Introduction

The probiotic concept has been defined by Fuller to mean "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (**Fuller, 1991; Guarner & Shaafsma, 1998**). The principle requisite for selection of a good probiotic includes product safety for human and animal consumption generally recognized as safe (GRAS) and survival in the gastrointestinal tract (GIT) (**Hyronimus *et al.*, 2000**). The probiotic strains must possess the ability to overcome the extremely low pH and the detergent effect of bile salts, and arrive at the site of action in a viable physiological state (**Chou & Weimer, 1999**). They should be capable of co-aggregation, resistant to gastro intestinal fluid and adhere to the intestinal mucosa (**Jacobsen *et al.*, 1999; Dunne *et al.*, 2001**). However, besides the various essential characteristics, the organisms should exhibit health benefits with functional properties. Various functional characteristics have been developed by the organisms. Clinically proven, various health effects have been reported for *Lactobacilli*, such as cholesterol reduction, diarrhea prevention, enhancement of lactose intolerance symptoms, anticancer effects, synthesis and enhancing the bioavailability of nutrients and immune-modulatory effects, all of which are considered functional aspects of probiotic criteria. In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host, and provide safety for the consumer (**De-Vries *et al*., 2006**)

During the last decade, the market of functional food products containing probiotics has been undoubtedly the fastest growing area of new food product development. For continuous manufacturing of these functional foods, the food industries need new probiotic cultures with more beneficial physiological effects beyond those of good nutrition without adverse effects for innovations. (**Zambou *et al*., 2004, 2008**)

In the development of probiotic foods intended for human consumption, strains of lactic acid bacteria, such as *Lactobacillus* as well as *Bifidobacterium* have been used most commonly (**Dunne *et al*., 2001).** Lactic acid bacteria are a large group of Gram positive rods occurring naturally in a variety of niches including the gastrointestinal tract (**Hammes & Hertel, 2006; Mohania *et al*., 2008).**

Nutritional and health aspects of functional foods incorporating probiotic bacteria, especially lactic acid bacteria and *Bifidobacteria,* have received considerable attention. Potential benefits may result from growth and action of cultured foods. These microorganisms have been used in food preservation for centuries (**Shahani & Ayebo, 1980).**

There are many products in the market containing viable lactic acid bacterial cells. It is extensively used in food processing, such as dairy and meat fermented products. The most common medium for growth is the Mann Rogosa Sharpe (MRS) medium (**De Man *et al*., 1960)**, however its high cost make them unsuitable for a large-scale biomass production. On the other hand, some cheap raw materials such as molasses and whey have been reported as culture media.

At present, these microorganisms are used in traditional food process and in probiotic therapy and their beneficial properties are widely recognized (**Ghrairi *et al*., 2008).** Because the beneficial effects of probiotics can vary between strains, the selection of the most suitable ones will be vital their use in making foodstuff (**Shid & Nanno, 2008).** A number of fundamental factors influence the survival of probiotics in foods during processing and storage. Probiotics could be protected using different methods such as microencapsulation, which provides protection of the cells from mild heat treatment in food processing and unfavorable environmental conditions such as pH and moisture (**Gong *et al*., 2009).**

The aim of the work

- *In vitro* tests to characterize and select lactic acid bacterial strains according to their preliminary probiotic properties including: tolerance to acid and bile salts; resistance to toxic phenol compounds, lysozyme and antibiotics; production of antimicrobial substances which inhibit some important food-borne pathogens and various adhesion attributes.
- Develop an alternative low-cost medium for a semi-industrial scale production of LAB biomass from cheap raw materials comparable with the conventional synthetic MRS media aiming to reduce the costs and the environmental pollution.
- Examine and evaluate the utilization of the selected proper probiotic species which has significant bio- preservative properties to prepare probiotic yoghurt.
- Finally, improvement of probiotic viability by cell microencapsulation and prebiotic addition. In addition, assess the physico-chemical, organoleptic and sensory properties of the bio-yoghurt during the end of shelf-life.

Review of Literature

1. Biological preservation

Food preservation is carried out to maintain the quality of raw material, the physical and chemical properties of the product in addition to the efficient quality. Consumers demand high quality, preservative-free, safe with extended shelf life **(Peck, 1997; Brul & Coote, 1999**). Consumers interest in high quality products with improved nutritional quality, at the same time maintaining microbial safety (**Kalchayanand *et al*., 1998**).

Biological preservation involves a new scientific approach to advance the microbial safety of foods. By explanations, this idea refers to utilization of antagonistic microorganisms or their metabolic products to inhibit or destroy unfavorable microorganisms in foods (**Holzapfel *et al*., 1995**).

