolated by Flórez et al. (2005) have shown resistance to metronidazol (MIC ≥

32 μg/ml), clindamycin (MIC ≥ 32 μg/ml), tetracyclin (MIC = 64 μg/ml) and sensitive to penicillin (MIC ≥ 16 μg/ml). Other authors such as Bayer et al. (1978); Vescovo et al. (1982); Charteris et al. (1998); Virginia et al., (2006) and Kheadr, (2006); have found that isolated lactobacilli were resistant to metronidazol, kanamycin (MIC = 125 to 500 μg/ml), nalidixic acid (MIC = >500 μg/ml) and were sensitive to amoxicillin and some author have found moderate sensitive to clindamycin and tetracyclin. From the study we have concluded that *Lactobacillus spp.* resistance to antibiotics could be used concomitantly while patients undergoing antibiotic treatment if the statements of Curragh and Collins (1992) is true that the antibiotic resistances of LAB are intrinsic and nontransmissible. Therefore, extensive study need to unveil the mechanism whether antibiotic resistances of LAB species transmissible or not. Our investigation findings recommend, *Lactobacillus spp.* intended for the use in the food systems as starter or probiotics should therefore be carefully examined for antimicrobial susceptibility, especially those isolated during so called antibiotic challenged environment.

6. SUMMARY AND CONCLUSION

The experimental results showed that isolated *Lactobacillus spp.* are able to tolerate inhibitory substances such as 0.05-0.3% bile acid, 0.4% phenol, 1-8% NaCl and also able to survive in simulated gastric (pH 2.22) condition, as well as in alkaline (pH 8.5) condition. The isolated lactobacilli from regional yoghurts were able to produce organic acid in milk. From the MIC results, we have found that the *Lactobacillus spp.* are sensitive to amoxicillin, gentamycin, clindamycin, azithromycin resistant to kanamycin, nalidixic acid, metronidazol, cefradine and tetracyclin which are most frequently used antibiotics in Bangladesh regarding treatments of human and animals. Although different results have found in case of *Lactobacillus spp.* isolated from yoghurt of Khulna region of Bangladesh which was resistant to amoxicillin (MIC = 256 μg/ml), azithromycn (MIC > 1024 μg/ml) with higher MIC. From all of our conducted experiments we have observed that our isolated *Lactobacillus spp.* could be used as a good candidate of probiotics. According to World Health Organization (WHO) and Food and Agriculture Organization (FAO, 2002) working group guidelines the probiotic organisms should possess the characteristics of resistance to gastric acidity and bile acid with other attributed criteria. Our isolated *Lactobacillus spp.* had aforementioned characteristics.

Our experimental results recommend that species specific probiotics properties of regional LAB need to study for up-scaling of technological benefits, better efficacy and advancement of food biotechnological researches. So far from our conducted study we could assume that there is a potential variation among the probiotic properties of LAB from country to country and region to region. Therefore, species level isolation, identification and probiotic properties of regional lactobacilli need to study for technological interest and product development with better efficacy.

References

REFERENCES

Andreja, C.M. and B.B Matijaši. 2001. Antibiotics influence on lactic acid bacteria inhabiting gastrointestinal tract. Mljekarstvo. vol. 51, pp. 119-134.

Andrews, M.J. **2001.** Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy. Vol. 48, pp. 5-16.

Aso, Y. and H. Akazan. **1992.** Prophylactic effect of a Lactobacillus casei preparation on the recurrence of superficial bladder cancer. Urol Int. vol. 49, pp. 125-9.

Axelsson, L.T., S.E. Ahrné, M.C. Andersson and S.R. Stahl. **1988.** Identification and cloning of a plasmid-encoded erythromycin resistance determinant from *Lactobacillus reuteri*. Plasmid. Vol. 20, pp. 171–174.

Azcarate-Peril, A.M. and R.R. Raya **2001.** Food Microbiology Protocols. (J.F.T. Spencer and A.L.R.D. Spencer, Ed.), Totowa, New Jersey: Humana Press Inc., chap. 17(Methods for Plasmid and Genomic DNA Isolation from Lactobacilli), p. 135.

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clinic Path. Vol. 45, pp. 493-496.

Bayane, A., R. Dominique, R. D. Dauphin, J. Destain, B. Diawara, P. Thonart. 2006. Assessment of the physiological and biochemical characterization of a *Lactic acid bacterium* isolated from chicken faeces in sahelian region. African J. of Biotech. vol. 5, pp. 629-634.

