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Influence of Different Capsule Materials on the Physiological Properties of Microencapsulated *Lactobacillus acidophilus*

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Was wir wissen, ist ein Tropfen
Was wir nicht wissen, ein Ozean

(Issak Newton)

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Einfluss verschiedener Kapselmaterialien auf die physiologischen Eigenschaften mikroverkapselter *Lactobacillus acidophilus*

Die Ziele der vorliegenden Arbeit bestehen in der Bewertung Stabilität von mikroverkapselten *Lactobacillus acidophilus* während des Zyklus von der Verkapselung (mit verschiedenen Kapselmaterialien) über die Lagerung, Verhalten in Lebensmitteln bis hin zum Verdauungstrakt.
Um die Überlebensrate von *L. acidophilus* nach der Sprühtrocknung zu untersuchen, wurde mit folgenden Kapselmaterialien einzeln und in Kombination verwendet: **Gummi Arabicum** und Gummi Arabicum in Kombination mit Mannitol, Pektin, Maltodextrin, Magermilch, Gum Guar, Gum Karya, Carrageen, Alginat, Lecithin, Glycerol, Tomatensaft, Tween 20, Sojabohnenmehl, Gelatine; **Sojaprotein** und Sojaprotein in Kombination mit Alginat, Pektin; **Molkeprotein** und Molkenprotein in Kombination mit Alginat, Pektin; **Sojamilch** und Sojamilch in Kombination mit Alginat, Pektin, Gummi Arabicum+Alginat, Gummi Arabicum+Pektin; Sojaprotein in Kombination mit Gummi Arabicum, Gummi Arabicum+Alginat, Gummi Arabicum+Pektin; Molkeprotein in Kombination mit Gummi Arabicum, Gummi Arabicum+Alginat, Gummi Arabicum+Pektin; **Gelatine** und Gelatine in Kombination mit Mannitol, Pektin, Maltodextrin, Magermilch, Alginat, Lecithin, Glycerol, Tween 20, Tomatensaft, Sojabohnenmehl, Sojaprotein, Molkeprotein, Sojamilch. Es wurde herausgefunden, dass die Überlebensrate nach der Sprühtrocknung in Abhängigkeit vom Kapselmaterial stark variiert. Es ist offensichtlich, dass bei allen verwendeten Kapselmaterialien die Zellzahl durch die Sprühtrocknung sinkt. Der Abfall reicht dabei von etwas weniger als eine Zehnerpotenz bis zu annähernd zwei Zehnerpotenzen.
Die Ergebnisse zeigten eine große Variabilität in der Überlebensfähigkeit mikroverkapselter *L. acidophilus* während der Lagerung bei 5 °C, die zwischen 4 bis 15 Wochen lag und mit hoher Sicherheit auf die unterschiedlichen Kapselmaterialien zurückzuführen ist. Die Ergebnisse der Untersuchung ergaben, dass mit Gummi Arabicum+Sojaprotein, Gummi Arabicum+Molkeprotein oder Gummi Arabicum+Sojamilch mikroverkapselte *L. acidophilus* Zellkulturen bei Kühllagerung 10 bis 11 Wochen lang eine Zellzahl aufwiesen, die auf dem für eine gesundheitsfördernde Wirkung empfohlenen Niveau lag. So lassen die gegenwärtigen Ergebnisse darauf schließen, dass mikroverkapselte Zellen eine Möglichkeit zur Reduzierung von Verlusten bei der Kühllagerung darstellen.
L. acidophilus wurden Temperaturen von 37, 40, 45, 50, 55 und 60°C jeweils 30 Minuten ausgesetzt und 60°C wurde aufgrund der extremen Reduktion der Kolonie bildenden Einheiten als Letaltemperatur gewählt. Dabei zeigten mikroverkapselte Zellen bei 60°C eine höhere Wärmetoleranz als freie Zellen. Bei höheren Temperaturen (63°C und 65°C) ist die Wärmetoleranz der verkapselten Zellen aber wieder niedrig.
Ein weiteres Ziel dieser Arbeit war die Bestimmung der Lebensfähigkeit von *L. acidophilus* in verschiedenen Saccharosekonzentrationen (5; 10; 15; 20 und 25%). Die Stabilität verkapselter Zellen wird für alle Saccharosekonzentrationen kaum beeinflusst. Nur bei 20 und 25% Saccharose konnte ein geringer negativer Effekt auf die verkapselten Zellen beobachtet werden.
Es wurde die Überlebensfähigkeit von freien und mikroverkapselten *L. acidophilus* Zellen in 1; 2; 3; 4 und 5%-iger Natriumchlorid-Lösung während einer Lagerung bei 5°C bestimmt. Mit Gummi Arabicum+Sojamilch verkapselte *L. acidophilus* hatten im Vergleich zu allen anderen mikroverkapselten Zellen die höchste Stabilität gegenüber Kochsalz.
Des weiteren wurde in der vorliegenden Studie die Stabilität freier und verkapselter *L. acidophilus* während einer Kühllagerung in verschiedenen Milchsäure-, Essigsäure, und Zitronensäurelösungen mit verschiedenen pH-Werten (3; 4 und 5) bestimmt. Insgesamt können Kapselmaterialien, die Proteine oder Sojamilch enthalten, *L. acidophilus* einen hohen Schutz bei niedrigen pH-Werten bieten.
In weiteren Untersuchungen wurden die Mikroorganismen in Salzsäurelösungen mit pH-Werten von 1; 2 und 3 und Gallesalzgemisch-Konzentrationen von 1; 2 und 4% aufgenommen. Es konnte gezeigt werden, dass mikroverkapselte Zellen bei allen Kapselmaterialien eine leicht höhere Säuretoleranz bei einem pH 1 und eine deutlich höhere Säuretoleranz bei pH-Werten von 2 und 3 aufweisen. Die mikroverkapselten Zellen werden durch die Proteinträgermatrix gepuffert und daher nicht den extremen pH-Werten im Magen ausgesetzt. Die Stabilität der verkapselten Mikororganismen gegenüber den Gallesalzen ist bei den mit Gummi Arabicum+Sojamilch verkapselt Porben am größten, gefolgt von Gummi Arabicum+Molkeprotein, Gummi Arabicum+ Sojaprotein und Gelatine.
Die vorliegende Arbeit gibt erstmals einen Überblick über die Stabilität hinsichtlich der Überlebensfähigkeit und der physiologischen Eigenschaften für den Gesamtzyklus von probiotischen Mikroorganismen von der Herstellung über die Lagerung bis in den Verdauungstrakt unter vergleichbaren Bedingungen. Es konnte gezeigt werden, das die Mikroverkapselung ein technologisches Konzept darstellt, Lebensmittel mit physiologisch aktiveren Probiotika herstellen zu können.

Influence of Different Capsule Materials on the Physiological Properties of Microencapsulated *Lactobacillus acidophilus*

This work aimed at evaluating the viability and physiological changes of *L. acidophilus* after the microencapsulation process and over a period of storage time; influence of heat treatments; viability and stability to some deliberately simulated conditions in the carrier foods (including the presence of salt, sugar, and organic acids); and the resistance of microencapsulated *L. acidophilus* to some simulated conditions of the human intestinal tract (including gastric juice and bile salts).

To investigate the survival of *L. acidophilus* after spray drying, *L. acidophilus* spray dried with different carrier material mixtures including: **gum arabic** and gum arabic with: mannitol, pectin maltodextrin, skim milk, gum guar, gum karaya, carrageenan, alginate, lecithin, glycerol, tomato juice, tween 20, soybean flour, and gelatin; **soy protein** and soy protein with: alginate, pectin, gum arabic, gum arabic+alginate, and gum arabic+pectin; **whey protein** and whey protein with alginate, pectin, gum arabic, gum arabic+alginate, and gum arabic+pectin; **soy milk** and soy milk with: alginate, pectin, gum arabic, gum arabic+alginate, and gum arabic+ pectin; **gelatin** and gelatin with: mannitol, pectin, maltodextrin, skim milk, alginate, lecithin, glycerol, tween 20, tomato juice, soybean flour, soy protein, whey protein, and soy milk. It was found that the survival of *L. acidophilus* in different capsule materials highly varied after spray drying. It is evident that, in general, the number of survivors decreased after spray drying for all capsule materials tested. The decreases ranged from less than 1 log cycle to 2 log cycles approximately.

The results showed a great variability in the survival ability of microencapsulated *L. acidophilus* during storage at 5°C ranging from 4 to 15 weeks, which could be highly dependent on the different kinds of capsule materials. The results showed that after 10-11 weeks refrigerated storage, microencapsulated *L. acidophilus* using gum arabic+soy milk, gum arabic+ whey protein, and gum arabic+soy protein could maintain their viability which corresponds to the advised therapeutic-minimum dose. However, the present results concluded that microencapsulated cells may present another approach for reducing viability losses under refrigerated storage.

Free *L. acidophilus* exposed to 37, 40, 45, 50, 55, and 60°C/30 min. 60°C was chosen as a lethal temperature. On the other hand, the results for microencapsulated cells demonstrated more thermotolerance at 60°C as compared to the free cells. While, microencapsulated cells were less thermotolerant at higher temperatures (63 and 65°C) used.

This study determined the viability of *L. acidophilus* during storage in different sucrose concentrations (5, 10, 15, 20, and 25%). The stability of microencapsulated cells was weakly affected by the different sucrose concentration used. 20 and 25% sucrose had moderate effect on the microencapsulated cells.

The survival of free and microencapsulated *L. acidophilus* in 1. 2. 3. 4. and 5.% sodium chloride during storage at 5°C was investigated. Microencapsulated *L. acidophilus* with soy milk+gum arabic showed the best stability to salt compared to the other capsule materials.

The present study evaluated the stability of free and microencapsulated *L. acidophilus* during refrigerated storage in different lactic, citric, and acetic acids solutions with different pH values (3, 4, and 5). Little variability was observed between the different acids used at the same pHs. The protein- and soy milk-containing capsule materials could highly protect and help *L. acidophilus* to survive better the low pHs harsh conditions.

Cells inoculated into HCl solutions with pH values of 1, 2 and 3. Free cells exhibited intolerance to pH 1 and 2 and were more acid tolerant at pH 3. Nevertheless, microencapsulated cells in all capsule materials were found to be slightly more acid tolerant at pH 1, and obviously more acid tolerant at pH 2 and 3. Microencapsulated cells were likely to be buffered by the protein carrier matrix and were thus not likely to be exposed to the low pH extremes. The bile concentrations tested were 1, 2 and 4%.Microencapsulated cells with soy milk+gum arabic survived best in bile, followed by whey protein+gum arabic, soy protein+gum arabic, gelatin, and gum Arabic.

The present study is the first investigation dealing with the effect of using different capsule materials on protecting and stabilizing the viability and stability of probiotics concerning the whole line: microencapsulation process stress; stress caused by the storage time and conditions; Influence within the food product until consumption; and stress within the intestinal tract.

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1. Introduction

Foods are no longer considered by consumers only in terms of taste and immediate nutritional needs but also in terms of their ability to provide specific benefits above and beyond their basic nutritional value. Functional foods have become an important and rapidly expanding segment of the food market as processed food manufactures seek to improve market share by promoting the health benefits provided by functional ingredients in their products. Nutritional science has been expanded the knowledge of how foods influence consumers in relation to specific health parameters. Functional foods targeted towards improving the balance and activity of the intestinal milieu and currently provide the largest segment of functional food market [Saarela et al., 2002].The oldest and still most widely used way to increase the numbers of advantageous bacteria in the intestinal tract is the direct consumption of food containing live bacteria. Such bacteria are called probiotics [Fuller, 1989; Salminen et al., 1998] and have to date been predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, both of which constitute part of the normal human intestinal or mucosal microbiota. Fig.1.1 shows the proposed health benefits stemming from probiotic consumption [after Saarela et al., 2002].

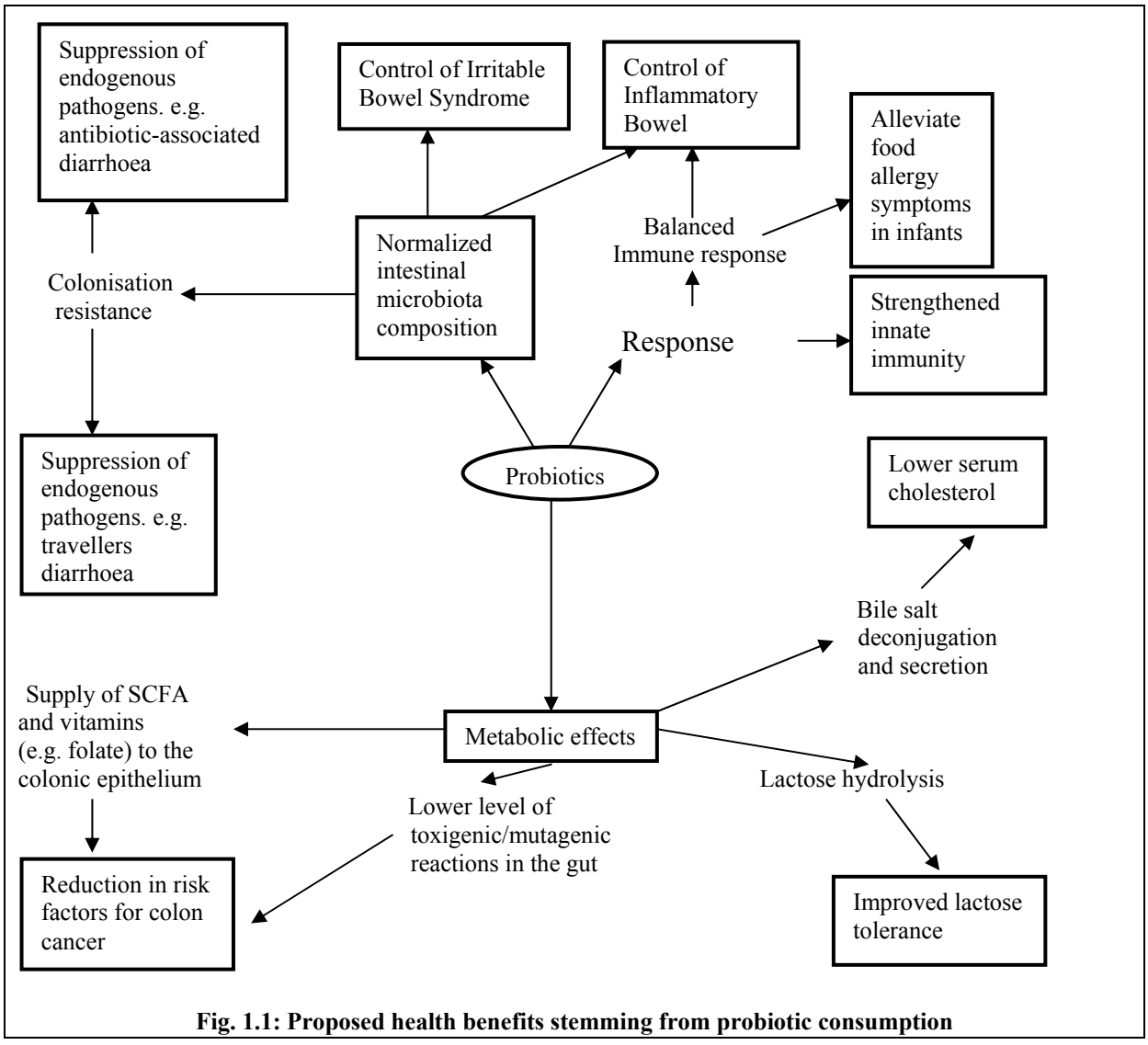


Fig. 1.1: Proposed health benefits stemming from probiotic consumption

Probiotics are defined as cultures of live microorganisms that, applied to animals or humans, benefit the host by improving properties of indigenous microflora [Havenaar and Huis, 1992]. Live cultures of probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* spp. in the diet are claimed to provide several therapeutic benefits [Modler, 1990; Kurmann and Rasic, 1991; Mital and Garg, 1992; Ishbashi and Shimamura, 1993].The viability and stability of probiotics has been both a marketing and technological challenge for industrial producers. Probiotic foods should contain specific probiotic strains and maintain a suitable level of viable cells during the product's shelf-life. The technological demands placed on probiotic strains are great and new manufacturing process and formulation technologies may often be required for bacteria primarily selected for their functional health properties. Before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions, then survive and retain their functionality during storage as frozen, freeze-dried or dried cultures, and also in the food products into which they are finally formulated. The probiotic strains should also survive the gastrointestinal stress factors and maintain their functionality within the host. Additionally, they must be able to be incorporated into foods without producing off-flavours or textures and they should be viable but not growing. The conditions under which the products are stored are also important for the quality of products.

Encapsulation methods have been applied to increase the survival and delivery of bacterial cultures. Several methods have been developed for the encapsulation of bacteria for use in fermentation, as well as for incorporating into products. Encapsulation helps in segregating the bacterial cells from the adverse environment (for example, of the product, of the gastrointestinal tract) thus potentially reducing cells loss. The encapsulation process and the capsule material influence the viability of bacteria, under different conditions as compared to when bacteria were in the non encapsulated state [Rao et al., 1989; Kebary, et al., 1998]. In regard to the utility of microencapsulation there are a few publications dealing with the effect of different capsule materials especially polysaccharides (e.g., alginate). The present study used several different encapsulating materials and their effects on protecting and stabilizing the survival of *Lactobacillus acidophilus* in some adverse environments and conditions were evaluated.

The better the capability of the product in retaining probiotics viability, the higher its beneficial effect upon the final consumer. For consumers to significantly benefits, probiotic cultures require some preservation or stabilization treatments in order that the cells maintain their viability and fermentative activity. The objectives of this study are to evaluate: the viability and physiological changes of *Lactobacillus acidophilus* after the micoencapsulation procedure and over a period of storage time; influence of heat treatments; viability and stability to some simulated conditions in the carrier foods (including the presence of salt, sugar, and organic acids); and the resistance of microencapsulated *Lactobacillus acidophilus* to some simulated conditions of the human intestinal tract (including gastric juice and bile salts); which could be improved and protected by microencapsulation procedure. The present study is the first investigation dealing with the effect of using different capsule materials on protecting and stabilizing the viability and stability of probiotics concerning the whole line: microencapsulation process stress; stress caused by the storage time and conditions; Influence within the food product until consumption; and stress within the intestinal tract.

2. Theoretical background

2.1 Biotechnology

The word biotechnology is derived from „bio," meaning life or living systems, and „technology," defined as scientific methods for achieving practical purpose. Biotechnology is the integrated use of biochemistry, microbiology and process engineering to manufacture products by utilizing the potential of microorganisms [Mittal, 1992]. Biotechnology is not an original science or technology, but is characterized by its interdisciplinary position as shown in Fig. 2.1.

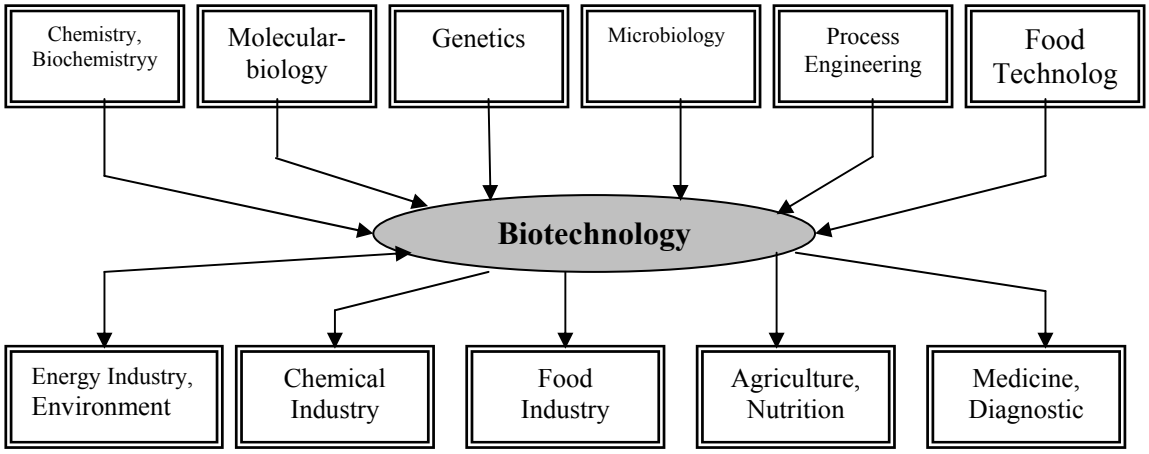


Fig. 2.1: Interdisciplinary position of biotechnology

Biotechnology is not new to the agricultural and food sector, since humans have been exploiting living systems for the production, processing, and preservation of food for centuries. Microorganisms including bacteria, yeast, and mold have been used since the beginning of the recorded history for the production of fermented dairy, meat, and vegetable products as well as for the fermenting of beverages such as wine and beer. Many ingredients used in foods as vitamins, stabilizers, flavors and flavor enhances, colors, and preservatives are produced by microorganisms. Additionally, microorganisms have been used to degrade the waste products generated during the processing of food [Kunz and Bauer, 1988; Harlander, 1992].