Foods preservation by natural and microbiological methods may be a suitable approach to resolve the economic losses due to microbial spoilage of food products and raw materials, as well as to reduce the incidence of food borne illnesses microorganisms (**Galvez *et al*., 2008**). The increasing consumer awareness of the dangers coming not only from microbial food-borne pathogens, but also most importantly from the synthetic chemical preservatives used to control them. This has led to improved and renewed the interest in so-called ‘‘green technologies’’ (**Abee *et al.*, 1995; Papagianni, 2003**).

Furthermore, an increasing needs for safe foods, with low level of chemical additives, has amplified the interest in changing the artificial chemical compounds by natural products, which are not harmful to the environment or the host. In consequence, bio-preservation of food has appeared as an attractive and safe approach (**Cocolin *et al.*, 2007).** In addition, food safety has been an international concern and larger consideration is being drawn towards application of natural and safe metabolites of LAB in foods as bio-preservatives.

In the last 19 century, microbiologist describe that microflora found in the gastrointestinal tract (GIT) of healthy individuals is different from those in diseased individual. They termed the beneficial microflora found in the GIT as probiotic. Probiotics are commonly defined as living microorganisms which, when managed in adequate amounts, give health benefit to the host (**FAO / WHO, 2002).**

The majority of probiotics commercially available these days belong to the genera *Lactobacillus* and *Bifidobacterium*, they belonging to lactic acid bacterial family. However, because they have been in use for many years, their safety has been confirmed (**Gillliland, 1990; Shah & Jelen, 1990**). Lactic acid bacteria are the most important group of microorganisms used in preservation, they inhibit food spoilage by producing antimicrobial substances (lactic acid, hydrogen peroxide, bacteriocin), which help to extend the shelf life of food products **(Phillip *et al.*, 2012; Noordiana *et al.*, 2013**).

2. Characteristics of probiotic bacteria

The gastrointestinal tract of humans is inhabited by over 500 bacterial species, staying in dynamic balance. Various factors can influence this balance (antibiotic therapy, disease, age, stress or diet), leading to increased counts of potentially harmful bacteria. One of the ways to restore bacterial balance is application of probiotics (**Gill & Guarner, 2004**).

Lactic acid bacteria are widely distributed in the nature. The lactic acid fermentation, which these bacteria perform has long been recognized and applied by the humans for producing diverse foodstuff. In recent years, probiotic activity of LAB has been emphasized (**Gill & Guarner 2004; Noriega *et al*., 2006**).

The LAB importance is related essentially with their physiological effects such as production of some digestive enzymes and vitamins, production of antibacterial substances **(Holzapfel & Schillinger, 2002**). Their general occurrence in foods and their long historical use contributes to their acceptance as GRAS for food fermentation and human consumption (**Silva *et al.*, 2002; Lemos *et al.*, 2008**).

Probiotics are usually defined as microbial food supplements with beneficial effects on the consumer, some of the beneficial effect of LAB consumption include, improving intestinal tract health by alter the intestinal microflora balance, enhancing immune system and enhancing bioavailability of nutrients **(Parvez *et al*., 2006),** as well as prevention of colon cancer **(Wollowski *et al*., 2001).** They can also produce antimicrobial compounds such as bacteriocins that have the capability to reduce or inhibit the growth of pathogenic and food spoilage bacteria (**Rattanachaikunsopon & Phumkhachorn, 2010**)**.**

It is well documented that probiotics are beneficial bacteria in that they favorably alter the intestinal microflora equilibrium, inhibit the growth of detrimental bacteria, support good digestion, and enhance resistance to infection **(Helland *et al*., 2004).** In order to exert their beneficial effect, probiotics must survive in the gastrointestinal tract, persist in the host, and verify safety for consumer **(De-Vries *et al*., 2006).**

Over the world, the research of novel probiotic strain is important in order to satisfy the increasing request of the market to obtain new functional products. These new functional products must contain probiotic cultures more active and with better probiotic characteristics comparing to those already present (**Sieladie *et al*., 2011**).

The selection of bacterial isolates to be used as an effective probiotic strain is a complex process, isolates should possess several physiological and biochemical criteria **(Al-Awwad *et al*., 2009).** Therefore, before a probiotic can benefit human health it must fulfill several criteria:

2.1. Antimicrobial activity

Antimicrobial activity is one of the most significant selection criteria for probiotics (**Dunne *et al*., 2001**). Lactic acid bacteria are widely used for preservation of wide range of foods e.g.: meat, fermented foods and milk **(Zhu *et al*., 2000**).