Bibel, D.J. **1988.** Elie Metchnikoff's bacillus of long life. ASM News. vol. 54, pp. 661–665.

Biller, et al., **1995.** Treatment of recurrent Clostridium difficile colitis with Lactobacillus GG. Journal of Pediatric Gastroenterology & Nutrition, Vol. 21, pp. 224-226.

Birollo, G.A., J.A. Reinheimer, C.G. Vinderola. **2000.** Viability of lactic acid microflora in different types of yoghurt. Food Res. Int. vol. 33 pp. 799–805.

Catanzaro, J. and Green. L. 1997. Microbial ecology and probiotics in human medicine (Part II). Altern Med Rev., Vol. 2(4), pp. 296-305.

Charteris, W.P., P.M. Kelly, L. Morelli and J.K. Collins. **1998.** Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Protect. Vol. 61, pp. 1636-1643.

Charteris, W.P., P.M. Kelly, L. Morelli, and J.K. Collins. **2001.** Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. J. Food Prot. vol. 64, pp. 2007–2014.

Church, D.L., R.D. Bryant, V. Sim, and E.J. Laishley. **1996.** Metronidazole susceptibility and the presence of hydrogenase in pathogenic bacteria. Anaerobe. vol. 2, pp. 147–153.

Condon, S. **1983.** Aerobic metabolism of lactic acid bacteria. Ir. J. Food Sci. Technol. vol. 7, pp. 15–25.

Curragh, H.J, M.A. Collins. **1992.** High levels of spontaneous drug resistance in Lactobacillus. Journal of Applied Bacteriology. vol. 73, pp. 31-36.

Dairy Council of California. **2000.** Probiotics – Friendly Bacteria with a Host of Benefits. pp. 1-4.

Danielsen, M. **2002.** Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. Plasmid. vol. 48, pp. 98–103.

Danielsen, M. and A. Wind. **2003.** Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int. J. Food Microbiol. vol. 82, pp. 1–11.

Danielsen, M., and A. Wind. **2003.** Susceptibility of *Lactobacillus* spp. to antimicrobial agents. Int. J. Food Microbiol. vol. 82, pp. 1–11.

Dave, R.J. and N.P. Shah. **1997.** Effect of cysteine on the viability of yoghurt and probiotic bacteria in yoghurts made with commercial starter cultures. *Int. Dairy J.*, Vol. 7(8/9). pp. 537-545.

De Man, J. C., M. Rogosa, and M. E. Sharpe. **1960.** A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* Vol. 23, pp. 130–135.

Eaton, T.J., and M.J. Gasson. **2001.** Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. vol. 67, pp. 1628–1635.

Eduardo, L.C.Jr., V.P. Carmina, Ma. R.B. Rivera and E.C. Cabrera. **2003.** Antimicrobial Activity of Probiotics from Milk Products. Phil J Microbiol Infect Dis. Vol. 32, pp. 71-74.

Elizete, D. F.R. P. and R.S. Carlos. **2005.** Biochemical characterization and identification of probiotic *Lactobacillus* for swine. B.CEPPA, Curitiba, vol. 23. no. 2, pp. 299-310.

Emanuel, V., V. Adrian, P. Ovidiu and C. Gheorghe. **2005.** Isolation of a *Lactobacillus plantarum* strain used for obtaining a product for the preservation of fodders. African Journal of Biotechnology. Vol. 4, pp. 403-408.

FAO and WHO. **2002.** Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food London, Ontario, Canada. pp.1-11.

Felten, A., C. Barreau, C. Bizet, P.H. Lagrange and A. Philippon. **1999.** *Lactobacillus* species identification, H_2O_2 production, and antibiotic resistance and correlation with human clinical status. J. Clin. Microbiol. vol. 37, pp. 729–733.

Fernandes, C.F., K.M. Shahani, M.A. Amer. **1987.** Therapeutic role of dietary lactobacilli and *Lactobacillus* fermented dairy products. FEMS Microbiol., Vol. 46, pp. 343-356.

Flórez, B. A., S. Delgado, and B. Mayo. **2005.** Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. Can. J. Microbiol. vol. 51, pp. 51–58.

Fukushima, M., A. Yamada, T. Endo and M. Nakano. **1999.** Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* on delta6-desaturase activity in the livers of rats fed a fat- and cholesterol-enriched diet. Nutrition, Vol. 15, pp. 373-378.