Bacteria, yeasts, and molds have been used for the production of fermented foods for centuries. Fermented foods are defined as those foods that have been subjected to the action of microorganisms or enzymes to produce desirable biochemical changes. The microorganisms may be the microflora indigenously present on vegetable or animal products that serve as the substrates for fermentation or they may be added as starter cultures. Microbial metabolism is responsible for the production of preservative agents such as acids, carbon dioxide and alcohol, as well as for chemical and physical changes that alter the flavor,

texture, shelf-life, safety, digestibility, and nutritional quality of fermented foods. The microorganisms involved are multifunctional and form an integral part of the end product. Fermentation is a relatively simple, natural, efficient, inexpensive, and low-energy food preservation process that reduces the need for refrigeration [Harlander, 1992].

The end products of fermentation influence the character of the final product and depend on the particular microorganisms involved in the fermentation. Lactic acid bacteria belonging to the genera *Lactobacillus, Lactococcus, Streptococcus, Pediococcus*, and *Leuconostoc* are used for the production of fermented dairy, meat, and vegetable products, and produce lactic acid as the primary end product of fermentation. Fermentative yeasts from the genus *Saccharomyces*, used for the production of wine, beer, and bread, produce alcohol and carbon dioxide as primary end products of metabolism. Filamentous fungi such as *Aspergillus, Penicillium*, *Mucor*, and *Rhizopus* are equipped with a powerful arsenal of enzymes that contributes to the degradation of substrates during fermentation. Fermented foods make a major contribution to the diet in all parts of the world; the classes of fermented foods produced in different regions of the world reflect the diet in each region.

Microorganisms produce a variety of secondary metabolites via fermentation that can be purified for use as food ingredients. Microorganisms are metabolically diverse, small in size, and easy to grow in large quantities on diverse substrates, making them ideal candidates for production of secondary metabolites. The types of chemicals produced by microbial fermentation include acidulants, amino acids, vitamins, flavor and flavor enhancers, pigments, stabilizers, thickeners, surfactants, sweeteners, polymers, antioxidants and antimicrobial agents [Neidleman, 1986; Campbell, 1987; Wasserman et al., 1988; Neidleman, 1989; and Morris, 1990].

2.2 Functional Food

There is a clear relationship between the food we eat and our health. Foods which promote health beyond providing basic nutrition are termed `functional foods`. These foods have potential to promote health in ways not anticipated by traditional nutrition science. This field of functional foods has evolved rapidly and many new terms have emerged in response. Although in many cases these terms have no legal definition, common usage definitions are shown in Tab. 2.1. One issue seems clear, however, that is the domain of functional foods is promoting health, not curing disease [Sanders, 1998]. Functional food science by reference to the new concepts in nutrition, it is the role of *Functional Food Science* to stimulate research and development of functional foods (Fig. 2.2) [Roberfroid, 2000].

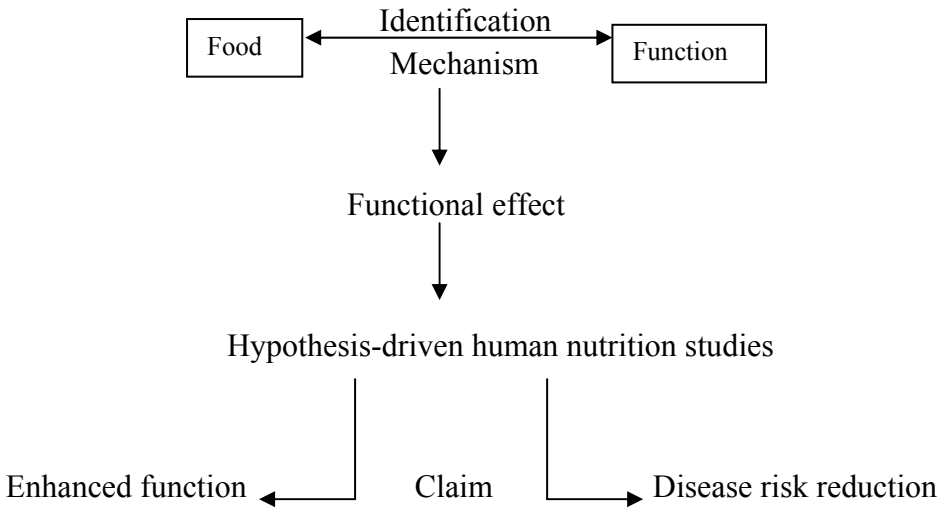


Fig. 2.2: The strategy for functional food development

Tab. 2.1: Definitions of Functional Food terms [after Sanders, 1998]

Term	***Definition***
Functional food	A modified food or food ingredient that provides a health benefit beyond satisfying traditional nutrient requirements.
Foods for specified health use (FOSHU)	An English language translation of a Japanese classification of functional foods. The Japanese government defines FOSHU as `foods which are expected to have certain health benefits, and have been licensed to bear a label claiming that a person using them for a specified health use may expect to obtain the health use through the consumption thereof.` As of June, 1997, 80 foods and 67 ingredients have been officially registered as FOSHU.
Nutraceutical	A nutraceutical is a food or part of food that offers medical and/or health benefits including prevention or treatment of disease.
Colonic food	Undigested food which reaches the colon, usually in the form of a non-digestible carbohydrate.
Prebiotic	A colonic food which encourages the growth of favorable intestinal bacteria (e.g., bifidobacteria or *lactobacilli*).
probiotic	A mono or mixed culture of microorganisms which when applied to animal or man affect the host beneficially.
Medical food	A special classification of food dictated in United States food law which: · Must be used under medical supervision · Must be for a disease with well defined, specific nutrient characteristics · Based on recognized scientific principles · Must provide medical evaluation.

2.2.1 Probiotics

The word`probiotic`, derived from the Greek language, means `for life` [Fuller, 1989] and had many definitions in the past. Definitions such as `organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance` were used. These general definitions were unsatisfactory because the term `substances` include chemicals such as antibiotics. The definition of probiotics has since then been expanded to stress the importance of live cells as an essential component of an effective probiotic. Most recently, Havenaar and Huis [1992] broadened the definition of probiotics as being `a viable mono- or mixed culture of microorganisms which, applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora `. This implies that the term `probiotic ` is restricted to products which (a) contain live microorganisms, e.g., as freeze-dried cells or in a fermented product; (b) improve the health status of man or animals and exert their effects in the mouth or gastrointestinal tract (e.g., included in food or administered as capsules), in the upper respiratory tract (aerosol) or in the urogenital tract (by local application) [Havenaar and Huis, 1992].

Probiotic bacteria are frequently used as the active ingredient in functional foods such as bio-yoghurt, dietary adjuncts and health related products [Brassart and Schiffrin, 1997]. The health benefits (for consumers) attributed to probiotic bacteria in the literature can be categorized as either nutritional benefits or therapeutic benefits. Nutritional benefits include: their role in enhancing the bio-availability of calcium, zinc, iron, manganese, copper and phosphorus [McDonough et al., 1983]; increasing the digestibility of protein in yoghurt [Breslaw and Kleyn, 1973], and synthesis of vitamins in yoghurt [Deeth and Tamine, 1981]. The therapeutic benefits of probiotics reported include: treatments of conditions including gastrointestinal disorders [Clements et al., 1983; Biller et al., 1995], hyper-cholesterolaemia [Mann and Spoerry, 1974; Noh et al., 1997], and lactose intolerance [Kilara and Shahani, 1976; Mustapha et al., 1997]; suppression of pro-carcinogenic enzymes [Goldin and Gorbach, 1977; McConnell and Tannock, 1991; Fujisawa and Mori, 1997]; inhibitory effects on Ehrlich ascites tumour cells [Reddy et al., 1983]; immunomodulation [Perdigon et al., 1995]; and the treatment of food-related allergies [Majamaa and Isolauri, 1997].

The majority of probiotic bacteria belong to two genera: *Lactobacillus* and *Bifidobacterium*. A stringent selection criteria for identification of probiotic strains is required in order to achieve consistent and positive probiotic effects. A consensus is emerging among practitioners as to what these criteria should be. Collins et al. [1998] have compiled a list of 12 important criteria for selecting a potential probiotic strain. Essentially, these criteria suggest that the selected strains must be safe, viable and metabolically active within the gastrointestinal tract in order to exert a beneficial impact on the host. Once a strain has been selected its unequivocal characterization is essential to allow elucidation of its contributions to the intestinal microbiota and for the control of any unique or beneficial properties [Havenaar et al., 1992].

2.2.1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) have been widely used as probiotics in various fermented foods since antiquity. LAB with probiotic activity are generally enteric flora, believed to play a beneficial role in the ecosystem of the human gastrointestinal tract. The probiotic spectrum of activity can be divided into nutritional, physiological, and antimicrobial effects (Tab. 2.2). These observations had led to the development of a variety of foods and feeds containing LAB cells for probiotic use in man and animals. [Naidu and Clemens, 2000].

Several investigations have demonstrated that various species of LAB exert antagonistic action against intestinal and food-borne pathogens [Gibson et al., 1997]. LAB are capable of preventing the adherence, establishment, replication, and/or pathogenic action of specific enteropathogens [Saavedra, 1995]. These antagonist properties may be manifested by (a) decreasing the luminal pH through the production of volatile short-chain fatty acids (SCFA) such as acetic, lactic or propionic acid; (b) rendering specific nutrients unavailable to pathogens; (c) decreasing the redox potential of the luminal environment [Krämer, 1997]; (d) producing hydrogen peroxide under anaerobic conditions; and/or (e) producing specific inhibitory compounds such as bacteriocins [Havenaar et al., 1992].

LAB strains for probiotic use must be representative of microorganisms that are Generally Recognized As Safe (GRAS microorganisms). The generally desired properties of LAB for human probiotic use are shown in Fig. 2.3 [Mattila and Saarela, 2000].

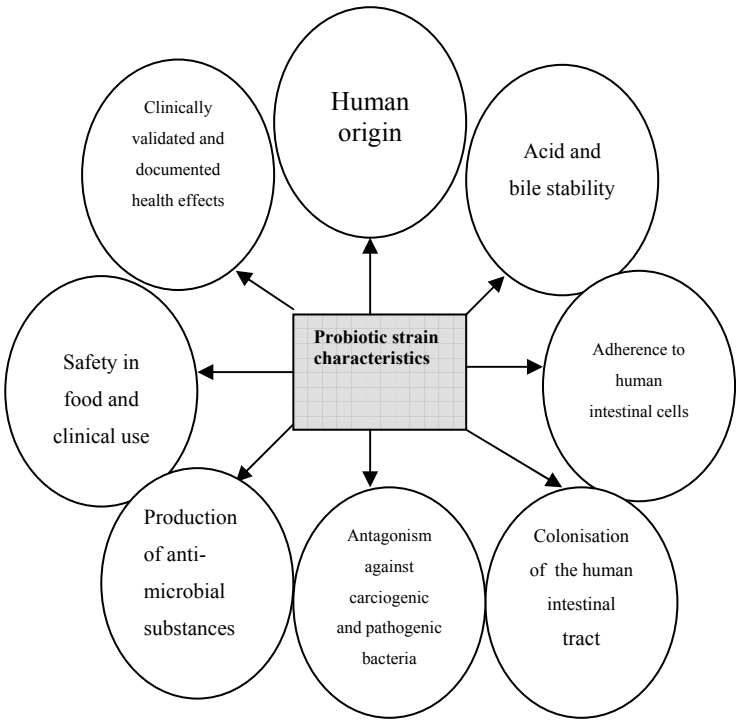


Fig. 2.3: The theoretical basis for selection of probiotic microorganisms

In recent years, consumers have become aware of probiotic properties of cultured milk and dosage specifications. A concentration of 1 x 105 cfu/g or ml of the final product has been suggested as the therapeutic-minimum. Most probiotic LAB, including the common yoghurt cultures.

L. acidophilus and *B. bifidum*, show a short stationary growth phase, followed by a rapid loss of cell viability, even in cold storage [Lee and Wong, 1993]. This short shelf life represents a logistical problem for both manufacturers and retailers, and a technical challenge for researchers. Thus, it is important to check the viability and resistance of the LAB during processing and storage. One of the first steps in probiotics productions is large-scale culturing, washing and drying of the microorganisms. Most of the LAB can be cultured in large-scale fermenters and are rather resistant to centrifugation [Havennar et al., 1992]. In general, LAB could withstand freezing and frozen storage at –20°C or lower [Klaenhammer and Kleeman, 1981], but are less resistant to freeze-drying and especially to spray-drying. In case of storage over long periods and/or under unfavorable conditions, encapsulation of LAB cultures should be considered. The viability of LAB could dramatically decrease during pelleting, thus preservation or protective measures are warranted during such processing. Contamination of probiotic products with undesirable microorganisms is also possible, especially in uncontrolled fermentation procedures and a stringent microbiological quality control is necessary.The quality control should insure the following stability and technical properties of probiotic LAB products:

- Ability to maintain viability through processing and storage
- Maintain good flavor, aroma profile and organoleptic qualities
- Maintain mild acidity throughout storage
- Retain intestinal colonizing properties throughout processing and storage
- Enhance the shelf life and storage stability of fermented products
- Demonstrate stability and functionality after freeze-drying and after drying methods
- Accurate strain identification and exclusion of undesirable contaminants
- Dose-response data for required effects [Naidu and Clemens, 2000].

Tab. 2.2: Potential health and nutritional benefits of functional foods prepared with probiotic bacteria [after Gomes and Malcata, 1999]

Beneficial effect	***Possible causes and mechanism***
Improved digestibility	Partial breakdown of proteins, fats and carbohydrates
Improved nutritional value	Higher level of B vitamins and certain free amino acids, viz. methionine, lysine and tryptophan
Improved lactose utilization	Reduced lactose in product and further availability of lactase
Antagonistic action towards enteric pathogens	Disorders , such as functional diarrhoea, mucous colitis, diverticulitis and antibiotic colitis controlled by acidity, microbial inhibitors and prevention of pathogen adhesion or pathogen activation
Colonisation in gut	Survival in gastric acid, resistance to lysozyme and low surface tension of intestine, adherence to intestinal mucosa, multiplication in the intestinal tract, immune modulation
Anticarcinogenic effect	Conversion of potential pre-carcinogens into less harmful compounds. Inhibitory action towards some types of cancer, in particular cancers of the gastrointestinal tract by degradation of pre-carcinogens, reduction of carcinogen-promoting enzymes and stimulation of the immune system
Hypocholesterolemic action	Production of inhibitors of cholesterol synthesis. Use of cholesterol by assimilation and precipitation with deconjugated bile salts
Immune modulation	Enhancement of macrophage formation, stimulation of production of suppresser cells and γ-interferon

2.2.1.1.1 *Lactobacillus acidophilus*

Lactobacillus acidophilus (*L. acidophilus*) is a gram-positive rod with rounded ends that occurs as single cells, as well as in pairs or in short chains. The typical size is 0.6-0.9 µm in length. It is non-flagellated, non-motile and non-sporeforming, and is intolerant to salt. In addition, it is microaerophilic, so surface growth on solid media is generally enhanced by anaerobiosis or reduced oxygen pressure and 5-10 % CO_2. Most strains of *L. acidophilus* can ferment cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and esculin [Nahaisi, 1986; Kunz, 1994]. Lactose is virtually the only sugar present in milk, yet *L. acidophilus* has been reported to utilize sucrose more effectively than lactose [Mital and Garg, 1992]; such observations may be ascribed to differences in ß-galactosidase and ß- fructofuranosidase activities. While ß-fructofuranosidase is a constitutive enzyme, ß-galactosidase may be induced in *L. acidophilus* [Nielsen and Gilliland, 1992]. Moreover, both glucose and fructose moieties of sucrose are utilized by *L. acidophilus,* whereas the galactose moiety of lactose cannot be metabolized to an appreciable degree. The glucose moiety is metabolized via the Embden-Meyerhof-Parnas pathway with lactic acid as essentially the sole end product. The yield of lactic acid is 1.8 mol/mol glucose, accompanied by minor amounts of other compounds. Acetaldehyde, a carbonyl flavoring molecule, may also result from metabolism of lactose, although in some instances it may be produced from metabolism of nitrogen-containing substances, e.g. threonine; a very high activity of threonine aldolase has been found in *L. acidophilus* [Marshall and Cole, 1983].

Growth of *L. acidophilus* may occurs at as high a temperature as 45°C, but optimum growth occurs within 35-40°C. It is acid tolerance varies from 0.3 % to 1.9 % titratable acidity, with an optimum pH 5.5-6.0 [Gomes and Malcata, 1999].

Lactobacilli have complex growth requirements. They require low oxygen tension [Nahaisi, 1986; and Klaver et al., 1993], fermentable carbohydrates, protein and its breakdown products [Marshall et al., 1982], a number of vitamins of the B-complex [Rogosa, 1974], nucleic acid derivatives, unsaturated free fatty acids [Gyllenberg et al., 1956], and minerals such as magnesium, manganese and iron [Ledesma et al., 1977] for their growth. Increased amount of thiol groups present in whey protein-enriched milks favours the growth of *L. acidohilus*, whereas peptone and trypsin stimulate its acid production [Kurmann, 1998]. Addition of tomato juice (as a source of simple sugars, minerals and vitamins of the B-complex) to skimmed milk has provided evidence for enhancement of both growth (i.e., higher viable counts and shorter generation times) and activity (i.e., improved sugar utilization and lower pH) by *L. acidohilus* [Babu et al., 1992]. Use of soy milk during refrigerated storage of *L. acidohilus* was associated with higher survival rate more than cow's milk [Valdez and Giori, 1992]. These essential nutrients should, therefore, be available in the medium for establishment of a predominant microflora of *lactobacilli*.

Lactobacilli are distributed in various ecological niches throughout the gastrointestinal and genital tracts and constitute an important part of the indigenous microflora of man and higher animals. Their distribution is affected by several environmental factors, including pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions. They are rarely associated with cases of gastrointestinal and extraintestinal infection, and strains employed technologically are regarded as non-pathogenic and safe microorganisms. Furthermore, they have the reputation of health promoters, especially in the human gastrointestinal and genitourinary tracts [Salminen et al., 1996]. Tab. 2.3 shows *lactobacillus* species used as human probiotics.

Tab. 2.3: *Lactobacillus* species used as human probiotics [after Kunz, 1994; Sanders and Klaenhammer, 2001]

Species **Obligatory homofermentative**	**Species** **Facultatively heterofermentative**	**Species** **Obligatory heterofermentative**
L. acidophilus	*L. casei*	*L. fermentum*
L. crispatus	*L. paracasei* ssp. *paracasei*	*L. reuteri*
L. amylovarus	*L. paracasei* ssp. *tolerans*	
L. gallinarum	*L. plantarum*	
L. gasseri	*L. rhamnosus*	
L. johnsonii		
L. helveticus		
L. delbrueckii ssp. *bulgaricus*		
L. salivarius ssp. *salivarius*		

One important factor for *L. acidophilus* strain to produce beneficial effects on host intestinal tract is the number of normal viable cells. Many inherent and environmental factors of the host can eliminate or reduce considerably the number of *L. acidophilus* cells in the intestine. Some of these, mentioned earlier, are antibiotic intake, abusive and improper food habits, diseases and surgery of the digestive tract, pelvis irradiation and stress [Drasar &Hill, 1985; Gilliland, 1990; Sandine, 1990; Hentges, 1993]. These conditions not only reduce the numbers of beneficial bacteria, but also give a chance for the undesirable bacteria to flare up and enteric pathogens to invade the digestive tract.

In the event the population of *L. acidophilus* in the host gastrointestinal tract is reduced, daily oral consumption of a large number (about 109 cells/day) of healthy viable cells of *L. acidophilus* over a period of 2 weeks or more has advocated to raise their levels [Speck, 1978]. A large variety of commercial preparations containing viable *L. acidophilus*, many of which are dried, are now available that are intended to restore the population following oral administration. A product in which high levels of healthy viable *L. acidophilus* cells have been inoculated in refrigerated pasteurized milk will maintain the high population. However, acidic (low pH) fermented products and particularly the dried products, many of which are stored at room temperature (22 to 30°C or higher depending upon a country), most likely will not have high numbers of viable *L. acidophilus* cells. Also, many of the viable cells can be injured and thus will be killed by the low pH in stomach and bile salts and lysozyme in the intestine.