The preservative property of probiotic bacteria is mainly due to their ability to make a variety of antimicrobial substances as a natural competitive means to inhibit other microorganisms sharing the same niche, among them, ethanol, formic acid, acetone, hydrogen peroxide, diacetyl, and bacteriocins (**Olivera *et al.*, 2008**). Also, fungal inhibitory metabolites were produced by LAB including propionic, acetic and lactic acids (**Sauer *et al.*, 2008).**

Similar study was conducted by **Saran *et al. (*2012),** they reported that *L. acidophilus* NCDC 13 exhibited more inhibitory activity than *L. acidophilus* NCDC 291 against target organisms (*E. coli*, *Enterococcus faecalis*, *S. typhimurium* and *Sh. flexneri*) as determined by agar well assay technique.

Bassyouni *et al.* (2012) studied the antagonistic effects of a collection of LAB isolated from different dairy products in Egypt. Eight isolates were found to be effective against four clinically isolated pathogens (*E. coli*, *Salmonella* spp., *Staphylococcus* spp. and *Micrococcus* spp

Another research made by **Mahmoudi *et al.* (2013)** found that *Bif. longum, Bif. bréve* and *Bif. bifidum* had antagonistic effect against *St. aureus, E. coli, P. aeruginosa, Salmonella.* Sp. **Widodo *et al. (*2014)** detected that the growth of pathogenic *Sh. flexnerii*, *E. coli*, *St. aureus*, and *Enterococcus faecalis* was inhibited by *L. casei* strain AP and AG.

Mahrous & Abd-El- Salam (2016) reported that *L. acidophilus r1*, *L.acidophilus* r2 and *L. lactis* subsp lactis r3 inhibited the growth of *St. aureus; E. coli* ATCC 25922 and *Bac. subtilis* NCIB3610 and the highest inhibition was observed by *L. acidophilus* r2 against the growth of *St. aureus; E. coli* ATCC 25922 and *Bac. subtilis* NCIB3610.

2.2. Resistance to unfavorable conditions

Microorganisms ingested with food begin their route in the intestinal tract through the mouth, being exposed during their transit to successive stressful factors that will influence their survival rate. First of all, they should defend against enzymes from the oral cavity, as the lysozyme. After that, the aggressive environment in the stomach (**Berrada *et al*., 1991).** Thus, it is essential for bacteria to have protection systems to withstand digestive enzymes, bile salts, low pH and toxic compounds.

1. Acid and bile tolerance

Probiotic bacteria are mostly delivered in food system and must be acid and bile tolerant. The time from entrance to release from stomach has been estimated to be approximately 90 min. (**Berrada *et al*., 1991**). Bacteria which constitute part of functional food articles must be capable of setting the gastrointestinal tract of the host. It is, therefore reasonable to initially select probiotic bacterial strains on the basis of their resistance to undesirable physiological aspects in the gastrointestinal tract (**Holzapfel *et al.*, 1998**).

Goldin & Gorbach (1992) found that a concentration of 0.3% oxgall intimately as the bile level found in the gastrointestinal tract. Different reports recorded the bile tolerance of LAB, however, majority of them demonstrated bile tolerance up to 0.3% concentration (**Liong & Shah, 2005; Mcauliffe *et al.*, 2005**).

The bile which is a steroid produced by the liver and released in the small intestine in the form of bile salts, reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids **(Gilliland *et al.*, 1987).** Therefore, bile resistance is essential for an organism that expected to grow in the intestine. **Margolles *et al. (*2003)** noticed that *Lactobacillus* growth inhibition by bile salts may be overcome some times by progressive adaptation to increase concentrations of these compounds.

The acid and bile salt resistance results of two isolates (*L. acidophilus* and *Bif. infant*is) isolated by **Al-Awwad *et al*. (2009)** showed good acid tolerance at pH as low as 2. Furthermore, the isolates could tolerate bile salt of 0.3% concentration with decreased viable counts of both isolates.

At pH 2 in gastric juice, the viability of *L. casei* 01 and *L. acidophilus* La-5 were not found after 30 minutes. *Lactobacillus acidophilus La-5* was more resistant than *L. casei 01* in acidic environment. Different concentrations of bile salt (0.3%, 0.5%, 1%) affected *Lactobacillus casei* 01. For *L. acidophilus* La-5, the viable cells were decreased, but at low bile salt concentrations, there had the ability to survive **(Both *et al.*, 2010**).

The *in vitro* ability screening of the survival of *Lactobacilli* in simulated GIT conditions may only have value in expected the actual *in vivo* survival of a strain when consumed in a non-protected way. **Dardir (2012**) studied the probiotic properties of twenty LAB strains (*L. casi, L. acidophilus, L. plantarum and Bifidobacteria species*). Regarding the bile salt hydrolase activity, they concluded that, all the tested strains of *L. acidophilus*, *L. plantarum* and *Bifidobacterium* spp. demonstrated positive bile salt hydrolase activity. While, the tested strains differed considerably in their resistance to acid, best survival was observed with strains of *L. acidophilus* and *Bif. lactis.* While other, strains (*L. casei* and *L. plantarum*) displayed loss of viability

Twenty eight isolates of three group *Lactobacilli* including *L. plantarum*, *L. casei* and *L. delbruki* had no viable cells after the first hour which suggested that isolates were mostly inhibited by severe conditions (pH $\leq$ 2.0). Bacterial viability was enhanced when bile salts were added to the cultures. *Lactobacillus* *plantarum* and *L. casei* were generally resistant for 0.3% bile concentration. While other strains could not survive at the same concentration (**Hassanzadazar *et al*., 2012**).