Fuller, R. **1989.** Probiotics in man and animals. J. Appl. Bacteriol, Vol. 66, pp. 365-378.

Fuller, R. **1991.** Probiotics in human medicine. Gut 32, pp. 439-442.

Fuller, R. **1992.** Probiotics: The scientific basis. Chapman & Hall, London, p. 398.

Gardiner, G., C. Heinemann, M. Baroja, A. Bruce, D. Beuerman, J. Madrenas, G. Reid. **2002.** Oral administration of the probiotic combination *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 for human intestinal applications. Int. Dairy J., Vol. 12, pp. 191-196.

Gasser, F. **1994.** Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull. Inst. Pasteur. vol. 92, pp. 45–67.

Gevers, D., M. Danielsen, G. Huys, and J. Swings. **2003.** Molecular characterization of *tet(M)* genes in *Lactobacillus* isolates from different types of fermented dry sausages. Appl. Environ. Microbiol. vol. 69, pp. 1270–1275.

Gfeller, K.Y., M. Roth, L. Meile, and M. Teuber. **2003.** Sequence and genetic organization of the 19.3-kb erythromycin- and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. Plasmid. Vol. 50, pp. 190–201.

Gibson, G.R., G. Rouzaud, J. Brostoff and N. Rayment. An evaluation of probiotic effects in the human gut: microbial aspects. Final Technical report for FSA project. University of Reading and King's College, London. pp. 1-19.

Gilliland, S. E., T. E. Staey and L. J. Bush. **1984.** Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct. Journal of Dairy Science, Champaign, vol. 67, pp.30-45.

Goldin, B.R. **1990.** Intestinal microbiota: Metabolism of drugs and carcinogens. Ann. Med. Vol. 22, pp. 43-48.

Graciela, F. de V. and María, P. T. **2001.** Food Microbiology Protocols. (J.F.T. Spencer and A.L.R.D. Spencer, Ed.), Totowa, New Jersey: Humana Press Inc., chap. 21 (Probiotic Properties of Lactobacilli), pp. 173-181.

Haddadin, M.S.Y., S.S. Awaisheh and R.K. Robinson. **2004.** The Production of Yoghurt with Probiotic Bacteria Isolated from Infants in Jordan. Pakistan Journal of Nutrition, vol. 3 (5), pp. 290-293.

Hammes, W., N. Weiss and W. **1995.** Holzapfel. The genera *Lactobacillus* and *Carnobacterium*. In: Balows A, H. Tr¨uper, M. Dworkin, W. Harder and K.H. Schleifer, eds. The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Applications. Vol II. 2nd ed. New York, NY: Springer. pp. 1536–1594.

Hammes, W.P., **1986.** Starterkulturen in der Fleischwirtschaft. Chem. Microbiol. Technol. Lebensm. Vol. 9, pp. 131-143.

Harish, K., T. Varghese. **2006.** Probiotics in humans – evidence based review. Calicut Medical Journal. vol. 4, pp. 1-11.

Harris, L.J., M.A. Daeschel, M.E. Stiles and T.R. Klaenhammer, **1989.** Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. J. Food Protection. Vol. 52, pp. 384-387.

Hartemink R., V.R. Domenech, F.M. Rombouts. **1997.** LAMVAB – A new selective medium for the isolation of lactobacilli from faeces, J. Microbiol. Meth. Vol. 29, pp. 77–84.

Hasan, N.A. and J.F. Frank **2001.** Applied Dairy Microbiology (2nd Edition). (E.H. Marth and J.L. Steele, Ed.), New York: Marcel Dekker, Inc., chap. 6 (Starter Cultures and Their Use), pp. 152-155.

Holzapfel, W.H, P. Haberer, J. Snel, U. Schillinger, J.H Huis in't. **1998.** Overview of gut flora and probiotics. Int. J. Food Microbiol. vol. 41, pp. 85-101.

Holzapfel, W.H., P. Haberer, R. Geisen, J. Björkroth, U. Schillinger. **2001.** Taxonomy and important features of probiotic microorganisms in food nutrition. Am. J. Clin. Nutr. Vol. 73, 365S-373S.

Husni, R.N., S.M. Gordon, J.A. Washington, and D.L. Longworth. **1997.** *Lactobacillus* bacteriemia and endocarditis: review of 45 cases. Clin. Infect. Dis. vol. 25, pp. 1048–1055.