In a study it found that the population of *L. acidophilus* in these products sold by the „health food store“ and pharmacies have very low numbers of *L. acidophilus*. Some of these products not only contained *lactobacilli* that are non-indigenous and sensitive to gastrointestinal environment (*Lactobacillus delbrueckii* ssp. *bulgaricus*) and not accepted now as a species (*Lactobacillus caucacicus*) but also had coliforms and other Gram-negative bacteria, possibly due to unsanitary practices during production and handling [Brennan et al., 1983]. One reason for low viable population of *L. acidophilus* in the dried commercial preparations could be due to the viability loss of the cells during freezing, drying and subsequent storage, especially at higher temperatures for long times and some in the presence of air. Freezing and drying are known to cause viability loss as well as sublethal injury of *L. acidophilus* cells and other bacteria. Storage, even at refrigeration temperature can reduce the viability of surviving population in the dried products rapidly. Higher storage temperatures, long storage times, and presence of oxygen (especially after opening a sealed bottle during use) can accelerate the rate of viability loss. The injured cells develop sensitivity to low pH, bile salts and lysozyme and when consumed are rapidly killed. Thus, such a product quite often is not expected to supply high numbers of healthy viable cells of *L. acidophilus* as the need dictates [Brennan et al., 1986; Ray & Johnson, 1986].

A number of factors have been claimed to affect the viability of probiotic bacteria in food. For instance, *L. acidophilus* grows slowly in milk during product manufacture. Therefore, the usual production practice for example in yoghurt is to incorporate yoghurt cultures along with probiotic cultures. However, *L. delbrueckii* ssp. *bulgaricus* produces lactic acid during

fermentation and refrigerated storage. The latter process is known in the industry as `post-acidification.` Post-acidification is found to cause loss of viability of probiotic bacteria. It is important that the cells remain viable throughout the projected shelf life of a product so that when consumed the product contains sufficient viable cells [Shah et al., 1995].

There are many reports on the antagonistic action of *L. acidophilus* towards some pathogenic microorganisms such as *Staphylococcus aureus* [Vincent et al., 1955; Anderson, 1986; Dahiya and Speck, 1986;], *Pseudomonas putrefaciens* [Shahani and shandan, 1979] *Escherichia coli* [Gilliland and Speck, 1977; Hosono et al., 1977] and *Salmonella tiphymurium* [Gilliland and Speck, 1977]. The exact mechanism whereby dietary cultures of *L. acidophilus* may inhibit intestinal pathogens is not completely understood [Klaenhammer, 1982; Gilliland, 1989]. However, it is known that *L. acidophilus* produces bacteriocins in addition of others types of inhibitory compounds [Barefoot and Klaenhammer, 1984; Ferreira and Gilliland, 1988]. By definition, bacteriocins are active only against closely related species of bacteria and thus, may not be of much benefit in controlling intestinal pathogens. However, they can be very important to the establishment for selected strains of *L. acidophilus* in the intestinal tract in the presence of other *lactobacilli* [Gilliland, 1989].

In order to exert both its therapeutic and nutritional effects in the gastrointestinal tract, *L. acidophilus* has to be viable and able to adhere to intestinal cells [Conway et al., 1987]. The first barrier met by these microoganisms ingested with food is the low pH (hydrochloric acid) present in the stomach [Conway et al., 1987]. If they survive gastric digestion they become strong candidates for the interaction with the gastrointestinal microflora [Kilara, 1982]. After passing through the stomach barrier, the microorganisms reach the duodenum, where the secretion of bile salt takes place. Thus, resistance to bile salts is an important factor to guarantee the establishment and growth of microorganisms used as dietary adjuncts within the intestinal tract [Hill and drasar , 1968; Floch et al., 1972]. Additionally, certain enzymes of the gastrointestinal system, such as lysozyme, are also deleterious to microorganisms [Hawley et al., 1959; Sandine, 1979].

Probiotic strains can be successfully manufactured and incorporated into highly acceptable food products where they can retain their viability and functionality. Today, research efforts are being made in incorporating probiotic encapsulation technology into foods to insure the viability and stability of probiotic cultures [Myllärinen et al., 1998].

2.3 Microencapsulation

Ingredients are added to food for many reasons. There is a recent trend toward reducing permitted levels of many food additives and, where possible, replacing chemically derived substances with alternatives perceived to be of natural origin [Kirby, 1991]. However, many of the natural ingredients are less potent, or more restricted in their applicability, than their ”synthetic” counterparts. A novel strategy to increase the effectiveness and range of

application of many types of natural functional ingredients is to use microcapsular delivery systems. Because of the wide availability of encapsulated ingredients, many food products that were thought to be technically unfeasible are now possible. Such ingredients are products of process that totally envelopes the ingredient in a coating or ”capsule”, thereby conferring useful or eliminating unuseful properties to or from the original ingredient.

Microencapsulation began with the creation of living cells. Most single-called plants or animals are living examples of the wonders of microencapsulation. Their natural capsular membranes are remarkably successful in fulfilling specific functions.

Microencapsulation is defined as the technology of packaging solids, liquids, or gases in miniature, sealed capsules that can release their contents at controlled rates under specific conditions [Todd, 1970; Sparks, 1981].The miniature packages, called ”microcapsules” may range from submicron to several millimeters in size and have a multitude of different shapes depending on the materials and methods used to prepare them. Generally speaking, the microcapsule has the ability to modify and improve the apparent shape and properties of a substance. More specifically, the microcapsule has the ability to preserve a substance in the finely divided state and to release it as occasion demands.

Contents of microcapsules are released by a variety of mechanisms [Karel and Langer, 1988; Jackson and Lee, 1991; Reineccius, 1995a]. The coating may be mechanically ruptured, for example, by the act of chewing (physical release) [Hengenbart, 1993]. Coatings may melt when exposed to heat (thermal release) [Hengenbart, 1993] or dissolve when placed in solvents. Changes in pH may alter the permeability of polymer coatings and thereby control leaching. Water soluble core materials diffuse into aqueous media. Protein or lipid coatings may degrade by the action of proteases and lipases respectively. Several fundamental equations governing controlled release of active substances were described [Mehta, 1986; Lu and Chen, 1993; Pothakamury and Barbosa-Canovas, 1995; Watano et al., 1995e; and Washington, 1996].

General purposes for microencapsulation are to make liquids behave like solids; separate reactive materials; reduce material toxicity; provide environmental protection to compounds; alter surface properties of the materials; control release of materials; reduce volatility or flammability of liquids; and mask the taste of bitter compounds [Andres, 1977; Bakan, 1978; Dziezak, 1988; Jackson and Lee, 1991; and Hegenbart, 1993].

Consequently, microencapsulation can be employed to enhance, time or tune the effect of functional ingredients and additives such as processing aids (leavening agents and enzymes); preservatives (acids and salts); fortifiers (vitamins and minerals); flavors (natural and synthetic), and spices [Arshady, 1993].

Major benefits brought about by microencapsulation of food ingredients and additives are summerized in a number of reasons : To reduce the reactivity of the core in relation to the outside environment (e.g., light, oxygen, and water); To decrease the evaporation or transfer

rate of the core material to the outside environment; To promote easier handling of the core material; To control the release of the core material in order to achieve the proper delay until the right stimulus; To mask the core taste; and to dilute the core material when it is used in only very small amounts, but achieve uniform dispersion in the host material [andres, 1977; and Versic, 1988].

Microcapsules offer the food processor a mean to protect sensitive food compounds, ensure against nutritional loss, utilize otherwise sensitive ingredients, incorporate unusual or time-release mechanisms into the formulation, mask or preserve flavors and aromas, and transform liquids into solid ingredients that are easy to handle [Balssa and fanger, 1971]. The unusual properties inherent in encapsulated ingredients offer the food technologist greater flexibility and control in developing foods that are more flavorful and nutritious to meet the expectations of today's consumers.

Various microcapsule properties may be changed to suit specific ingredient applications, including their composition, mechanism of release, particle size, final physical form, and cost. Before considering the properties desired in encapsulated products, the purpose of encapsulation must be clear. In designing the encapsulation process, the following questions should be asked:
1. What functions must the encapsulated ingredients provide for the final product?
2. What kind of coating material should be selected ?
3. What processing conditions must the encapsulated ingredient survive before releasing its content ?
4. What is the optimum concentration of the active material in the microcapsule ?
5. By which mechanism will the ingredient be released from the microcapsule ?
6. What are the particle size, density, and stability requirements for the encapsulated ingredients ?
7. What are the cost constraints of the encapsulated ingredient ? [Shahidi and Han, 1993].

2.3.1 Microencapsulating materials

The core of microcapsules, also called the fill or internal phase, `is the mass to be encapsulated.` Core material may be in any physical state: liquid, solid, gases, dispersions in liquids or complex emulsions. The initial step in encapsulating a food ingredient is the selection of a suitable coating material, referred to as the encapsulation matrix. The coating material also has been referred to as the shell or wall material, encapsulating agent, microencapsulating agent or carrier in the literature [Bakan, 1978; Rao et al., 1989; Champagne et al., 1993; Hyndman et al., 1993; Sheu and Marshall, 1993; Kim et al., 1996; Jankowski et al., 1997; Charpentier et al., 1998].

Coating substances that are basically film forming materials can be selected from a wide variety of natural or synthetic polymers, depending on the material to be coated and the characteristics desired in the final microcapsules. The coating composition is the main

determinant of the functional properties of the microcapsule and of the method to be used to improve the performance of a particular ingredient. An ideal coating material should have the following properties:

1. Good rheological properties at high concentration and ease of manipulation during the process of encapsulation.
2. Ability to disperse or emulsify the active material and stabilize the emulsion produced.
3. Non-reactivity with the material to be encapsulated both during processing and on prolonged storage.
4. Ability to seal and hold the active material within its structure during processing or in storage.
5. Complete release of the solvent or other materials that are used during the process of encapsulation, under drying, or other desolventization conditions.
6. Ability to provide maximum protection to the active material against environmental conditions (e.g., heat, light, humidity).
7. Solubility in solvents acceptable in the food industry, e.g., water, ethanol, etc.
8. Chemical non-reactivity with the active material.
9. Ability to meet specified or desired capsule solubility properties and active material release properties.
10. Economy of food-grade substance.

Because almost no coating material can meet all the properties listed above, in practice they are used in combination with other coating materials and/or modifiers, such as oxygen scavengers, antioxidants, chelating agents, and surfactants. Generally, water-insoluble polymers are used to microencapsulate the aqueous core while the converse is true for organic core materials [Jackson and Lee, 1991]. Thickness of coat is manipulated to alter permeability and stability of microcapsules. Some types of coating materials are presented in Tab. 2.5.

Tab. 2.4: Coating materials for encapsulation of food ingredients [after Shahidi and Han, 1993]

Carbohydrates	Starch, maltodextrins, corn syrup, dextran, sucrose, cyclodextrins
Celluloses	Carboxy methylcellulose, methylcellulose, ethylcellulose, nitrocellulose, acetylcellulose, cellulose acetate-phthalate, cellulose acetate-butylate-phthalate
Gums	Gum acacia, agar, sodium alginate, carrageenan
Lipids	Wax, paraffin, beeswax, tristearic acid, diglycerides, monoglycerides, oils, fats, hardened oils
Proteins	Gluten, casein, gelatin, albumin, hemoglobin, peptides

One class of materials often exploited for its encapsulating abilities is hydrocolloids or more commonly, gums. These compounds are long-chain polymers that dissolve or disperse in water to give a thickening or viscosity-building effect [Glicksman, 1982]. Gums are usually used as texturing ingredients. They are also used for secondary effects, including encapsulation [Carroll et al., 1984], stabilization of emulsions, suspension of particulates, control of crystallization, and inhibition of syneresis (the release of water from fabricated foods) [Glicksman, 1982]. A few gums are able to form gels.

Food gums are obtained from a variety of sources. Most gums come from plant materials such as seaweed, seeds, and tree exudates; others are products of microbial biosynthesis; and still others produced by chemical modification of natural polysaccharides [Shahidi and Han, 1993].

Alginates, agar, and carrageenan are extracts from red and brown algae that are collectively called seaweeds [Dziezak, 1991].

Alginate occurs in the cell walls and intercellular spaces of brown algae. It provides both flexibility and strength to the plants. Alginic acid, the free acid from alginate, is the intermediate product in the commercial manufacture of alginates and has limited stability. In order to make stable water-soluble alginate products, alginic acid is transformed into a range of commercial alginates by incorporating different salts. This produces Na-, K-, NH_4-, Mg, and Ca- alginate [Ons∅yen, 1997].

Alginates include a variety of products made up of D-mannuronic acid and L-gluconic acid, (Fig. 2.4) [Ons∅yen, 1997] which arranged in regions composed solely of one unit or the other, referred to as M-blocks and G-blocks, and regions where the two units alternate [Dziezak, 1991]. Both the ratio of mannuronic acid to gluconic acid and the structure of the polymer determine the solution properties of the alginate. Monomeric M- and G-residues in alginates are joined together in sections consisting of homopolymeric M-blocks (MMMMM) and G-blocks (GGGGG) or heteropolymeric blocks of alternating M and G (MGMGMG). In the polymer chain, the monomers will tend to find their most energetically favorable structure. The rather bulky carboxylic group is responsible for a β-1,4 equatorial/equatorial glycosidic bond in M-M, and α-1,4 axial/axial glycosidic bond in G-G, and an equatorial/axial bond in M-G. The consequence of this is a buckled and stiff polymer in the G-block regions and a flexible ribbon-like polymer in the M-block regions. The MG-block regions have intermediate stiffness [Ons∅yen, 1997].

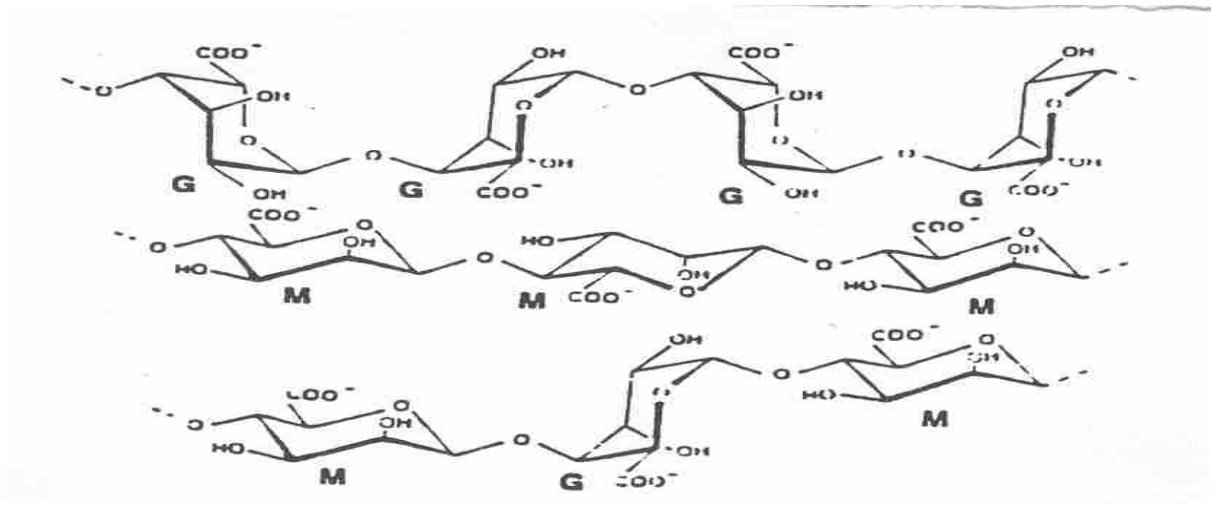


Fig. 2.4: Block types in alginate: top: G-blocks; middle: M-blocks; bottom: MG-blocks

Alginate properties utilized in food production are: gel forming (e.g., pudding), water-binding (e.g., soups), stabilizing (e.g., ice cream), and film forming (e.g., coatings) [Ons∅yen, 1997]. Alginate films are formed by evaporation of an aqueous alginate solution followed by ionic crosslinking with a calcium salt. They are impervious to oils and fats but are poor moisture barriers. Despite this, alginate gel coatings can significantly reduce moisture loss from foods sacrificially. In other words, moisture is lost from the coating before the food significantly dehydrates. Alginate coatings are good oxygen barriers, can retard lipid oxidation in foods, and can improve flavor, texture, and batter adhesion [Krochta and De Mulder-Johnston, 1997]. Alginates have found use in a variety of products, including the encapsulation of food ingredients since they were first used in the U.S. in the 1920s. It has been reported that water-soluble alginate is capable of forming encapsulated liquid capsules [Meiji Seika Kaisha, 1971]. Viscous high-fat food also can be encapsulated with calcium alginate [Veliky and Kalab, 1990].

Alginates have been used in microencapsulation of microbial cells [Sheu and Marshall, 1991; Sheu and Marshall, 1992; Shah and Ravula, 2000; Hansen et al., 2002]. Some encapsulated lactic acid bacteria cultures have successfully been used in food fermentations. For example, encapsulation of probiotic bacteria with alginate-starch improved the survival rate in simulated gastrointestinal conditions and in yoghurt [Khalida et al., 2000]. Alginate encapsulated bifidobacteria has been found to enhance the survival rate in mayonnaise [Khalil and Mansour, 1998]. Kebary et al. [1998] have improved the viability of bifidobacteria and their effect on frozen ice milk by microentrapped them in alginate or κ- carrageenan beads. Immobilization of *Bifidobacterium* in calcium alginate beads increased the survivability in simulated gastric juices and bile salt solution [Lee and Heo, 2000].

Carrageenan is a naturally occurring polysaccharide material which fills the voids in the cellulosic structure of red seaweeds. It is a high-molecular mass linear polysaccharide made up of repeating galactose units and 3,6-anhydrogalactose (3,6-AG), both sulfated and non-sulfated, joined by alternating α-1,3 and β-1,4 glycosidic linkages. Various naturally occurring arrangements of components create three basic types of carrageenan, commonly referred to as kappa (κ), iota (ι), and lambda (λ) [Thomas, 1997]. Variations of these components influence gel strengths, texture, solubility, synergisms, and melting temperatures of the carrageenan. All carrageenans are soluble in hot water, developing very low fluid-processing viscosities. Except for lambda, only the sodium salts of iota and kappa carrageenans are soluble in cold water. Potassium and calcium are essential for gelation and the gels are stable at room temperature. The gels can be remelted by heating to about 5-10 degrees above the gelling temperature (40-70°C) [Thomas, 1997].

Carrageenan consists of a family of hydrocolloids which have different properties and, therefore, it has a wide variety of uses. The most important uses are in water- and milk-based systems, which range from cake glazes to chocolate beverages (i.e., hot-processed gelling applications, hot-processed thickening applications, and cold-processed thickening applications) [Thomas, 1997]. Coatings that include carrageenan as a major part or sole component have been applied to a variety of foods to carry antimicrobials and reduce moisture loss, oxidation, or disintegration [Krochta and De Mulder-Johnston, 1997]. Gels from carrageenans are thermoreversible. Because of its reactivity with certain proteins, the gum has found use at low concentrations (typically 0.01 to 0.03%) in a number of food products [Hoashi, 1989]. Carrageenan has used also to encapsulate bacteria [Hammill and Crawford, 1997; Kebary et al., 1998; Adhikari et al., 2000].

Gum arabic (gum acacia) is a hydrocolloid produced by the natural excudation of acacia trees and is an effective encapsulating agent due to its high water solubility, the low viscosity of concentrated solutions relative to other hydrocolloid gums, and its ability to act as an oil-in-water emulsifier [Glicksman, 1983; Brian et al., 1998].

Gum arabic is composed of a highly branched arrangement (Tab. 2.5) of the simple sugars galactose, arabinose, rhamnose, and glucuronic acids [Anderson and Stoddart, 1966; Street and Anderson, 1983] and also contains a protein component (~2% w/w) covalently bound within its molecular arrangement (Fig.2.5) [Anderson et al., 1985]. The protein fraction plays a crucial role in determining the functional properties of gum arabic [Randall et al., 1988]. Tab. 2.6 shows gum arabic food applications.