Bif. animalis subsp. *Lactis* DSMZ 10140; *Bif. animalis* subsp. *animalis* DSMZ 20104; *Bif. Longum* subsp. *Infantis* DSMZ 20088; *Bif. breve* DSMZ 20213 and *Bif. bifidum* DSMZ 20456 were resistant to bile salts. Also, the cell count was mostly reduced under acidic pH. The viability loss was recorded ca. 100% for *Bif. bifidum* DSMZ 20456 and *Bif. breve* DSMZ 20213, 30% for *Bif. animalis* subsp *Lactis* DSMZ 10140 and 50% for *Bif. animalis su*bsp. *animalis* DSMZ 20104 and *Bif. longum* subsp. *Infantis* DSMZ 20088 **(Bevilacqua *et al*., 2012)**.

A comparative study on probiotic potential of three strains of *L. acidophilus* was done by **Dixit *et al.* (2013)**.The survival rates of all three strains were higher after 2 hr incubation period than 4 hr at pH 2.5. The ability of the *Lactobacillus* strains to grow in the presence of different concentrations of bile salts (0.2 to 0.5 %) was also studied. Results clearly revealed that the strains showed maximum growth at 0.2% bile, while negligible growth was detected at 0.5%. Thus, strain NCIM 2660 exhibited weak growth at 0.3% bile.

Lactobacillus fermentum and *L. casei* isolated from various curd samples were sensitive to pH 2. But they had the ability to grow in low pH condition (pH 3 and 4). Also, they had the capability of growing in MRS broth containing 0.2-0.3% bile salts **(Halder & Mandal, 2015)**.

2. Enzymes and toxic compounds

In addition to bile salts, there is also the presence of toxic compounds such as phenol in the intestine. This compound is produced by the intestine microflora, since this is formed by different bacterial species and reflect the conversion of various substances into products either beneficial or harmful (**Mitsuoka, 1996).**

Phenol tolerance is a desired characteristic for probiotic. In the intestines, some aromatic amino acids from the digested foods can be converted into phenols through the bacterial action **(Gilliland &Walker, 1990).**

Abd El-Salam *et al.* (2004) found that good tolerance to different concentration of phenol was exhibited by *L. acidophilus* TISTR450 and *L. johnsonii* ATCC 33200. However, **Xanthopoulos *et al*. (2012)** revealed that *Str. thermophilus* was sensitive to this compound. In presence of 0.4% phenol, *Str. thermophilus* ST8.01 growth was lost after 24 hr. but after 3 hr., the viable count was still retained **(Tuncer & Tuncer, 2014)**.

Pozza *et al.* (2011) evaluated 75 *Lactobacillus* strains for their tolerance ability to phenol; they observed that all strains were able to grow in the presence of 0.3% w/v phenol.

The lysozyme content in saliva varies from 10 to 200 µg and in gastric juice from 43 to 106 µg. Therefore, the ability of probiotics to survive in these concentrations can be additional parameter for selecting bacteria (**Saran *et al.*, 2012**). **Phillips (1966)** demonstrated that lysozyme enzyme is able to cleave the glycosidic bonds in peptidoglycan which is the major constituent of the bacterial cell walls**.**

Two *L. acidophilus* strains were assessed for their resistance to lysozyme in MRS media by **Saran *et al*. (2012).** Results indicated that no significant difference of viable counts was observed with or without lysozyme and even the viable counts got increased in the presence of lysozyme.

Suskovic *et al.* (1997) found that *L. acidophilus* M92 was resistant to100 µg/ml lysozyme. This concentration is higher than the physiological intestinal lysozyme concentration. *Lactobacillus acidophilus* strains were not affected by 100 µg/ml lysozyme. Moreover, the viable cells were improved **(Saran *et al*., 2012)**.

Orlowski & Bieleka (2006) studied seven *Lactobacillus* and two *Bifidobacterium* strains for their lysozyme tolerance. Results proved that growth of majority of the investigated strains was not affected by lysozyme. **Koll *et al*. (2008)** also investigated the lysozyme activity of oral *Lactobacilli* species. They found that all the tested strains of *Lactobacilli* showed high tolerance of lysozyme at concentrations of 0.2–10 mg/ml with no inhibitory effect on their growth.