Huys, G., K. D'Haene, J.-C. Collard, and J. Swings. **2004.** Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. Appl. Environ. Microbiol. vol. 70, pp. 1555–1562.

IDF. **1997.** Dairy Stater cultures of Lactic Acid Bacteria (LAB), Standard 149A, Int. Dairy Fed. Brussels, Belgium.

IDF. **1997.** Yoghurt-Enumeration of characteristic micro-organisms-colony count technique at 37 °C Standard 117 B, Int. Dairy Fed. Brussels, Belgium.

Isolauri, E., M. Juntunen, T. Rautanen, P. Sillanaukee, T. Koivula. **1991.** A human Lactobacillus strain (Lactobacillus casei sp. Strain GG) promotes recovery from acute diarrhea in children. Pediatrics. vol. 88, pp. 90-97.

Itoh, T., Y. Fujimoto, Y. Kawai, T. Toba and T. Saito. **1995.** Inhibition of food borne pathogenic bacteria by bacteriocins from *Lactobacillus gasseri*. Lett. Appl. Microbiol. vol. 21, pp. 137-141.

Jin, L.Z, Y.W. Ho, N. Abdullah, M.A. Ali and S. Jalaludin. **1996.** Antagonistic effects of intestinal Lactobacillus isolates on pathogens of Chicken. Lett. Appl. Microbiol. vol. 23, pp. 67-71.

Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food London, Ontario, Canada, April 30 and May 1, **2002.** Guidelines for the evaluation of probiotics in food. pp. 1-11.

Kacem, M. and K. Nour-Eddine. **2006.** *In vitro* preselection criteria for probiotic lactobacillus plantarum strains of fermented olives origin. International Journal of Probiotics and Prebiotics. Vol. 1. No. 1. pp. 27-32.

Kaenhammer, T.R. **2000.** Probiotic bacteria: today and tomorrow. *J. Nut.*, vol. 130, pp. 415-16.

Kandler, O. and N. Weiss. **1986.** Regular non-sporing Gram-positive rods, Genus *Lactobacillus* (Beijerinck 1901, 22), In: PHA Sneath, NS Mair, ME Sharpe, JG Holt (Eds.), Bergey's manual of systematic bacteriology, 8th ed, vol. 2, William and Wilkins Co, Baltimore, pp. 1208-1234.

Kandler, O., N. Weiss. **1984.** Genus *Lactobacillus*, in: Sneath P.H.A., N.S. Mair, M.E. Sharpe., J.G. Holt (Eds.), Bergey's Manual of Systematics Bacteriology, Williams and Wilkins, Baltimore M.D., USA, vol. 2, pp. 1209–1234.

Katla, A.K., H. Kruse, G. Johnsen, and H. Herikstad. **2001.** Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. Int. J. Food Microbiol. vol. 67, pp. 147–152.

Kheadr, E.E. 2006. Impact of acid and oxgall on antibiotic susceptibility of *Probiotic Lactobacilli.* African Journal of Agricultural Research. vol. 1 (5), pp. 172-181.

Kilara, A. **1982.** Influence of *in vitro* gastric digestion on survival of some lactic cultures. *Milchwissenschaft, Vol.* 37, pp. 129–132.

Kimoto, H., M. Nomura, M. Kobayashi, T. Okamoto and S. Ohmomo. **2004.** Identification and Probiotic Characteristics of *Lactococcus* Strains from Plant Materials. JARQ. Vol. 38, pp. 111-117.

Koebnick, C., I. Wagner, P. Leitzmann, U. Stern and H.J. Zunft. **2003.** Probiotic beverage containing Lactobacillus casei Shirota improves gastrointestinal symptoms in patients with chronic constipation. Can J Gastroenterol. vol. 17, pp. 655-659.

Kruse, H., H. Sorum. **1994.** Transfer of multiple-drug resistance plasmide between bacteria of diverse origin in natural microenvironments. Appl. Environ. Microbiol. vol. 60, pp. 4015-4021.

Kurland, C.G., B. Canback, and O.G. Berg. **2003.** Horizontal gene transfer: a critical review. Proc. Natl. Acad. Sci. USA. vol. 100, pp. 9658–9662.

Lankaputhra W.E.V., N.P. Shah. **1995.** Survival of *Lactobacillus* and *Bifidobacterium* spp. in the presence of acid and bile salts. Cult. Dairy Prod. J. vol. 30, pp. 2–7.