Tab. 2.5: Chemical composition of gum arabic [after Kravtchenko, 1998]

Compound	% in Gum
Galactose	36.0
Arabinose	30.0
Rhamanose	12.6
Glucuronic acid	19.2
Protein	2.2

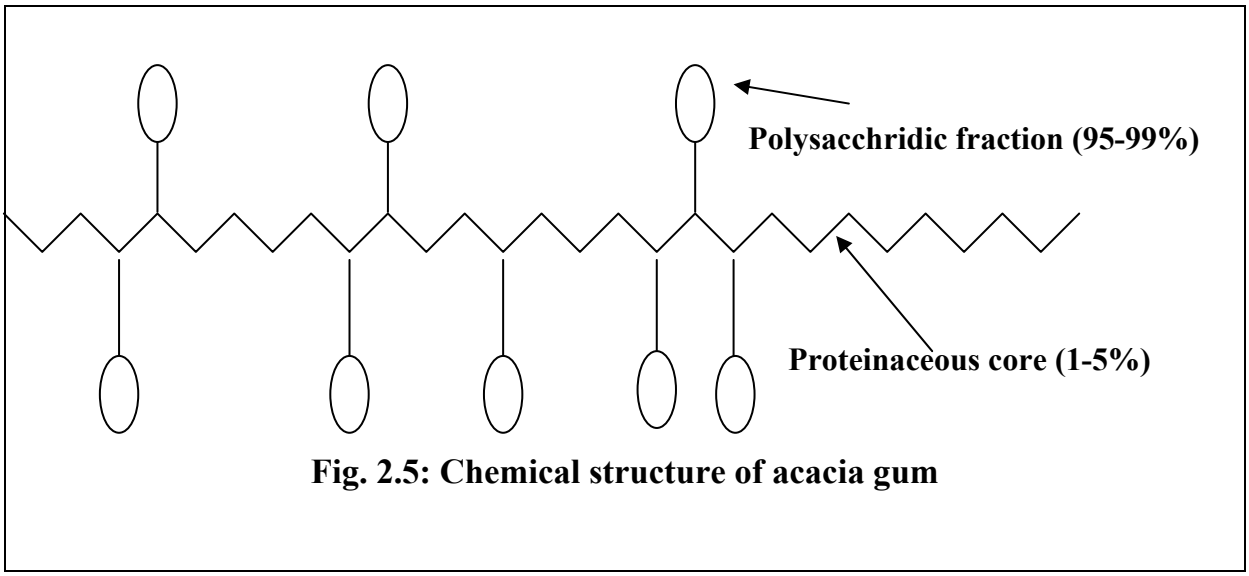


Fig. 2.5: Chemical structure of acacia gum

Tab. 2.6: Gum arabic food applications

Food	Function	Level
Meat flavoring	Carrier-stabilizer	50%
Carbonated artificial beverages	Thickener for CO2 retention	
Synthetic, dehydrated juice pulp	Hydrophilic adjuvant	50% (0.5% in final juice powder)
Synthetic orange juice powder	Texturizer	Not given
Pastry dough	Thickener	0.2-0.6%
quick-cooking cereal	Hydrophilic adjuvant	0.5-2.5%
Coffee bonbons (candies)	Texturizer and coating agent	0.2%
soluble fruit or vegetable powder	Flavor fixative	0.1-2% in the dehydrated juice
soluble meat powder	Thickener	12% in a subsequently dried paste
Candy	Emulsifier and sugar Crystallization retarder	40% or more
Meat	Thickener	
Sausage casing or other food-wrapping membrane	Water-soluble ingredient	1-5%
Milk products, specifically ice cream	Stabilizer	ca. 0.06%
Frozen desserts	Stabilizer and texturizer	
Dietetic confectionery cream candy centers	Gum adhesive	5-6%
Fats	Package coating	20-30% in the coating solution
Oil-containing foods, flavoring agents	Release control	1-77%
Whipping powder (for synthetic whipped cream, icing or cream filling)	Surfactant	ca. 2.3% in the reconst. cream
Table syrup (maple)	Emulsion stabilizer	0.1-0.5%
Ray and wheat flour	Baking improver	0.008-0.02%
Bread	Glazing agent	38%
Baking additives	Soluble-film pouch	Main ingredient
Bakery products	Stabilizer	
Dietetic foods (e.g., fruit drops)	Thickener, texturizer or bulking agent	Main ingredient
Gum candies	Bulking agent	55%
Citrus oil emulsion for baking	Emulsifier	
Processed baby foods	Protective colloid	
Beer	Foam stabilizer	0.025%
Wine	Clarifier	
Dry, oil-soluble-vitamin supplement	Stabilizer	
Polished rice	Coating agent	7.5% in the coating solution
Synthetic diabetic syrup	Thickener	15%

(From FDA, Scientific Literature Review of Gum Arabic, PB 221, NTIS, Department of Commerce, Washington, D.C., 1972).

Acacia gum has been known for many years by the scientific community as a potential source of dietary fibre. Its nutritional properties have been well demonstrated. In addition, acacia gum can be added in very large amounts while maintaining the original taste and texture of the food in which it is incorporated. And last but not least, because of its highly complex polymeric structure, acacia gum does not show any nutritional side-effects [Kravtchenko, 1998].

As compared with most other polysaccharides, acacia gum is extremely resistant to various physico-chemical treatments, especially in acidic conditions. For example, acacia gum does not show any loss of dosable dietary fibre during the pasteurization of fruit juices [Kravtchenko, 1998].

Due to its low viscosity and its absence of taste and odor, acacia gum can be added in large quantities without disturbing the organoleptic properties of the food product in which it is incorporated [Kravtchenko, 1998].

Pectin is a major cell wall component with a variety of important biological functions in plants. It plays a role in the control of cell growth, in defence against invasions of microorganisms and in maintaining the physical and sensory properties of fresh fruits and their processing characteristics [Wang et al., 2002]. A representative structure of pectin is illustrated in Fig. 2.6. Pectins are composed of a α-(1$\rightarrow$4) linked D-galacturonic acid backbone interrupted by single α-(1$\rightarrow$2) linked L-rhamnose residues [BeMiller, 1986]. One aspect of difference among the pectic substances is their content of methyl esters, or degree of esterification, which decreases somewhat as plant ripening takes place. The degree of esterification (DE) is defined as the (number of esterified D-galacturonic acid residues per total number of D-galacturonic acid residues) X 100 [Whistler and Daniel, 1990]. The best known property of pectin is that it can gel under suitable conditions. A gel may be regarded as a system in which the polymer is in an state between fully dissolved and precipitated [Flory, 1953].

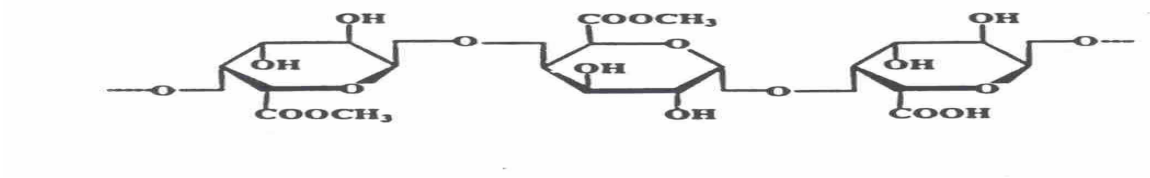


Fig. 2.6: Chemical structure of pectin

Pectins display a wide range of physiological and nutritional effects important to human nutrition and health. Pectin is a dietary fibre, because it is not digested by enzymes produced

by humans. Although not digested and absorbed in the upper gastrointestinal tract, pectin can be fermented by colonic microflora in the colon to CO_2, CH_4, H_2 and short chain fatty acids (SCFA), mainly, acetate, propionate and butyrate. These fatty acids are a potential energy source for the mucosal cells of the large intestine, and some may be absorbed from the colon, providing energy and having additional metabolic effects. The lowering of serum cholesterol by pectin was reported in rats and humans as early as 1960s [Keys et al., 1961; Wells and Ershof, 1961; Palmer and Dixon, 1966]. Since then, the short-term cholesterol-lowering effect of pectin has been demonstrated repeatedly in a wide variety of subjects and experimental conditions [Jenkins et al., 1975; Kay and Truswell, 1977; Judd and Truswell, 1982; Vargo et al., 1985; Schuderer, 1986; Haskell et al., 1992].

Proteins play several important roles in biological and food systems. Some of these include biocatalysts (enzymes), structural components of cells and organs (e.g., collagen, keratin, elastin, etc.), contractile proteins (actin, myosin, tubulin), hormones (insulin, growth factor, etc.), transport proteins (serum albumin, transferrin, hemoglobin), metal chelation (phosvitin, ferritin), antibodies (immunoglobulins), protective proteins (toxins, and allergens), and storage proteins (seed proteins, casein micelles, egg albumen) as nitrogen and energy source for embryos [Damodaran and Paraf, 1997].

Proteins are the basic functional components of various high protein processed food products and thus determine textural, sensory and nutritional properties. Food products include various proteins with different structural, physical, chemical and functional properties, and sensitivity to heat and other treatments. Functional properties of proteins are those physicochemical properties of proteins which affect their behavior in food systems during preparation, processing, storage, and consumption, and contribute to the quality and sensory attributes of food systems [Kinsella, 1976].The most important functional properties of proteins in food applications (Tab. 2.7) are: - hydrophilic, i.e., protein solubility, swelling and water retention capacity, foaming properties, and gelling capacity; - hydrophilic-hydrophobic, i.e., emulsifying, foaming; and –hydrophobic, i.e., fat binding properties. There is no generally accepted scheme of classification for the functionality of proteins with relation to specific physicochemical properties of protein molecules. Attempts to classify functionality of Soy and other proteins have been presented [Kinsella, 1976].

In a phenomenological sense, the various functional properties of food proteins are manifestations of two molecular aspects of proteins [Damodaran, 1989]: (1) protein surface-related properties and (2) hydrodynamic properties. The functional properties that are affected by these molecular aspects of proteins are listed in Tab. 2.8. The surface-related properties are governed by the hydrophobic, hydrophilic, and steric properties of the protein surface, and the properties related to hydrodynamic properties of proteins are governed by size, shape, and flexibility of proteins.

Tab. 2.7: Functional roles of food proteins in food systems [after Kinsella et al., 1985]

Function	Mechanism	Food system	Protein source
1. Solubility	Hydrophilicity	Beverages	Whey proteins
2. Viscosity	Water binding, hydrodynamic size and shape	Soups, gravies, salad dressing, desserts	Gelatine
3. Water binding	Hydrogen bonding, ionic hydration	Meat sausages, cakes, breads	Muscle and egg proteins
4. Gelation	Water entrapment and immobilization, network formation	Meats, gels, cakes, bakeries, cheese	Muscle, egg, and milk proteins
5. Cohesion / Adhesion	Hydrophobic, ionic, hydrogen bonding	Meats, sausages, pasta, baked goods	Muscle, egg, and whey proteins
6. Elasticity	Hydrophobic bonding, disulphide cross-links	Meats, bakery	Muscle and cereal proteins
7. Emulsification	Adsorption and film formation at interface	Sausages, bologna, soupe, cakes, dressings	Muscle, egg, and milk proteins
8. Foaming	Interfacial adsorption and film formation	Whipped toppings, ice cream, cakes, desserts	egg, and milk proteins
9. Fat and Flavor binding	Hydrophobic Bonding, entrapment		Milk, egg, and cereal proteins

Tab. 2.8: Functional properties of food proteins [afterDamodaran, 1989]

Surface-related properties	Hydrodynamic properties
Solubility	Viscosity
Wettability	Thickening
Dispersibility	Gelation
Foaming	texturization
Emulsification	
Fat and flavor binding	

Gelatin is the most commonly used protein for encapsulating food ingredients, even though other proteins also are used for this purpose [Shahidi and Han, 1993]. It is a valuable coating

material that is non-toxic, inexpensive, and commercially available. Gelatin is a high-molecular-mass polypeptide derived from collagen, the primary protein component of animal connective tissues, which include bone, skin and tendon. Commercially, its sources of practical importance are hides, bones and pigskin. Commercial gelatins can be divided into two groups: gelatin type A obtained by acid pre-treatment and gelatin type B obtained by basic pre-treatment [Poppe, 1997]. The main difference between these two types are shown in Tab. 2. 9.

Tab. 2.9: Characteristics of gelatin type A and type B [after Pope, 1997]

Type	A	B
Raw material	pigskin, bone	bone, hide
Pre-treatment	Acid	Lime
Isoelectric point	7 – 9.4	4.5 –5.3

All the amino acids that occur in proteins are present in gelatin Fig. 2.7 [Hudson, 1993] with the probable exception of tryptophan and cystine. Amino acids are linked together in gelatin by peptide bonds [Poppe, 1997]. A typical sequence for gelatin is: Gly-X-Y
Where, X is mostly proline and Y is mostly hydroxyproline. The structure of gelatin (Fig. 2.8) [Hudson, 1993] is somewhat fibril-like. Generally, the molecular mass distribution of gelatin ranges from 104 to 106 Dalton [Pope, 1997].

Gelatin swells when placed in cold water, absorbing 5 to 10 times its own volume of water. When heated to temperatures above its melting point, the swollen gelatin dissolves and forms a gel when cooled. This sol-gel conversion is reversible and can be repeated. The basic mechanism of gelatin gelation is the random coil-helix reversion on cooling. The ability to form a gel is without doubt one of the most important properties of gelatin. Gelatin is used in foods because of its unique physical properties rather than for its nutritional value as a protein (e.g., confectionery, dairy products, meat products, sauces, dressings etc.) [Poppe, 1997].

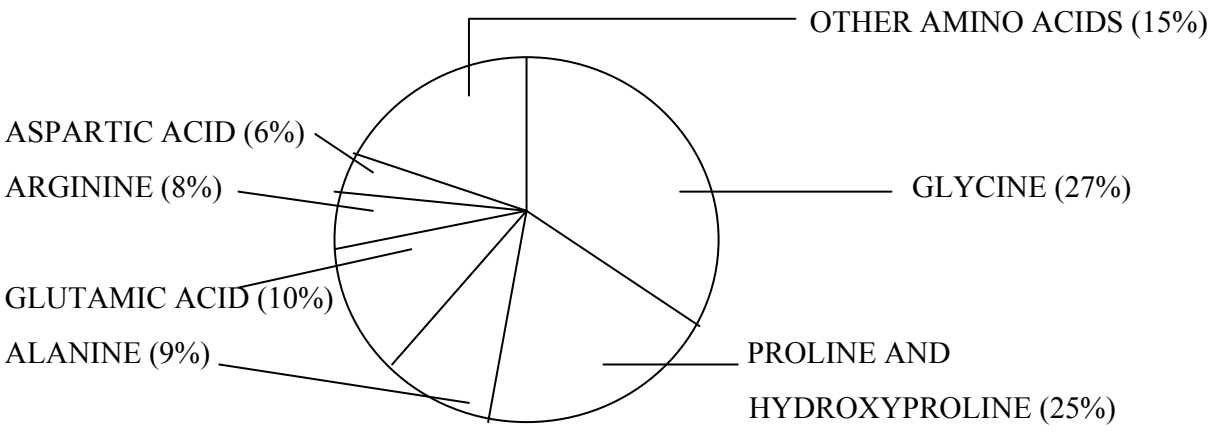


Fig. 2.7: Gelatin amino-acids composition

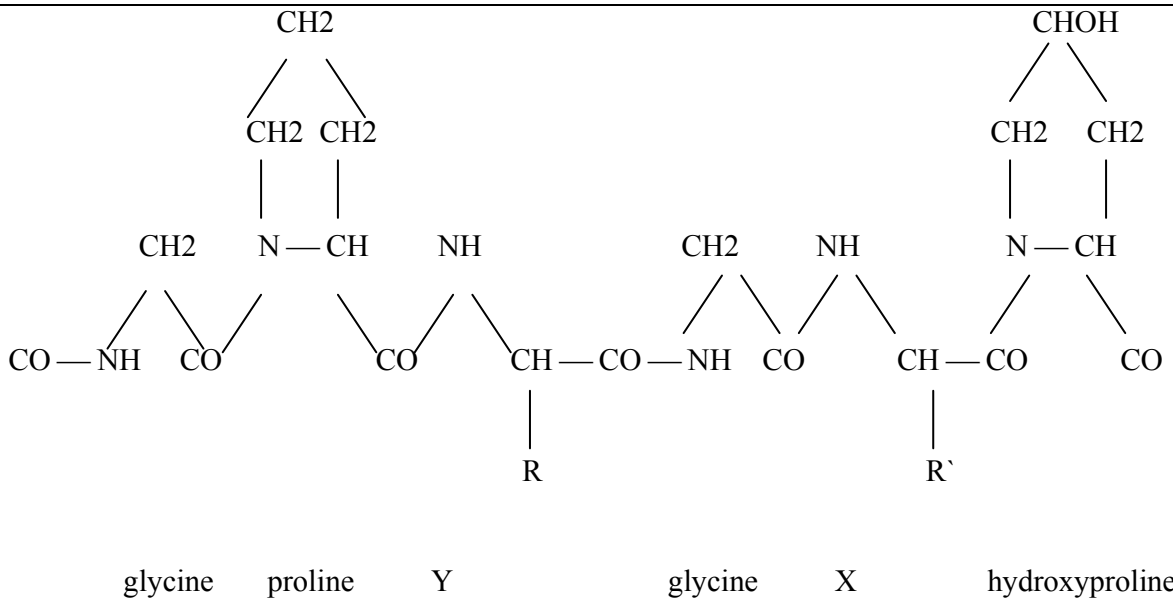


Fig. 2.8 : Gelatin structure chain

Gelatin has a good film forming property as well as other ideal chemical and physicochemical characteristics for the process of microencapsulation. Gelatin forms thermally reversible gels when warm aqueous suspensions of polypeptides are cooled. With an aqueous solution of gelatin, the change between the gel and solid state is quite definite. However, when the gelatin concentration in the aqueous solution is lower than about 1%, definite gelation cannot be observed even by cooling. These characteristic properties are effectively used for formation of capsules [Shahidi and Han, 1993].

Soy proteins have been utilized for many kinds of traditional foods. The use of soy protein products as functional ingredients is gaining increasing acceptance in food manufacturing from the standpoints of human nutrition and health. The applicability of soy proteins in foods is based on their functionality. Typical functions of soy proteins (Tab. 2.10) are gelation, emulsification, foaming, cohesion-adhesion, elasticity, viscosity, solubility, water absorption and binding, fat absorption, and flavor binding. These are influenced by environmental factors and the conditions of protein preparation as they affect the intrinsic physical and chemical properties of the protein [Kinsella, 1979].

The soy protein products used as ingredients can be divided into three categories: (1) soy flour (protein content less than 65%), (2) soy protein concentrate (protein content 65-89%) and (3) soy protein isolate (protein content more than 90%) [Fukushima, 2000].

Soy proteins were initially classified according to their sedimentation velocity into 2S, 7S, 11S, and 15S fractions [Naismith, 1955; Wolf and Briggs, 1956]. The 2S fraction contains

trypsin inhibitors and cytochrome and constitutes about 8% of the protein. The 7S fraction contains globulins and enzymes (lipoxygenase and amylase) and constitutes about 35% of the protein. The 11S fraction is considered to be a single protein and constitutes about 52% of the total protein [Wolf et al., 1961; Wolf and Cowan, 1975]. The 15S fraction is a polymer form of 11S fraction and constitutes about 5% of the protein. The major soy globulins were also classified into glycinin, α-, β-, and γ-conglycinin based on their different immunological responses [Catsimpoolas, 1969b]. The 11S fraction is believed to be identical to glycinin and the globulin portion of the 7S fraction to conglycinin [Rhee, 1994].

Tab. 2.10: Functional properties performed by soy protein preparations in actual food systems [after Kinsella, 1979]

Functional property	Mode of action	Food system
Solubility	Protein solvation, pH dependent	Beverages
Water absorption and binding	Hydrogen-bonding of HOH, entrapment of HOH , no drip	Meats, sausages, breads, cakes
Viscosity	Thickening, HOH binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Cohesion-adhesion	Protein acts as adhesive material	Meats, sausages, baked goods, pasta products
Elasticity	Disulphide links in gels deformable	Meats, bakery
Emulsification	Formation and stabilization of fat emulsions	Sausages, blogna, soupe, cakes
Fat adsorption	Binding of free fat	Meats, sausages, donuts
Flavor-binding	Adsorption, entrapment, release	Stimulated meats, bakery
Foaming	Forms stable films to entrap gas	Whipped toppings, chiffon desserts, angel cakes
Color control	Bleaching of lipoxygenase	Breads

Vegetable proteins usually exhibit a hypocholesterolemic effect in comparison with animal proteins. Soybean proteins are some of the most typical vegetable proteins, in which the hypocholesterolemic activity has generally been established [Huff and Carrol, 1980; Nagata et al., 1982; Sugano et al., 1990; Wang et al., 1995; Gatchalian-Yee et al., 1997]. Soybean proteins have a strong ability to bind with sterols, such as bile acid, because of their high hydrophobicity. Therefore, a mechanism is proposed by which the peptide-bound bile acid is excreted into faeces without being reabsorbed from the intestinal tract, thereby lowering the serum cholesterol level [Fukushima, 2000].

Whey from the cheese industry is increasingly being used either as animal feed or as a source of protein for human nutrition. Two types of whey can be distinguished: (1) soft whey, which comes from rennet coagulation of milk at pH 6.6 (e.g., in cheddar or emmental manufacturing) and (2) the acid whey obtained from fresh soft cheese production (e.g., cream cheese, Camembert, or Petit Suisse), after acid coagulation of milk, and, in the case of cottage cheese, following heating of the curd. Marketed wheys come in liquid and powder forms, with different compositions [Cayot and Lorient, 1997].

Whey proteins are mixture of proteins with numerous and diverse functional properties and therefore may have many potential uses. The main proteins are β-lactoglobulin and α-lactoalbumin. They present approximately 70% of the total whey proteins and are responsible for the hydration, gelling, and surface-active properties (emulsifying and foaming properties) of the whey protein ingredients [Cayot and Lorient, 1997]. Products containing more than 35% protein on a dry basis are called whey protein concentrates, and whey protein isolates which have 90% protein or higher [Kilara, 1994]. Several industrial application and uses of whey products in the food industries are listed in Tab. 2.11. Whey proteins have been considered as functional and nutritional ingredients in many foods. These protein ingredients are also used for their nutritional and therapeutic properties in low-calorie diets and in intensive care enteral nutrition [Cayot and Lorient, 1997].

Tab. 2.11: Uses of whey proteins in human foods [after Cayot and Lorient, 1997]

Industrial application	Functional properties expected	Proteins used
Bread making	Waterholding	WPC or WPC +caseinates
Biscuit manufacturing	Fat dispersibility	WPI
Breakfast cereals	Emulsion stabilization, Overrun of foam, Gelling properties, Browning, Aroma enhancement	WPI, coprecipitates whey
Pasta	Binding and texturing effect, Browning	Coprecipitates
Confectionary	Emulsion manufacturing	WPC + hydrolyzed caseinates
Chocolate Confectionery	Overrun of foam, Browning, Aroma, Antioxidizing effect	WPC Whey Coprecipitates
Ice cream	Emulsion stabilization, Overrun of foams, Gelling properties	WPC + caseinates and total milk proteins
Meat products	Emulsion making, Waterholding (creamy and smooth texture), Adhesive or binding properties	WPC, WPI alone or in Mixture with caseinate
Sauces	Emulsion stability, Waterholding	WPC + caseinates + Egg yolk
Soups	Emulsion stability, Waterholding	WPC + caseinates + Egg yolk
Ready-to-eat food	Texturing	WPC + caseinates + whole egg
Milk products	Emulsion stability, Waterholding, Gelling properties	Caseinates WPC + caseinates WPI
Alcoholic beverages	Cream stabilization, Cloudy aspect	WPC + caseinates WPC or WPI
Nutritional uses	Protein intake, Enteral nutrition	Whey, WPC, WPI, WPC hydrolysates
Cosmetics	Skin protection, Antimicrobial properties	Lactoferrin, WPC Hydrolysates Lactoferrin, lactoperoxidase

The functionality profile of wall materials for spray drying includes high solubility, effective emulsification and film forming characteristics, and different drying properties. In addition, concentrated solutions of wall material should have low viscosity [Reineccius, 1988; Sheu and Rosenberg, 1993]. Whey proteins have such properties and were reported as an effective basis for microencapsulation by spray drying of anhydrous milkfat or volatiles [Moreau and Rosenberg, 1993; Rosenberg and Young, 1993; Sheu and Rosenberg, 1993; Young et al., 1993a,b].

2.3.2 Microencapsulation techniques

A variety of encapsulation techniques are used in the food and pharmaceutical industry. Virtually any material that needs to be protected, isolated or slowly released can be encapsulated [Rish, 1995]. Many investigators classify encapsulation processes as either chemical or mechanical but Thies [1996] preferred to classify them as type A or type B processes, since so-called mechanical processes may actually involve a chemical processes may rely exclusively on physical phenomena. Tab. 2.12 lists representative examples of both type of processes. Generally type B encapsulation processes are more common methods of encapsulating food ingredients and additives.

Tab. 2.12: List of types A and B encapsulation processes [after Thies, 1996]

Type A (chemical processes)	Type B (mechanical processes)
Complex coacervation	Spray drying
Polymer-polymer incompatibility	Spray chilling
Interfacial polymerization in liquid media	Fluidized bed
In situ polymerization	
In-liquid drying	Electrostatic deposition
Thermal and ionic gelation in liquid media	Centrifugal extrusion
Desolation in liquid media	Spinning disk or rotational suspension separation
	Polymerization at liquid-gas or solid-gas interface
	Pressure extrusion or spraying into solvent extraction bath

2.3.2.1 Spray drying

Microencapsulation by spray drying is most widely used in the food industry due to its low costs [Dziezak, 1988]. The process is economical and flexible, uses equipment that is readily available, and produces particles of good quality [Heath, 1985; Dziezak, 1988].

The process of microencapsulation by spray drying involves:
- Formation of an emulsion or suspension of coating and core material;
- Nebulization of the emulsion into a drying chamber containing circulating hot dry air;
- Evaporation of moisture from the emulsion droplets while the remaining solids of the coating material entrap the core [Jackson and Lee, 1991].

Advantages and disadvantages of spray drying encapsulation are summerized in Tab. 2.13. Food ingredients entrapped by spray drying include fats, oils and flavor compounds [Dziezak, 1988; Jackson and Lee, 1991]. Zhao and Whistler [1994] reported the ability of small starch granules to combine into interesting and potentially useful porous spheres, when spray dried with small amounts of bonding agents such as proteins or a wide range of water-soluble polysaccharides. These spheres can carry a variety of food ingredients such as flavors for controlled release from the porous structure of the granules. Matsuno and Adachi [1993] reviewed lipid encapsulation technology by means of drying. Lactic acid bacteria have been widely spray dried [Prajapati et al., 1987; Abd-El-Gawad et al., 1989;Teixeira et al., 1995; Mauriello et al., 1999; Gardiner et al., 2000; Awad et al., 2001; Desmond et al., 2001; Desmond et al., 2002; Silva et al., 2002].

Tab. 2.13: Advantages and disadvantages of spray drying microencapsulation

Advantages	Disadvantages
Well-established technology [Dziezak, 1988; Jackson and Lee, 1991; Risch, 1995; Thies, 1996]	Only water-soluble shell materials with a low to moderate viscosity [Thies, 1996]
Production of large amount of capsules [Thies, 1996]	20-30% core loading [Thies, 1996]
Many shell materials approved for Food use [Thies, 1996]	Oxidation of unencapsulated oil [Jackson and Lee, 1991; Thies, 1996]
Variety of particle sizes [Jackson and Lee, 1991]	Loss of low boiling compounds [Dziezak, 1988; Jackson and Lee, 1991; Thies, 1996]
Useful for heat sensitive food ingredients [Dziezak, 1988; Jackson and Lee, 1991]	A necessary supplementary agglomeration step [Dziezak, 1988; Risch, 1995]
Excellent dispersibility of the microcapsules in aqueous media [Jackson and Lee, 1991]	

3 Objectives

The intestinal tract is home to trillions of bacteria comprising hundreds of beneficial species. These “friendly” microorganisms are called probiotics. Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance [Fuller, 1989]. They are an essential link in proper health and well-being. Probiotics above all help the body to get more nutrition out of the food we eat.

While many microorganisms are found to be sensitive to the stomach high acidity, their numbers increase dramatically the further down the intestinal tract we go. The greatest numbers and variety are found in the large intestine. When these microbial populations are in balance, we reap the benefits toward optimum health. For instance, probiotics keep harmful pathogenic species in check. Without sufficient numbers of viable probiotics, our intestinal ecology is thrown off balance, resulting in a wide range of possible health problems and disease conditions.

Probiotics attach themselves to the intestinal wall and produce a mildly acidic environment (primarily lactic acid) that curbs the growth of harmful, disease-causing bacterial species. Probiotics are also essential in nutrient assimilation, producing many important enzymes and increasing the bioavailability of vitamins, particularly the Bs and K, fatty acids, lactase, and calcium. Among other benefits are the strengthening of the immune system, neutralization of toxins, normalization of bowel movements, control of cholesterol, the countering of allergies and skin problems, and the prevention of yeast and fungal infections [Collins et al., 1998; Prasad et al., 1998; Dunne et al., 1999].

Modern consumers are increasingly interested in their personal health, and expect the food that they eat to be healthy or even capable of preventing illness. Gut health in general has shown to be the key sector for functional foods in world.

Live cultures of probiotic bacteria such as *L. acidophilus* and *Bifidobacterium* spp. in the diet are claimed to provide several therapeutic benefits [Modler, 1990; Kurmann and Rasic, 1991; Mital and Garg, 1992; Ishbashi and Shimamura, 1993].

The viability and stability of probiotics have been both a marketing and technological challenge for industrial producers. Probiotic foods should contain specific probiotic strains and maintain a suitable level of viable cells during the product’s shelf life. The technological demands placed on probiotic strains are great and new manufacturing process and formulation technologies may often be required for bacteria primarily selected for their functional health properties. Before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions, and then survive and retain their functionality during storage as frozen, freeze-dried or dried cultures, and also in the food products into which they are finally formulated. The probiotic strains should also survive the

gastrointestinal stress factors and maintain their functionality within the host. Additionally, they must be able to be incorporated into foods without producing off-flavours or textures and they should be viable but not growing. The conditions under which the products are stored are also important for the quality of products.

Future technological prospects exist in innovations finding solutions for the stability and viability problems of probiotics in new food environments. Current research on novel probiotic formulations and microencapsulation technologies exploiting biological carrier and barrier materials and systems for enteric release provides promising results (Mattila-Sandholm et al., 2002).

L. acidophilus shows a short stationary growth phase, followed by a rapid loss of cell viability, even in cold storage. This short shelf life represents a logistical problem for both manufacturers and retailers, and a technical challenge for researchers. Thus, it is important to check the viability and resistance of *L. acidophilus* after processing and during storage.

Spray drying which has high production rate and low operation cost, is a well-known technology in the food industry. Spray drying is used for the preservation and concentration of microorganisms. However, microorganisms are subjected to heat and dehydration damage during spray drying. Therefore, the survival of microorganisms becomes crucial if spray drying is employed for the preparation of microbial culture. *L. acidophilus* should insure ability to maintain viability through processing and storage; demonstrate stability and functionality after drying methods. One reason for low viable population of *L. acidophilus* in the dried commercial preparations could be due to the viability loss of the cells during drying and subsequent storage. Drying is known to cause viability loss as well as sublethal injury of *L. acidophilus* cells and other bacteria.

One very important property of the culture to be used as a dietary adjunct is that the organism should remain viable during storage before consumption. However, such dietary cultures will not play an effective biological role in products unless they are present in sufficient viable numbers by the time of consumption. For this reason, changes in the population of viable bacteria during the expected shelf life of product in question should be known to some extent and taken as a basis for selection criteria of such strains. Storage, even at refrigeration temperatures can reduce the viability of surviving population in the dried products rapidly. The injured cells develop more sensitivity to stomach low pH and intestine bile salts and when consumed are rapidly killed. Thus, such a product quite often is not expected to supply high numbers of healthy viable cells of *L. acidophilus* as needed. *L. acidophilus* may die quickly during refrigerated storage and the count of viable cells sufficient to be consumed by consumers were maintained only for one week [Kosikowski, 1977]. There was much doubt about the real numbers of viable cells that reach consumers. Therefore, it is important to deal with the stability of *L. acidophilus* under refrigerated storage.

It is well known that heat treatment influences the survival and biochemical activity of lactic acid bacteria. *L. acidophilus* and other *lactobacilli* are utilized world-wide for the manufacture of cheeses, yoghurts and fermented foods. Processing conditions often subject these bacteria to adverse environmental conditions, including temperature extremes. The most thoroughly characterized stress response in bacteria and higher cells is heat shock.

It is important that the cells remain viable throughout the projected shelf life of a product, so that the product will contain sufficient viable cells when consumed. The probiotic cultures must tolerate the manufacturing process so as to prepare a bio-product and maintain cell viability during product storage. Strain survival in the product will depend on many factors such as pH, presence of preservatives, and even the occurrence of potential microbial growth inhibitors. Some common additives used in the food and dairy industry are salts, sugars, organic acids etc. The effect of additives on the growth of lactic acid starter and probiotic bacteria have not been extensively studied. Beyond the additives used, some products of the lactic acid starter metabolism, such as mainly lactic acid, could be associated with the loss of viability of added probiotic bacteria.

One important factor for *L. acidophilus* strain to produce beneficial effects on host intestinal tract is the number of viable cells. Many inherent and environmental factors of the host can eliminate or reduce considerably the number of *L. acidophilus* cells in the intestine. Many of the viable cells can be injured and thus will be killed by the low pH in stomach and bile salts in the intestine. Acid and bile resistance are important characteristics to be considered when selecting a culture, which should be used as a dietary adjunct. In order to exert both its therapeutic and nutritional effects in the gastrointestinal tract, *L. acidophilus* has to be viable and able to adhere to intestinal cells. The first barrier met by these microorganisms ingested with food is the low pH present in the stomach. If they survive gastric digestion they become strong candidates for the interaction with the gastrointestinal microflora. Although *L. acidophilus* tolerates acidity, a rapid decrease in their numbers has been observed under acidic conditions both *in vitro* and *in vivo*. After passing through the stomach barrier, the microorganisms reach the duodenum, where the secretion of bile salt takes place. Thus, resistance to bile salts is also an important factor to guarantee the establishment and growth of microorganisms used as dietary adjuncts within the intestinal tract in order to provide real health benefits.

We need to encourage the growth of probiotic flora in our intestines. It must be abundant for proper health. Just as roots are the foundation for the growth of plants, our intestinal tract is the root at the foundation of the health of our whole body.

Encapsulation methods have been applied to increase the survival and delivery of bacterial cultures. Several methods have been developed for the encapsulation of bacteria for use in fermentation, as well as for incorporating into products. Encapsulation helps in segregating the bacterial cells from the adverse environment, for example, of the product, of the

gastrointestinal tract, thus potentially reducing cells loss. The encapsulation process and the capsule material influence the viability of bacteria, under different conditions as compared to when bacteria were in the non encapsulated state [Rao et al., 1989; Kebary, et al., 1998]. In regard to the utility of microencapsulation there are a few publications dealing with the effect of different capsule materials especially polysaccharides (e.g., alginate). The present study used many different encapsulating materials and their effect for protecting and stabilizing of *L. acidophilus* survival in some adverse environments and conditions were evaluated.

Generally, probiotic bacteria have to overcome barriers: during manufacturing process, storage time and conditions, carrier food conditions, as well as in the digestion tract, which influence their physiological activity having therefore limited effects on their functionality. To be significantly beneficial to consumers, probiotic cultures require some preservation or stabilization treatments in order that the cells retain their viability and to overcome the mentioned barriers. The objectives of this study are to evaluate: the viability and physiological changes of *L. acidophilus* after the micoencapsulation procedure and over a period of storage time; influence of heat treatments; viability and stability to some simulated conditions in the carrier foods (including the presence of salt, sugar, and organic acids); and the resistance of microencapsulated *L. acidophilus* to some simulated conditions of the human intestinal tract (including gastric juice and bile salts); which could be improved and protected by microencapsulation procedure.

4. Materials and Methods

4.1 Materials

4.1.1 Bacterial culture

The bacterial strain used in this study was pure freeze dried culture of *Lactobacillus acidophilus* La-5 (from Hansen).

4.1.2 Chemicals

Tab. 4.1: Chemicals applied within the experiments

Acetic acid	Fluka	Maltodextrin	ICN Biochemicals
Agar	Poeper	Manganese sulphate	Merck
Alginate	Fluka	Mannitol	ICN Biochemicals
API CHL 50 kit	BioMerieux	Meat extract	Fluka
Bile salts	Sigma	Methylene blue	Merck
Carrageenan	Aldrich	MRS broth	Merck
Citric acid	Fluka	Pectin	Fluka
Crystal violet	Merck	Peptone	Fluka
D-Glucose	Fluka	Chloramphenicol	Fluka
Dipotassium hydrogen phosphate	Merck	Safranin	Merck
Ethanol	Commercial	Skim milk powder	Oxoid
Gelatin	Fluka	Sodium acetate	Riedel-de Haen
Glycerol	Merck	Sodium chloride	Fluka
Gram`s iodine	Merck	Soy milk	Rettenmaier
Gum arabic	Rettenmaier	Soy protein	Sigma
Gum guar	Sigma	Soybean flour	Sigma
Gum karaya	Sigma	Sucrose	Merck
Hydrochloric acid	Merck	Tomato juice	Reform Haus
Hydrogen peroxide	Fluka	Triammonium citrate	Fluka
Lactic acid	Fluka	Tryptone	Oxoid
Lecithin	Fluka	Tween 20	Fluka
Magnesium sulphate	Merck	Tween 80	Fluka
Malachite green	Fluka	Whey protein	MD Food ingredients
Malt extract	Extraktchemie	Yeast extract	Fluka

4.1.3 Media

Plate count agar (Tryptone Glucose Yeast Extract Agar) [Harrigan, 1998**]**
(A non-selective medium for general viable counts of bacteria in food)

Tryptone	5.0 g
Yeast extract	2.5 g
D-Glucose	1.0 g
Agar	15.0 g
Distilled water	1 litre

MRS broth [de Man, Rogosa and Sharpe, 1960**]**
(for the culturte of lactobacilli)

Peptone	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
Tween 80	1.0 g
Dipotassium hydrogen phosphate	2.0 g
Sodium acetate	5.0 g
Triammonium citrate	2.0 g
Magnesium sulphate, hydrated (MgSO4.7H2O)	0.2 g
Manganese sulphate, hydrated (MnSO4.4H2O)	0.05 g
Distilled water	1 litre

MRS Agar
(for the culture of lactobacilli)
MRS broth plus agar.

Malt extract agar [Harrigan, 1998**]**
(For the culture of yeasts and molds)

Malt extract	30.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1 litre

4.1.4 Equipements and Instruments

- **Spray dryer**

Spray dryer Nubilosa was used (Fig. 4.1).

Fig. 4.1: Spray dryer of Institute of Food Technology, Bonn University

Tab. 4.2: Spray dryer parameters

Parameter	Value
Inlet-temperature	170°C
Outlet-temperature	55°C
Air pressure	4.5 bar
Nozzle diameter	0.8 mm
Feed concentration	Mostly 6-9% (dry weight)
Drying yield	35-50%

- **Autoclave:**

Varioklav: H+P Labortechnik GmbH
Sanoclav: M-ECZ Wolf

- **Water bath:**

Julabo PC

- **Microscope:**

Raster electronic microscope, modell XL 20

- **pH-meter:**

Knick pH-meter 765 Calimatic

- **Centrifuge:**

Heraeus 17RS SEPATECH

- **Balance:**

Sartorius

- **Spectrophotometer:**

PERKIN – ELMER Lambada 2 UV/VIS spectrophotometer

- **Incubator:**

Member
GFL 3033

- **Laminar Flow:**

GELAIRE Flow Laboratories HF 72

- **Mixer:**

IKAMAG RCT
IKA Labortechnik VF2

- **Homogenizer:**

ULTRA TURRAX, IKA T18 basic

- **Viscosimeter:**

Bohlin visco 88 BV

4.2 Methods

4.2.1 Culture activation and maintenance

L. acidophilus strain was rehydrated in MRS broth and incubated for 24 hours at 37°C. Cells were then cultured in the same conditions for three successive transfers in MRS broth at 37°C for 20-24 hours. It was then properly activated and served as the inoculum. The culture was grown for 20-24 hours at 37°C and was transferred three times weekly using 1% inoculum. *L. acidophilus* culture was held at 4°C between transfers.

4.2.2 Estimation of growth kinetics

For growth analysis, an overnight culture was inoculated (1%) into MRS broth (100 ml) and incubated aerobically at 37°C. Samples were collected hourly for pH and optical density (OD_{660}) determinations.

4.2.3 Microencapsulation procedure

The microencapsulation of *L. acidophilus* in different capsule materials (Tab. 4.3) was carried out by spray drying following the methodology summarized in Fig. 4.2. The culture was subcultured in MRS perior to use. The cells were harvested and washed once by centrifugation at 10.000 rpm for 10 minutes at 4 °C and mixed with sterilized capsule material solutions then spray dried and the resulted powder storaged at 5°C. Viable cell counts in the

microencapsulation feed were determined before spray drying (recalculated) and immediately after spray drying (0 time) then weekly during storage.

Example for the recalculation method, which used to equipoise the viable cell count numbers in microencapsulation feed (before spray drying cfu/ml) to powder (after spray drying as cfu/g).

8 % gum arabic solution (as microencapsulation feed)

1. 1 ml microencapsulation feed (8% gum arabic in water) has → 7.2×10^9 cfu
2. 100 ml microencapsulation feed has ⟶ 8g gum arabic
3. 1 ml microencapsulation feed has ⟶ x g gum arabic
4. x has ⟶ 8/100 = 0.08 g gum arabic
5. 1 ml microencapsulation feed has → 0.08 g and has → 7.2×10^9 cfu
6. 0.08 g has → 7.2×10^9 cfu
7. 1 g has → $7.2 \times 10^9 / 0.08 = 9 \times 10^9$ cfu.