Lilia, T., S. Stefanova and T. Yordanova. **2002.** Identification of lactic acid bacteria isolated from Katyk, goat's milk and cheese. Journal of Culture Collections. Vol. 3, pp. 48-52.

MacFaddin, J. F. **1985.** Media for the isolation-cultivation-identification-maintenance of medical bacteria, Ed 2nd, Williams & Wilkins, Baltimore, M.D, USA, vol. 1, pp. 483-623.

Majamaa, H. and E. Isolauri. **1997.** Probiotics: a novel approach in the management of food allergy. J Allergy Clin Immun. vol. 99, pp. 179-185.

Majhenic, A.C. and B.B. Matijašic. **2001.** Antibiotics influence on lactic acid bacteria inhabiting gastrointestinal tract. Mljekarstvo, vol. 51 (2), pp. 119-134,

Mann, G.V., A. Spoerry. 1974. Studies of a surfactant and cholesterolemia in the Massai. Am J Clin Nutr., Vol. 27, pp. 464- 469.

Marteau, P, J.C. Rambaud. **1993.** Potential of using lactic acid bacteria for therapy and immunomodulation in man. FEMS Microbiol Rev. vol. 12, pp. 207-220.

Mattila-Sandholm, T., J. Mättö and M. Saarela. **1999.** Lactic acid bacteria with health claims-interactions and interference with gastrointestinal microflora. Int Dairy J. vol. 9, pp. 25-35.

Mckinley, M.C. **2005.** The nutrition and health benefits of yoghurt. International Journal of Dairy Technology. vol. 58, pp. 1-12.

Noh, D.O., S.H. Kim and S.E. Gilliland, **1997.** Incorporation of cholesterol into the cellular membrane of *Lactobacillus acidophilus* ATCC 43121. J. Dairy Sci., Vol. 80, pp. 3107-3113.

Nyam news, **2005.** Probiotics. Caribbean Food and Nutrition Institute, UWI Campus Mona, Kingston 7. pp. 1-3.

Oksanen, P.J., S. Salminen, M. Saxelin, P. Hamalainen, A. Ihantola-Vormisto, L. Muurasniemi-Isoviita, S. Nikkari, T. Oksanen, I. Porsti, E. **1990.** Salminen. Prevention of travelers diarrhea by Lactobacillus GG. Ann Med. vol. 22, pp. 53-56.

Oskar, A.; S.N. Meydani, and R.M. Russell. **2004.** Yogurt and gut function. Am J Clin Nutr, Vol. 80, pp. 245–56.

Ouwehand, A.C., H. Lagstrom, T. Suomalainen, S. Salminen. **2002.** Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. Ann Nutr Metab. vol. 46, pp. 159–62.

Oyetayo, V.O. **2004.** Phenotypic characterisation and assessment of the inhibitory potential of *Lactobacillus* isolates from different sources. African Journal of Biotechnology. Vol. 3, pp. 355-357.

Ozawa, Y., K. Tanimoto, T. Nomura, M. Yoshinaga, Y. Arakawa, and Y. Ike. **2002.** Vancomycin-resistant enterococci in humans and imported chickens in Japan. Appl. Environ. Microbiol. vol. 68, pp. 6457–6461.

Pennacchia, C., D. Ercolini, G. Blaiotta, O. Pepe, G. Mauriello and F. Villani. 2004. Selection of Lactobacillus strains from fermented sausages for their potential use as probiotics. Meat Science, Barking. Vol. 67, pp. 309-317.

Perreten, V., F. Schwarz, , L. Cresta, M. Boeglin, G. Dasen, and M. Teuber. **1997.** Antibiotic resistance spread in food. Nature (London). vol. 389, pp. 801–802.

Prescott, M. L., J. P. Harley, D.A. Klein. 2001. Laboratory Exercises in Microbiology. 5th Ed. Exercise 40 (P^H). McGraw-Hill Higher Education, pp. 243-244.

Raibaud, P., M. Coulet, J. V. Galpin, and G. Mocquot. **1961.** Studies on the bacterial flora of the alimentary tract of pigs. II Streptococci: selective enumeration and differentation of the dominant group. J. Appl. Bacteriol. vol. 24, pp. 285–291.

Rashid, M.H.U., K. Togo, M. Ueda, and T. Miyamoto.**2007.** Probiotic Characteristics of Lactic Acid Bacteria Isolated from Traditional Fermented Milk 'Dahi' in Bangladesh. Pakistan Journal of Nutrition. Vol. 6 (6), pp. 647-652.