Tab. 4.3: Comprises all used capsule material mixtures

Gum arabic*

Gum arabic (8%)
Gum arabic (8%) + Mannitol (1%)
Gum arabic (7%) + Pectin (1%)
Gum arabic (7%) + Maltodextrin (1%)
Gum arabic (7%) + Skim milk (1%)
Gum arabic (7%) + Gum guar (0.5%)
Gum arabic (7%) + Gum karaya (0.5%)
Gum arabic (7%) + Carrageenan (0.5%)
Gum arabic (7%) + Alginate (1%)
Gum arabic (7%) + Glycerol (1%)
Gum arabic (8%) + Tomato juice (5.8/3%)
Gum arabic (7%) + Tween 20 (1%)
Gum arabic (7%) + Lecithine (1%)
Gum arabic (5%) + Soybean flour (3%)
Gum arabic (4%) + Gelatin (4%)

Gum arabic + Proteins*

Gum arabic (4%) + Whey protein (4%)
Gum arabic (4%) + Soy protein (4%)
Gum arabic (5%) + Whey protein (2%) + Soy protein (2%)
Gum arabic (5%) + Whey protein (2%) + Alginate (0.5%)
Gum arabic (5%) + Whey protein (3%) + Pectin (0.5%)
Gum arabic (4%) + Soy protein (4%) + Alginate (0.5%)
Gum arabic (4%) + Soy protein (4%) + Pectin (0.5%)

Gum arabic + Soy milk*

Gum arabic (5%) + Soy milk (4%)
Gum arabic (5%) + Soy milk (3%) + Alginate (0.5%)
Gum arabic (4%) + Soy milk (4%) + Pectin (0.5%)

Whey protein*

Whey protein (5%)
Whey protein (5%) + Aginate (1%)
Whey protein (5%) + Pectin (1%)

Soy protein*

Soy protein (7%)
Soy protein (6%) + Alginate (1%)
Soy protein (6%) + Pectin (1%)

Soy milk*

Soy milk (4%)
Soy milk (4%) + Alginate (0.5%)
Soy milk (4%) + Pectin (0.5%)

Gelatin*

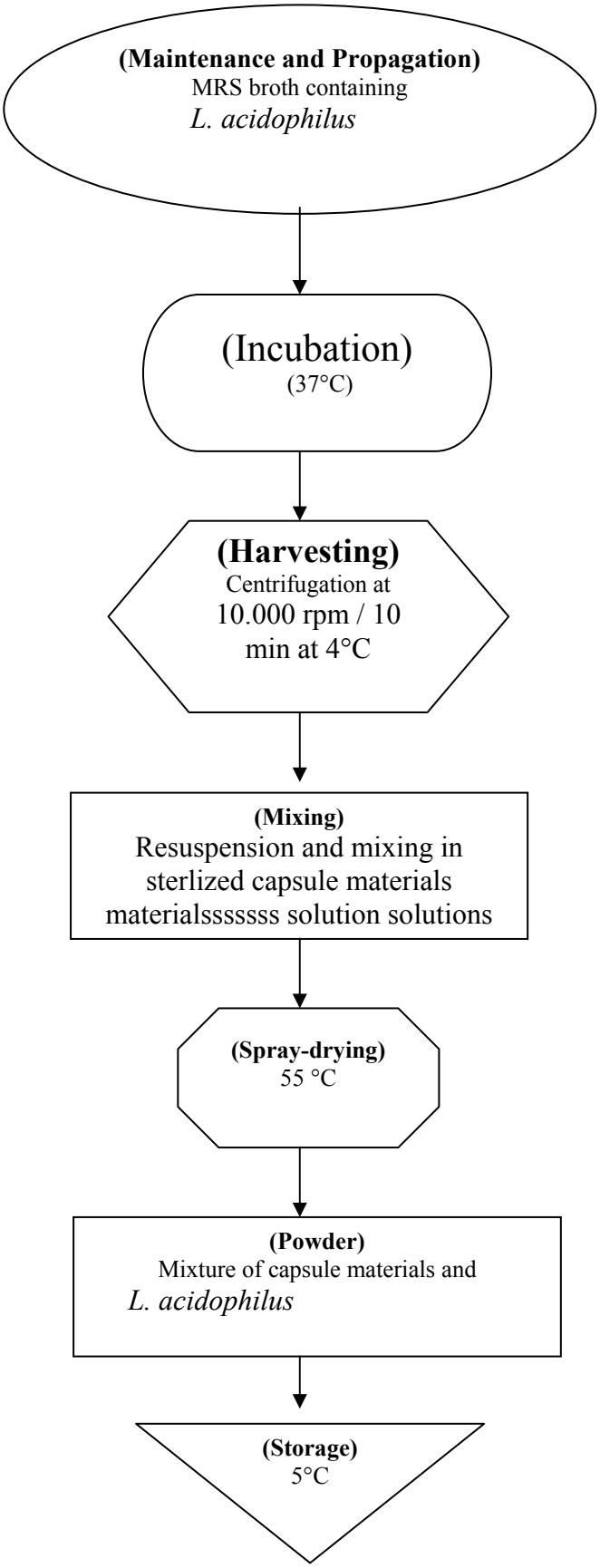
Gelatin (6%)
Gelatin (6%) + Mannitol (1%)
Gelatin (6%) + Pectin (1%)
Gelatin (6%) + Maltodextrin (1%)
Gelatin (6%) + Skim milk (1%)
Gelatin (6%) + Gum guar (0.3%)
Gelatin (6%) + Gum karaya (0.5%)
Gelatin (6%) + Carrageenan (0.4%)
Gelatin (6%) + Alginate (1%)
Gelatin (6%) + Glycerol (1%)
Gelatin (6%) + Tomato juice (5.8/3%)
Gelatin (6%) + Tween 20 (1%)
Gelatin (6%) + Lecithine (1%)
Gelatin (5%) + Soybean (3%)
Gelatin (4%) + Soy milk (3%)

Gelatin + Proteins*

Gelatin (5%) + Whey protein (3%)
Gelatin (5%) + Soy protein (4%)

* Main capsule material(s)

Fig. 4.2: Flow sheet of the microencapsulation procedure of *L. acidophilus*



4.2.4 Evaluation of cultured powder purity and identity

4.2.4.1 Methylene blue staining

Methylene blue is a homologue of Toluidine Blue O. This stain is used to visualize intracellular metachromatic granules.

Procedure:

1. Smears are fixed and allowed to cool.
2. Stain with the methylene blue solution two to seven minutes.
3. Wash slide and blot dry.

By this method, the intracellular metachromatic granules stain a ruby-red to black color; with the remainder of the cell staining a pale blue color.

4.2.4.2 Gram staining

The Gram staining method, is one of the most important staining techniques in microbiology. It is almost always the first test performed for the idintification of bacteria. The primary stain of the Gram`s method is crystal violet. The microorganisms that retain the crystal violet-iodine complex appear purple brown under microscopic examination. These microorganisms that are stained by the Gram`s method are commonly classified as Gram positive. Others that are not stained by crystal violet are referred to as Gram negative, and appear red.

Procedure:

1. Cover the smear with crystal violet. Wait 20 seconds.
2. Wash with water until the water runs clear.
3. Add gram`s iodine; wait 1 minute.
4. Decolorize with Gram`s decolorizer.
5. Rinse with water to stop decolorization.
6. Add safranin. Wait 20 seconds.
7. Rinse with water.
8. Blot with bibulous paper and observe under oil immersion.

4.2.4.3 Spore staining

The spore stain, is a differential stain used to detect the presence or location of spores in bacterial cells. Only few genera produce spores such as *Bacillus* and *Clostridium.*

Procedure:

1. Prepare and fix a smear.
2. Cover the smear with a blotting paper strip, and then saturate the strip with malachite green staining solution.
3. Heat the slide gently until it steams; allow solution to remain 2 or 3 minutes (more solution may be added to prevent drying)
4. Wash slide with tap water, and then apply safranin solution for 30 seconds.
5. Wash slide with tap water, drain, blot dry, and examine using the oil immersion objective. The spores stain green and the vegetative cells stain red.

4.2.4.4 Catalase test

The catalase test determines whether bacteria produce "catalase", an enzyme that breaks down hydrogen peroxide into water and oxygen gas, which appears as a bubbly product (Fig. 4.3).

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Procedure:

1. Dip a capillary tube into 3% H_2O_2.
2. Touch a colony.
3. Observe the tube for bubble indicating a positive reaction.

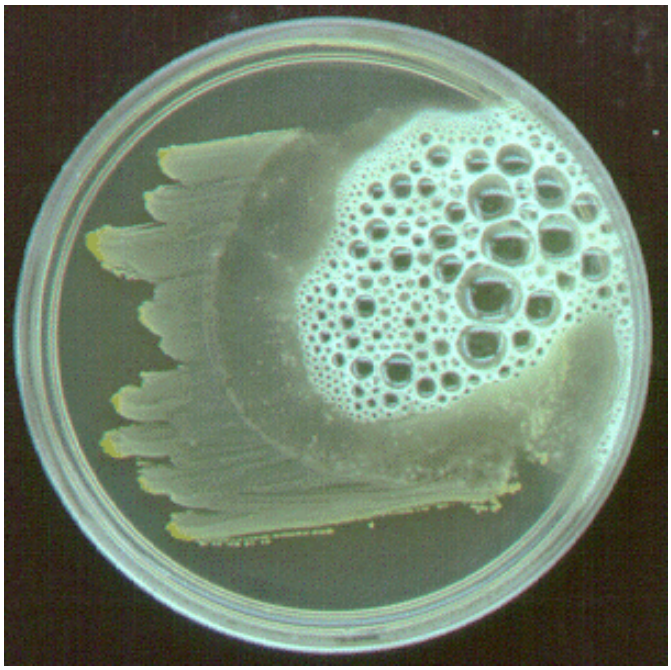


Fig. 4.3: Breakdown of hydrogen peroxide into water and oxygen by the enzyme catalase causes foaming as the oxygen bubblesd through the water.

4.2.4.5 API test

API 50 CHL medium is a ready-to-use medium that enables the fermentation of 49 carbohydrates on the API 50 CHL strip to be studied. A suspension is made in the medium with the microorganism to be tested and each tube of the strip is inoculated. During incubation, carbohydrates are fermented to acids which produce a decrease in the pH, detected by the color change of the indicator. The results make up the biochemical profile of the strain and are used in its identification or typing.

Procedure:

1. Check the purity of the strain.
2. Culture it on MRS agar medium, and incubate anaerobically for 24 hours at 37°C.
3. Pick up all the bacteria of the culture with the help of a swab in order to make a heavy suspension (in 2 ml sterilized distilled water) (S1).
4. In 5 ml sterilized distilled water (S2), obtain a suspention with a turbidity equivalent to 2 McFarland (reference medium) by transferring a certain number of drops: record this number (n).
5. Inoculate the API 50 CHL medium by adding twice this number of drops of suspension S2 (ie 2n). Homogenize.
6. Fill the tubes with the inoculated API 50 CHL medium, and overlay all of the tests with mineral oil.
7. Incubate aerobically at 37°C, for 48 hours.
8. All the tests are read after both 24 and 48 hours of incubation. A positive test corresponds to acidification revealed by the bromocresol purple indicator contained in the medium changing to yellow. For the Esculin test (tube No. 25), a change from purple to black is observed. Record the results and compare it with the Manufacturer`s recommended identification table.

Tab. 4.4: Composition of the media for API test

Suspension medium 2 or 5 ml	Demineralized water	
API 50 CHL medium 10 ml	Polypeptone	10 g
	Yeast extract	5 g
	Tween 80	1 ml
	Dipotasium phosphate	2 g
	Sodium acetate $3H_2O$	5 g
	Diammonium citrate	2 g
	Magnesium sulphate $7H_2O$	0.20 g
	Manganese sulphate 4 H_2O	0.05 g
	Bromocresol Purple	0.17 g
	Demineralized water to make	1000 ml

Tab. 4.5: Composition of the strip of API test

Strip No. 1	Strip No. 2	Strip No. 3	Strip No. 4	Strip No. 5
0 Control	10 Galactose	20 α-Methyl-D-Mannoside	30 Melibiose	40 Turanose
1 Glycerol	11 Glucose	21 α-Methyl-D-Glucoside	31 Sucrose	41 D-Lyxose
2 Erythritol	12 Fructose	22 N-Acetyl-Glucosamine	32 Trehalose	42 D-Tagatose
3 D-Arabinose	13 Mannose	23 Amygdalin	33 Inulin	43 D-Fucose
4 L- Arabinose	14 Sorbose	24 Arbutin	34 Melezitose	44 L- Fucose
5 Ribose	15 Rhamnose	25 Esculin	35 Raffinose	45 D-Arabitol
6 D-Xylose	16 Dulcitol	26 Salicin	36 Starch	46 L- Arabitol
7 L-Xylose	17 Inositol	27 Celiobiose	37 Glycogen	47 Gluconate
8 Adonitol	18 Mannitol	28 Maltose	38 Xylitol	48 2-Keto- Gluconate
9 ß-Methyl-D-Xyloside	19 Sorbitol	29 Lactose	39 Gentibiose	49 5-Keto- Gluconate

4.2.4.6 Total counts on MRS agar

Viable cell samples (1g) were serialy diluted 10^{-1} to 10^{-8} in peptone water (0.1%) and 0.1 ml of the samples from the appropriate dilutions were spread plated onto MRS agar. Viable cells count, performed in duplicate, was determined after 48-72 hours incubation at 37°C. For every sample two separate dilutions were enumerated and averaged for the viable cell count.

4.2.4.7 Total bacterial counts on plate count agar

Viable cell samples (1g) were serialy diluted 10^{-1} to 10^{-8} in peptone water (0.1%) and 0.1 ml of the samples from the appropriate dilutions were spread plated onto plate count agar. Viable cells count, performed in duplicate, was determined after 48-72 hours incubation at 30°C. For every sample two separate dilutions were enumerated and averaged for the viable cell count.

4.2.4.8 Yeasts and molds count

Viable cell samples (1g) were serialy diluted 10^{-1} to 10^{-8} in peptone water (0.1%) and 0.1 ml of the samples from the appropriate dilutions were spread plated onto malt extract agar. Viable cells count, performed in duplicate, was determined after 5 days incubation at 25°C. For every sample two separate dilutions were enumerated and averaged for the viable cell count.

4.2.5 Viability and stability tests

4.2.5.1 Stability to some technological conditions:

4.2.5.1.1 Viability of free and microencapsulated *L. acidophilus* at high temperatures

Free *L. acidophilus* (10%) in sterile distilled water was exposed to different temperatures 37, 40, 45, 50, 55, and 60°C/30 minutes in water bath to determine viability at each temperature.10 % from the powder after the microencapsulation procedure by spray drying added to test tubes with sterile distilled water and exposed to 60°C/30 min, 63°C/30 min and 65°C/15 and 65°C/30 min. in water bath. Enumeration of *L. acidophilus* after each treatment was accomplished using MRS agar. The plates were inverted and incubated at 37°C for 72 hours. Following incubation, colony forming units were counted and recorded.

4.2.5.2 Stability to some deliberately conditions in carrier foods:

4.2.5.2.1 Viability of free and microencapsulated *L. acidophilus* in high sucrose concentrations

To determine the survival of microencapsulated *L. acidophilus* with high sucrose concentrations, sterile sucrose solutions at concentrations of 5.0, 10.0, 15.0, 20.0 and 25.0% were added to test tubes containing powder (10%) containing approximately 10^8–10^9 cfu/g *L. acidophilus.* The samples were enumerated immediately (0 time) and then stored at 5°C for weeks. The viable counts of microencapsulated *L. acidophilus* were determined at weekly intervals by sampling the contents of individual tubes. Free cells culture was subjected to the same conditions as powder to serve as a control.

4.2.5.2.2 Viability of free and microencapsulated *L. acidophilus* in high NaCl concentrations

To evaluate the survival of microencapsulated *L. acidophilus* with high NaCl solutions, sterile sodium chloride solutions at concentrations of 1.0, 2.0, 3.0 and 5.0% were added to test tubes containing powder (10%) containing approximately 10^8–10^9 cfu/g *L. acidophilus* and then plated immediately (time 0) on MRS agar. Serial dilutions were made using distilled water and 0.1% peptone. The NaCl solutions containing *L. acidophilus* were then stored at 5°C for weeks. The viable counts of microencapsulated *L. acidophilus* were determined weekly by sampling the contents of individual tubes. Free cells culture was subjected to the same conditions as powder to serve as a control.

4.2.5.2.3 Viability of free and microencapsulated *L. acidophilus* under acidic conditions

To determine the survival of microencapsulated *L. acidophilus* cells under acidic conditions. Solutions of lactic, acetic and citric acids in double-distilled water were adjusted to pH levels at 3.0, 4.0 and 5.0. Sterile double-distilled water (pH 6.3-6.5) served as the control. The solutions were prepared in 100 ml volumes, sterilized, and stored at room temperature until needed.

Stored solutions of each pH were thoroughly mixed and transferred into sterile test tubes containing microencapsulated *L. acidophilus* (10%) containing approximately 10^8–10^9 cfu/g. *L. acidophilus* were then plated immediately (time 0) on MRS agar. Serial dilutions were made using distilled water and 0.1% peptone. The pH solutions containing *L. acidophilus* were then stored at 5°C for weeks. The viable counts of microencapsulated *L. acidophilus* were determined at weekly intervals by sampling the contents of individual tubes. Free cells culture was subjected to the same conditions as powder to serve as a control.

4.2.5.3 Stability to some simulated conditions of human intestinal tract:

4.2.5.3.1 Survival of free and microencapsulated *L. acidophilus* in simulated gastric juice

Preparation of solutions to stimulate pH of human stomach:
Solutions of 37% HCl in double-distilled water were adjusted to pH levels of 1.0, 2.0, and 3.0. Sterile double-distilled water (pH 6.3-6.5) served as the control (Fig. 4.3). The solutions were prepared in 100 ml volumes, sterilized, and stored at room temperature until needed.

Enumeration of *L. acidophilus* in the pH solutions:

Stored solutions of each pH were thoroughly mixed and transferred into sterile test tubes containing microencapsulated *L. acidophilus* (10%) containing approximately 10^8–10^9 cfu/g. *L. acidophilus* were then plated immediately (time 0) on MRS agar. Serial dilutions were made using distilled water and 0.1 peptone. The pH solutions containing *L. acidophilus* were then incubated at 37°C followed by intermitten plating after 1.0, 2.0, 3.0 and 4.0 hours to simulate survival of *L. acidophilus* under pH conditions common to the human stomach. Enumeration of *L. acidophilus* after each storage intervals were accomplished using MRS agar. The plates were inverted and incubated at 37°C for 72 hours. Following incubation, colony forming units were counted and recorded. Free cells culture was subjected to the same conditions as the powder as a control. The experiment for both microencapsulated and free cells was with two replicates.

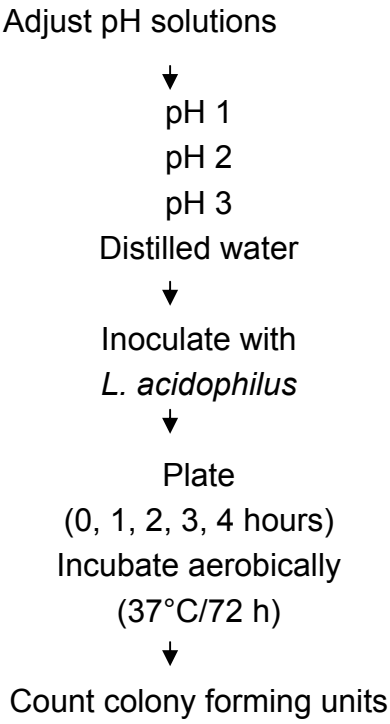


Fig. 4.3: Method for determining the effect of simulated stomach pH on *L. acidophilus*

4.2.5.3.2 Survival of free and microencapsulated *L. acidophilus* in bile

Preparation of bile solutions:

To evaluate the survival of *L. acidophilus* in bile salts, 1.0, 2.0, and 4.0 % concentrations of bile salts in distilled water were prepared. Distilled water without bile salts was used as the control. All solutions were sterilized at 121°C for 15 minutes. After sterilization, bile solutions and distilled water (control) were stored at room temperature until needed.

Enumeration of *L. acidophilus* in bile:

Stored solutions of each bile salts concentration were transferred into sterile test tubes containing microencapsulated *L. acidophilus* (10%). The mixtures were then plated with MRS agar for initial counts (0 times). After plating for initial counts, mixtures were incubated for four hours at 37°C. *L. acidophilus* were then enumerated again to test for survival rates after 4 hours incubation, by intermittent plating after 1.0, 2.0, 3.0, and 4.0 hours. Free cells culture was subjected to the same conditions as the powder as a control. Following incubation, colony forming units were counted and recorded. Two replications were conducted with each concentration.

5 Results

5.1 Growth kinetics of *L. acidophilus*

L. acidophilus was cultivated in MRS broth to determine the times taken to reach different growth phases. For that, pH value and O.D were estimated. It took approximately 3 hours to reach the log phase and 12 hours to establish the stationary phase. When growth of *L. acidophilus* became stationary, as indicated in Fig. 5.1, cells were harvested, mixed with capsule material solutions and spray dried.

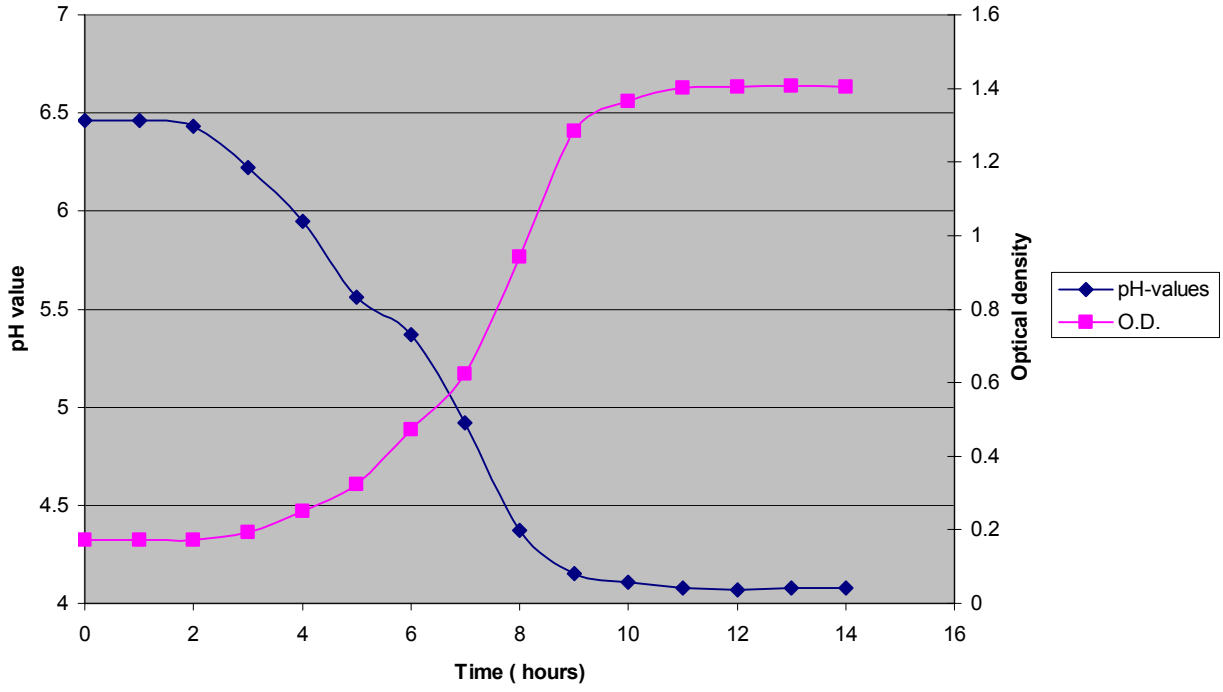


Fig. 5.1: Growth kinetics of *L. acidophilus*

5.2 Survival of *L. acidophilus* after spray drying with different capsule materials

It is evident that, in general, the number of survivors decreases mostly after spray drying for all capsule materials. Figs. 5.2-5.13 show the survival of L. acidophilus after spray drying with different carriers as compared to the numbers before spray drying.

The numbers for, gum arabic (G.A), G.A+maltodextrin, G.A+skim milk, G.A+gum karaya, G.A+alginate, G.A+whey protein, G.A+soy milk, soy protein, whey protein+pectin, G.A+ soy milk+pectin, G.A+whey protein+pectin, G.A+soy milk+alginate, gelatin+skim milk,

gelatin+alginate, and gelatin+soyprotein decreased about less than 1 log cycle after spray drying.

Decreases of about 1 log cycle were also observed after spray drying with, G.A+mannitol, G.A+ carrageenan, G.A+glycerol, G.A+tomato juice, G.A+tween 20, G.A+lecithin, G.A+ soybean flour, G.A+soy protein, soy protein+alginate, whey protein+alginate, soy milk, whey protein, soy protein+pectin, soy milk+pectin, G.A+soy protein+pectin, G.A+whey protein+alginate, G.A+soy protein+alginate, gelatin+mannitol, gelatin+pectin, G+lecithin, gelatin+tween 20, gelatin+tomato juice, gelatin+soybean flour, gelatin+soy milk, gelatin+whey protein, and G.A.+gelatin

Further decreases in number of about 2 log cycle for, G.A+pectin, soy milk+alginate, gelatin, gelatin+maltodextrin, and gelatin+glycerol were observed after spray drying.

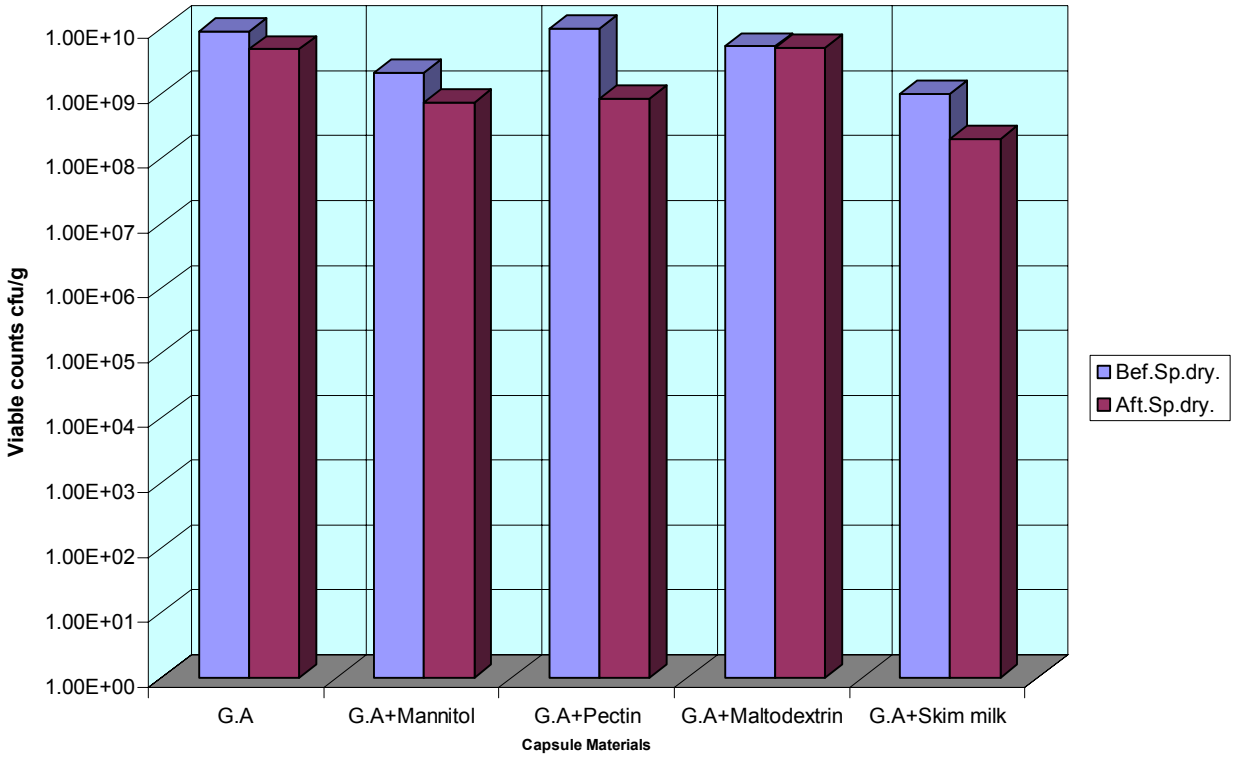


Fig. 5.2: Viable counts of *L. acidophilus* before and after spray drying using gum arabic as a main capsule material

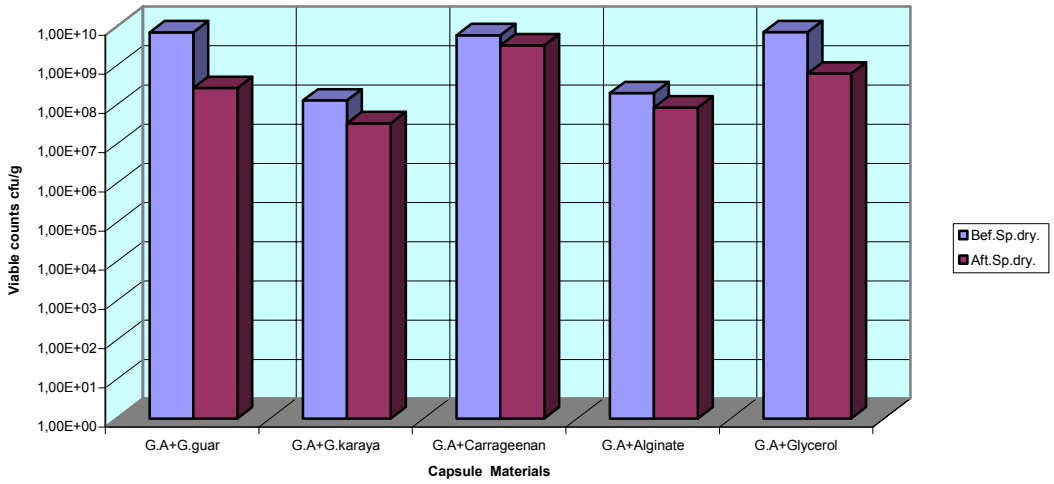


Fig. 5.3: Viable counts of *L. acidophilus* before and after spray drying using gum arabic as a main capsule material

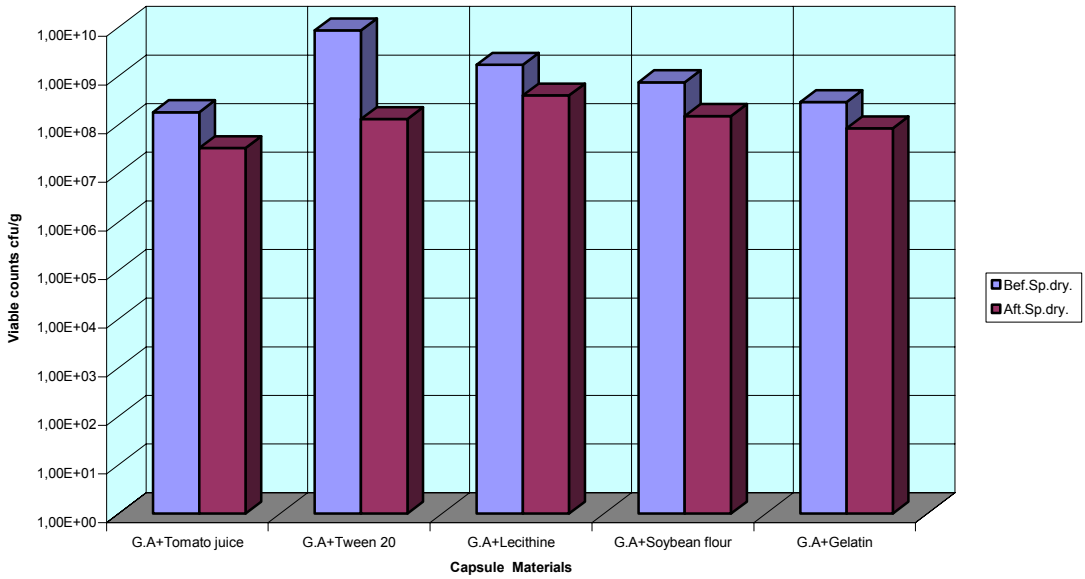


Fig. 5.4: Viable counts of *L. acidophilus* before and after spray drying using gum arabic as a main capsule material

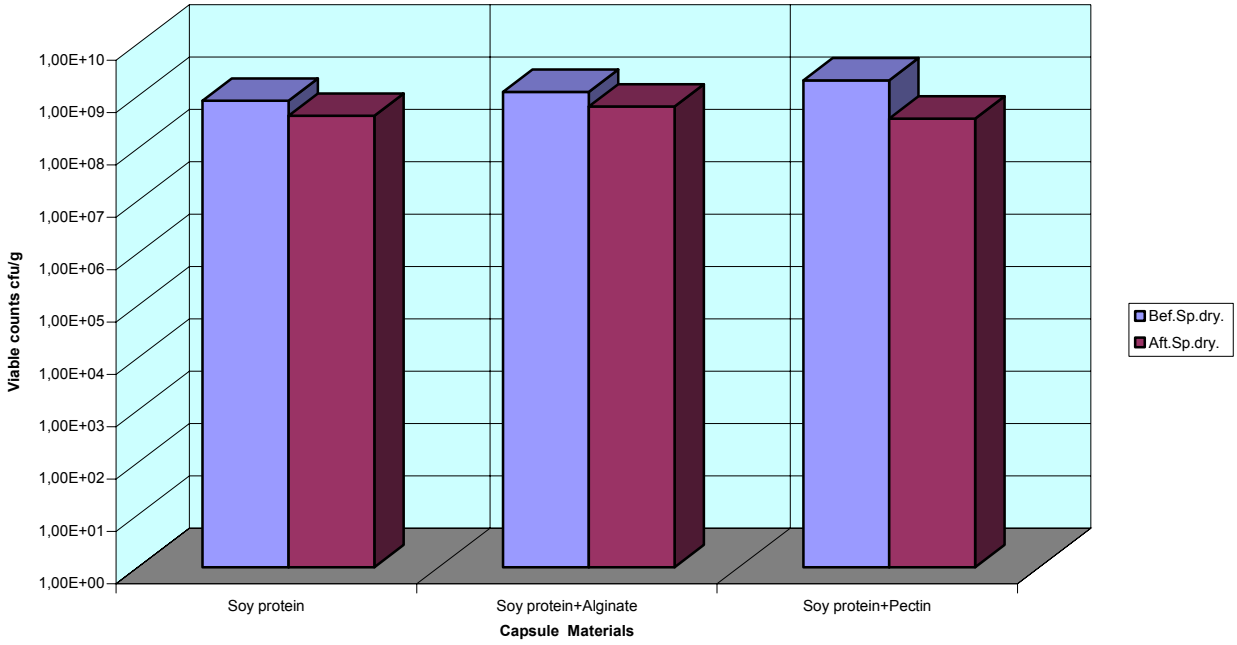


Fig. 5.5: Viable counts of *L. acidophilus* before and after spray drying using soy protein as a main capsule material

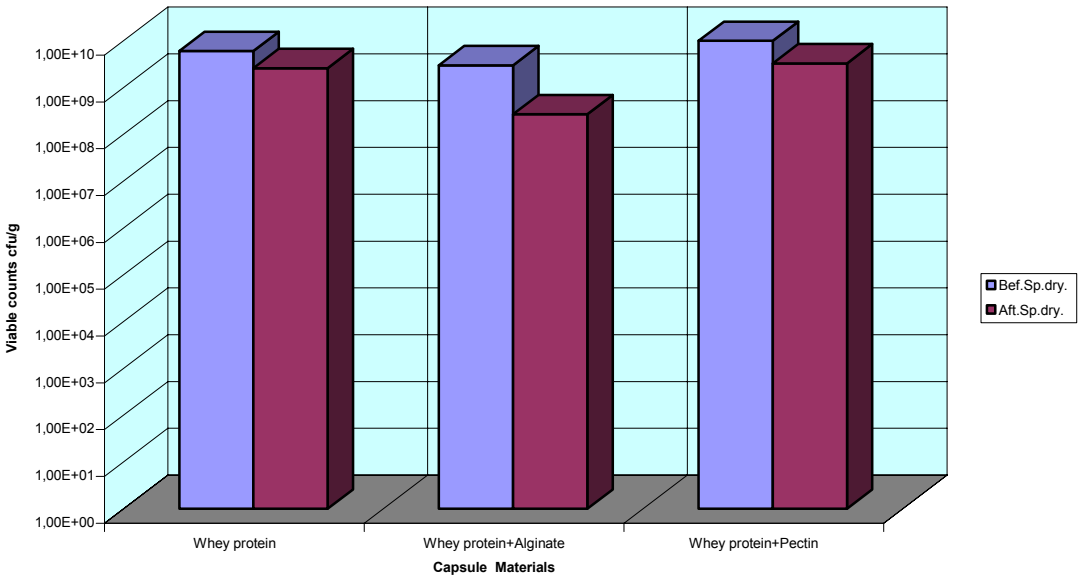


Fig. 5.6: Viable counts of *L. acidophilus* before and after spray drying using whey protin as a main capsule material

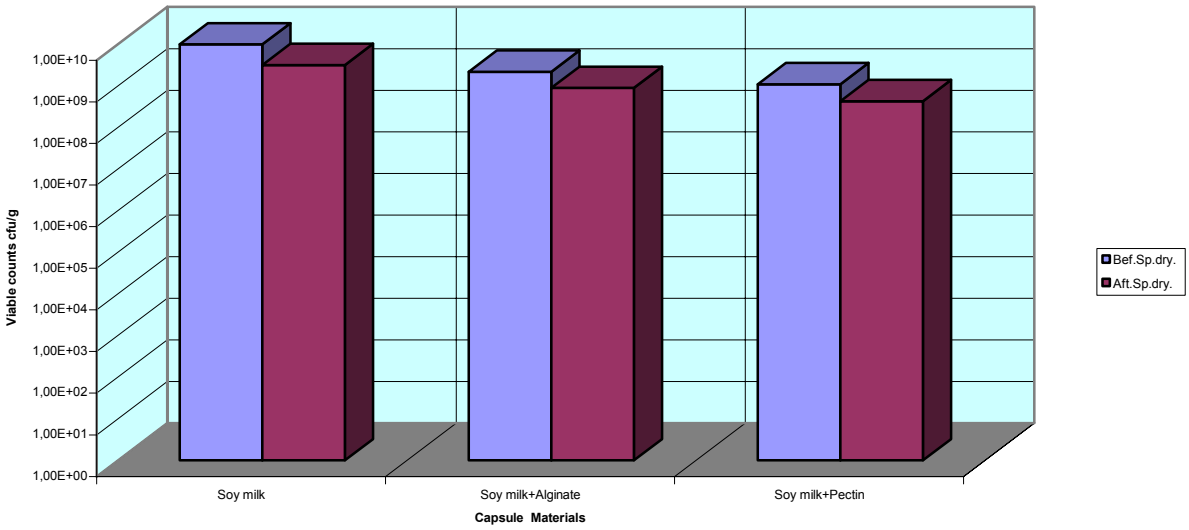


Fig. 5.7: Viable counts of *L. acidophilus* before and after spray drying using soy milk as a main capsule material

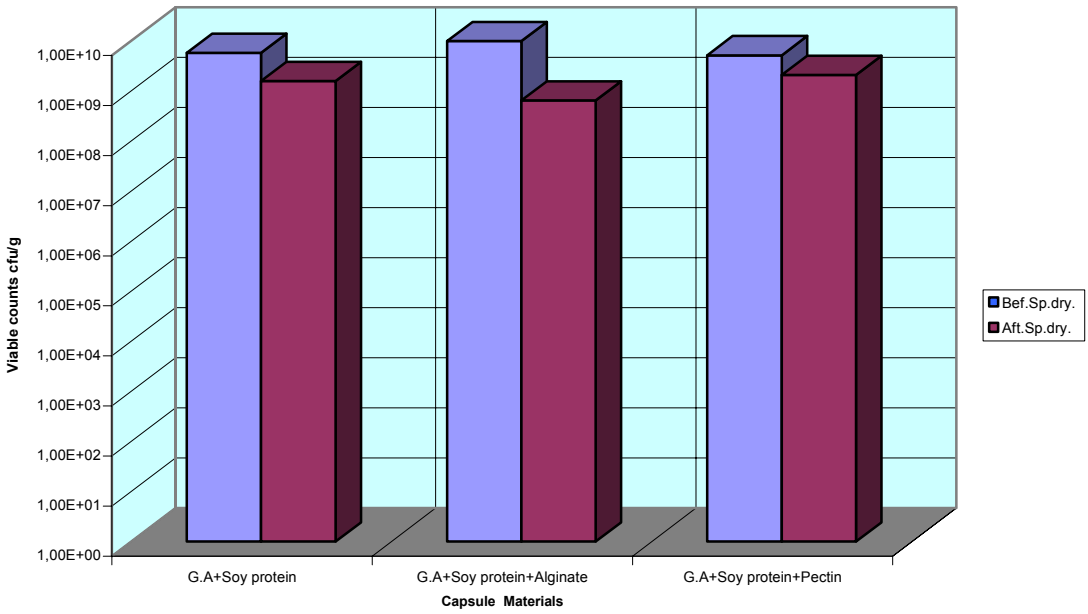


Fig. 5.8: Viable counts of *L. acidophilus* before and after spray drying using gum arabic+ soy protein as main capsule materials