Reid, G. **2001.** Probiotic agents to protect the urogenital tract against infection. Am. J. Clin. Nutr. Vol. 73, 437S-443S.

Reuter, G. **2001.** Probiotics-Possibilities and limitations of their application in food, animal feed, and in pharmaceutical preparations for men and animals. *Berl Munch Tierarztl Wochenschr*. vol. 114 p.410.

S. Ganesh. **2006.** A novel yogurt product with *Lactobacillus acidophilus.* Thesis work, submitted to the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science. p. 2.

Saavedra, et al., **1994.** Feeding of Bifidiobacterium bifidum and Streptococcus thermophilus to infants in hospital for the prevention of diarrhea and shedding of rotavirus. Lancet, Vol. 344, pp. 1046-1049.

Saito, H., H. Tomioka and K. Nagashima, **1987.** Protective and therapeutic efficacy of *Lactobacillus casei* against experimental infections due to *Mycobacterium fortuitum* complex. J. Gen. Microbiol., Vol. 133, pp. 2843-2851.

Salminen, S., A.V. Wright and L. Morelli. **1998.** Demonstration of safety of probiotics-a review. International Journal of Food Microbiology. Vol. 44, pp. 93-106.

Salminen, S., C. Bouley, M.C. Boutron-Ruault, J.H. Cummings, A. Franck, G.F.R. Gibson, E. Isolauri, M.C. Moreau, M. Roberfroid, and I. Rowland. **1998.** Functional food science and gastrointestinal physiology and function. Brit. J. Nutr. vol. 80, pp. 147–S171.

Sanders, E.M. **1999.** Probiotics: a publication of the institute of food technologists' expert panel on food safety and nutrition. vol. 53, no. 11, pp. 67-77.

Sanders, M.E. **1999.** Probiotics. Food Technology. vol. 53 pp. 67-77.

Sartor, R.B. **2004.** Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: Antibiotics, probiotics and prebiotics. Gastroenterology. vol. 126, p. 1620.

Schillinger, U. and F.K. Lucke. **1987.** Identification of lactobacilli from meat and meat products. Food Microbiology. Vol. 4, pp. 199-208.

Schrezenmeir, J., M. de Vrese. **2001.** Probiotics, prebiotics, and synbiotics – approaching a definition. Am. J. Clin. Nutr. Vol. 73, 361S-364S.

Sellars, R.L. **1991.** Acidophilus products. In: Therapeutic properties of fermented milks. Robinson, R.K. (Ed.) Elsevier Applied Science, London. pp. 81-116.

Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, J. Dore. **2000.** Quantification of bacterial groups within human fecal microbiota by oligonucleotide probe hybridization. Appl. Environ. Microbiol. Vol. 66, pp. 2263-2266.

Shah N.P. **2000.** Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci., vol. 83 pp. 894–907.

Siddlingiaya, G.P., K.B.P.K. Reddy, P. Raghavendra, B.G. Kumar and M.C. Misra. **2007.** Screening of probiotic properties of lactic acid bacteria isolated from *Kanjika*, an ayruvedic lactic acid fermented product: An in-vitro evaluation. *J. Gen. Appl. Microbiol.*, Vol. 53, pp. 207–213.

Siitonen, S. H. Vappatalo, S. Salminen, A. Gordin, M. Saxelin, R. Wikberg and A.L. Kirkkola. **1990.** Effect of Lactobacillus GG yogurt in prevention of antibiotic associated diarrhea. Annals of Medicine, Vol. 22, pp. 57-59.

Skytta, E. and T. Mattila-Sandholm, 1991. A quantitative method for assessing bacteriocins and other food antimicrobials by automated turbidometry. J. Microbiol. Methods, Vol. 14, pp. 77-88.

Stuart, H. **2005.** Essential Microbiology. John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, England. Chapter 14 (Antimicrobial agents). Pp. 366-367.

Tannock, G. **2004.** A special fondness for lactobacilli. Appl. Environ. Microbiol, Vol. 70, pp. 3189-3194.

Teuber, M., L. Meile, , and F. Schwarz. **1999.** Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Leeuwenhoek. vol. 76, pp. 115–137.

Tharmaraj, N. and N. P. Shah. **2003.** Selective Enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus*, Bifidobacteria, *Lactobacillus casei, Lactobacillus rhamnosus,* and Propionibacteria. J. Dairy Sci. vol. 86, pp. 2288–2296.