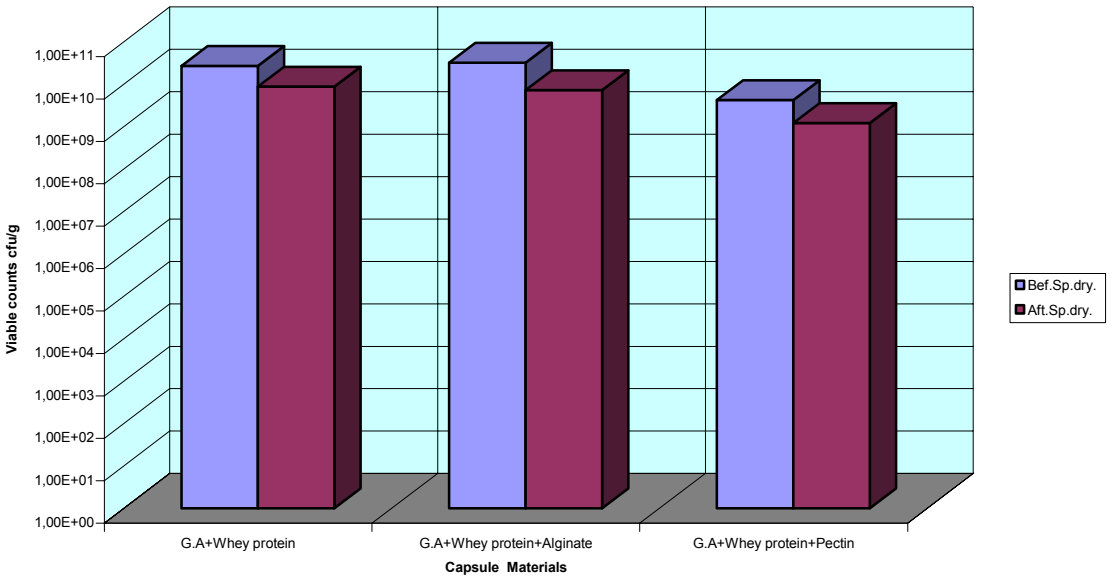


Fig. 5.9: Viable counts of *L. acidophilus* before and after spray drying using gum arabic+ whey protein as main capsule materials

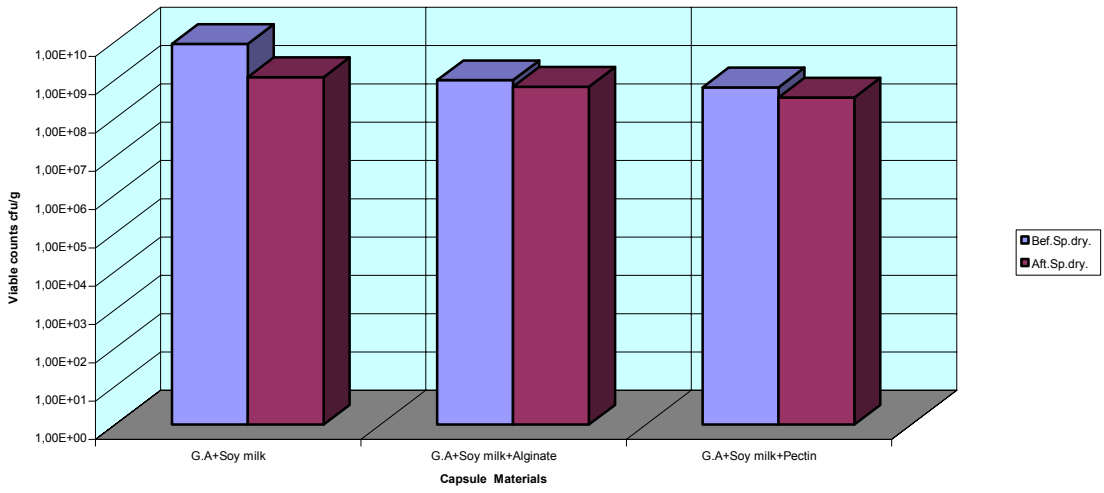


Fig. 5.10: Viable counts of *L. acidophilus* before and after spray drying using gum arabic+soy milk as main capsule materials

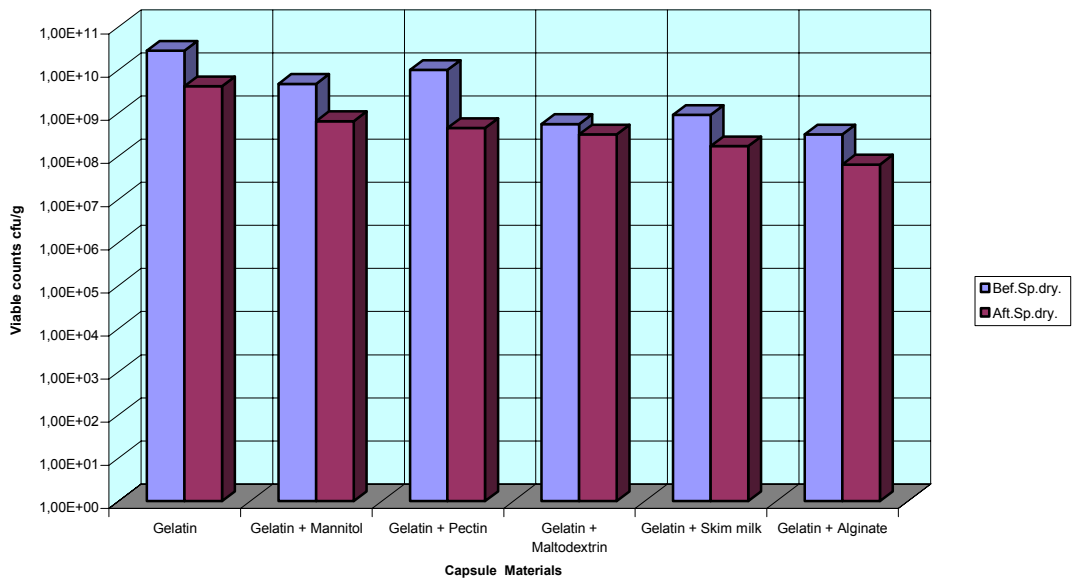


Fig. 5.11: Viable counts of *L. acidophilus* before and after spray drying using gelatin as a main capsule material

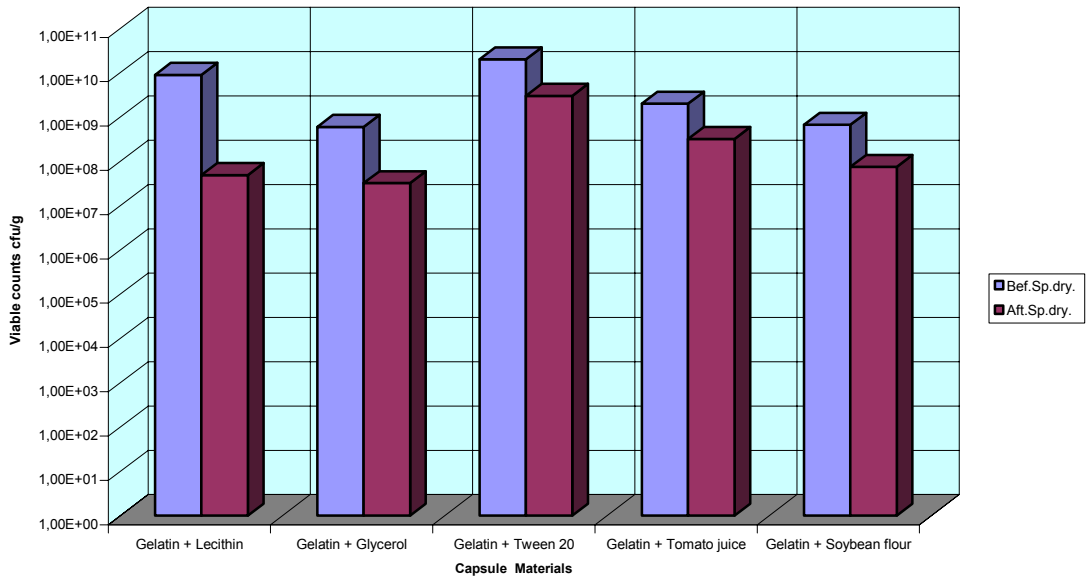


Fig. 5.12: Viable counts of *L. acidophilus* before and after spray drying using gelatin as a main capsule material

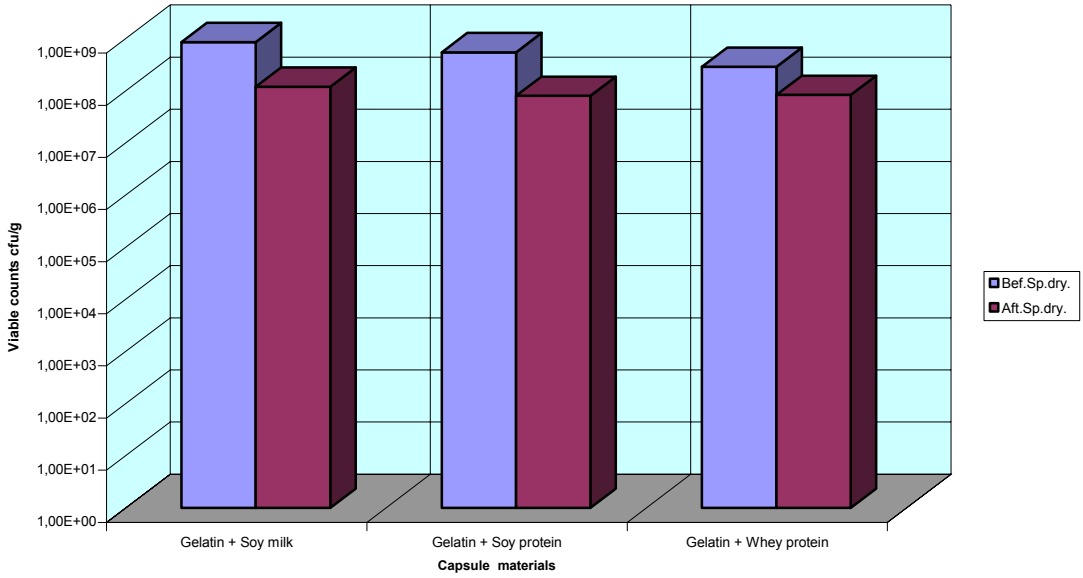


Fig. 5.13: Viable counts of *L. acidophilus* before and after spray drying using gelatin+ proteins or soy milk as main capsule materials

5.3 Evaluation of cultured microencapsulated powder purity and identity

The purity and identity of the cultured microencapsulated powder after spray drying was proved by: Api test; plating and counting on MRS agar (for *lactobacilli*), plate count agar (for general viable counts of bacteria), and malt extract agar (for yeasts and molds). The data given in Tab. 5.1 show the morphological and biochemical characteristics of the applied strain. The tests revealed that the used strain is rod like, Gram (+), non-sporeforming, catalase (-), and grows on MRS agar. This strain could utilize and ferment the following carbohydrates: L-Arabinose, Galactose, D-Glucose, D-Fructose, Mannitol, N-Acetylglucosamine, Amygdalin, Arbutin, Esculin, Salicin, Cellobiose, Maltose, Lactose, Melibiose, Saccharose, Trehalose, D-Raffinose, Amidon, β-Gentibiose, D-Tagatose. And could not utilize and ferment the following carbohydrates: Glycerol, Erythritol, D-Arabinose, Ribose, D-Xylose, L-Xylose, Adonitol, β-Methylxyloside, L-Sorbose, Rhamnose, Dulcitol, Inositol, Sorbitol, α-Methyl-D-Mannoside, α-Methyl-D-Glucoside, Inulin, Melezitose, Glycogen, Xylitol, D-Turanose, D-Lyxose, D-Fucose, L-Fucose, D-Arabitol, L-Arabitol, Gluconate, 2-Ketogluconate and 5-Ketogluconate.

As shown Tab. 5.2, the results were, $31x10^8$ cfu/g, $14x10^7$ cfu/g, and $<10^1$ cfu/g for *lactobacilli*, general viable counts of bacteria, and yeasts and molds counts respectively.

Tab. 5.1: Experimental comparison of microbiological characteristics of the microencapsulated culture with literature data

Test items	Experimental Results	Kandler & Weiss (1986)♦▲	BioMerieux **	Test items	Experimental Results	Kandler & Weiss (1986) ♦ ▲	BioMerieux **
Morphology	Rods	Rods	Rods	Arbutin	+		+
Gram staining	+	+	+	Esculin	+	+	+
Spore staining	–	–	–	Salicin	+	+	+
Catalase	–	–	–	Cellobiose	+	+	+
Carbohydrates utilization✪ Glycerol	–	ND	–	Maltose	+	+	+
Erythritol	–		–	Lactose	+	+ *	+
D-Arabinose	–	–	–	Melibiose	+	(D)	+
L-Arabinose	–	–	+	Saccharose	+	+ *	+
Ribose	–	–	–	Trehalose	+	(D)	+
D-Xylose	–	–	–	Inulin	–	ND	(+)
L-Xylose	–	–	–	Melezitose	–	–	–
Adonitol	–	ND	–	D-Raffinose	+	(D)	+
β-Methylxyloside	–	ND	–	Amidon	+	ND	+
Galactose	+	+ *	+	Glycogen	–	ND	(+)
D-Glucose	+	+ *	+	Xylitol	–	ND	–
D-Fructose	+	+ *	+	β-Gentibiose	+	ND	+
D-Mannose	+	+	+	D-Turanose	–	ND	[+]
L-Sorbose	–	ND	–	D-Lyxose	–	ND	–
Rhamnose	–	–	–	D-Tagatose	+	ND	+
Dulcitol	–	ND	–	D-Fucose	–	ND	–
Inositol	–	ND	–	L-Fucose	–	ND	
Mannitol	–	–	+	D-Arabitol	–	ND	–
Sorbitol	–	+	–	L-Arabitol	–	ND	–
α-Methyl-D-Mannoside	–	ND	–	Gluconate	–	–	–
α-Methyl-D-Glucoside	–	ND	–	2-Ketogluconate	–	ND	–
N-Acetylglucosamine	+	ND	+	5-Ketogluconate	–	ND	–
Amygdalin	+	+	+				

♦▲ data obtained by Bergey`s Manual of Systematic Bacteriology (1986).
✪ data obtained by API 50 CHL kit, +: positive, –: negative.
* The same data obtained by Srinivas et al. (1990).
** data obtained from mannual of Api 50 CHL medium, BioMerieux, France.
D 11 – 89% strains positive.
(+) 1– 20% strains positive.
[+] 1 – 4% strains positive.
ND not determined.

Tab. 5.2: Microencapsulated *L. acidophilus* powder purity

Test item	***cfu / g***
MRS agar	31×10^8
Plate count agar	14×10^7
Malt extract agar	$< 10^1$

5.4 Kinetics of acidification

Acidification kinetics of microencapsulated and non-microencapsulated cells were established hourly after inoculating in MRS. The initial pHs were in the range of approximately 5.57 – 5.68. The pH value at the end of the incubation time was approximately 4.06 (the results are shown in Figs. 5.14 - 5.16).

The rate of acidification for the microencapsulated cells was slower than that observed for free cells incubated under similar conditions. The time taken for the microencapsulated cells to arrive at the same end point of pH change was longer than that reached by the free cells. For example, the free cells achieved the pH of 5.0 after 6 hours, while it took more than 8, 8, 9, 12, and 13 hours for microencapsulated cells in: gum arabic, gelatin, G.A + whey protein, G.A + soy protein, and G.A + soy milk respectively.

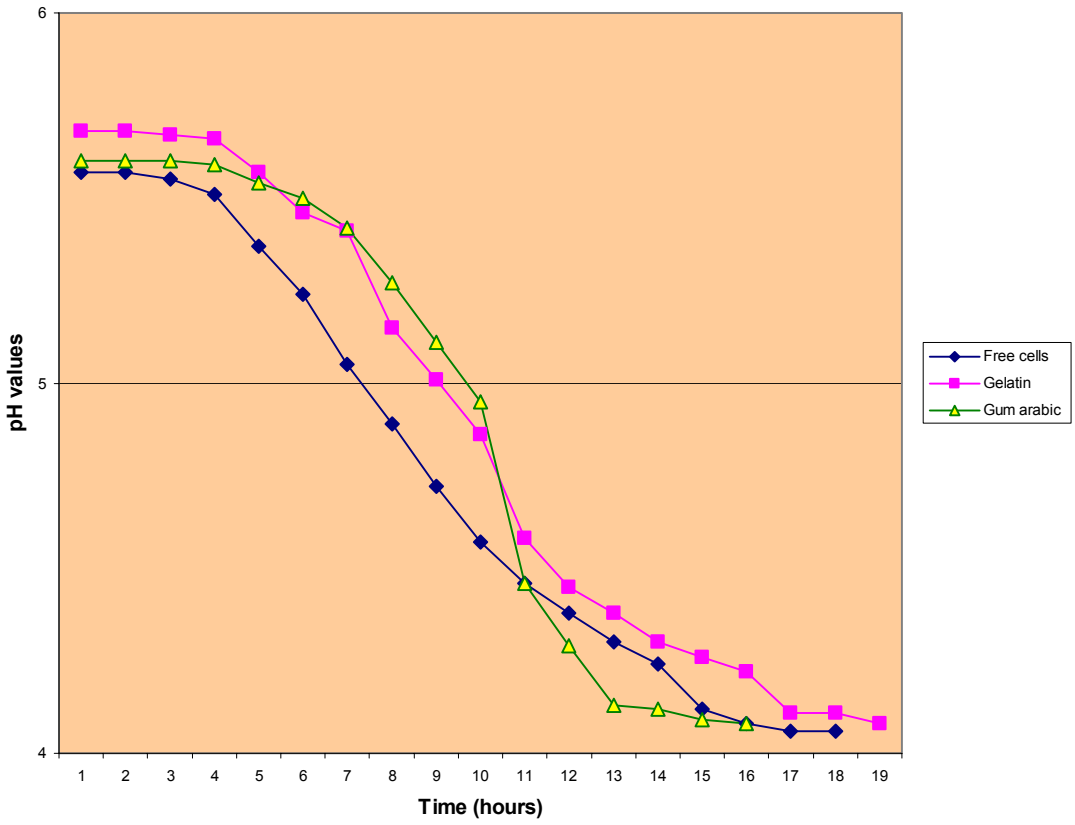


Fig.5.14: Changes of the pH values of MRS broth fermented with free and microencapsulated *L. acidophilus*

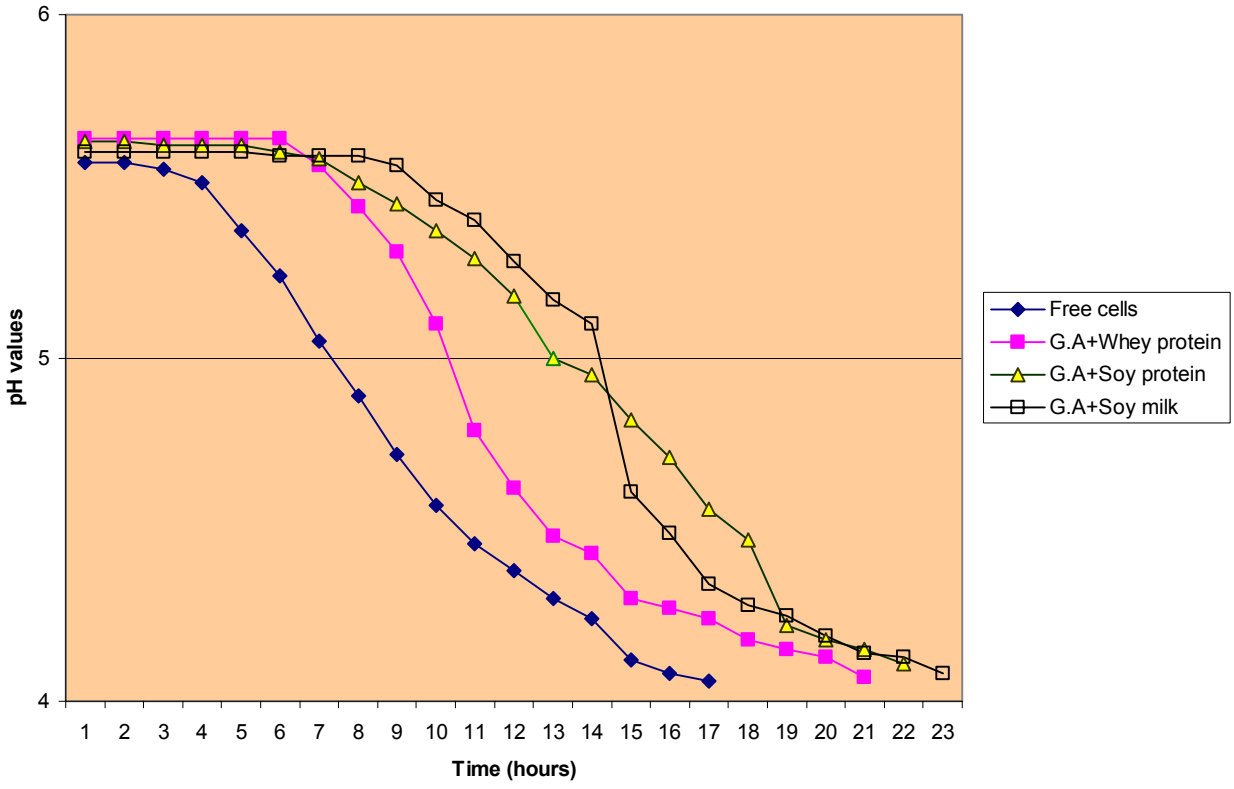


Fig. 5.15: Changes of the pH values of MRS broth fermented with free and microencapsulated *L. acidophilus*

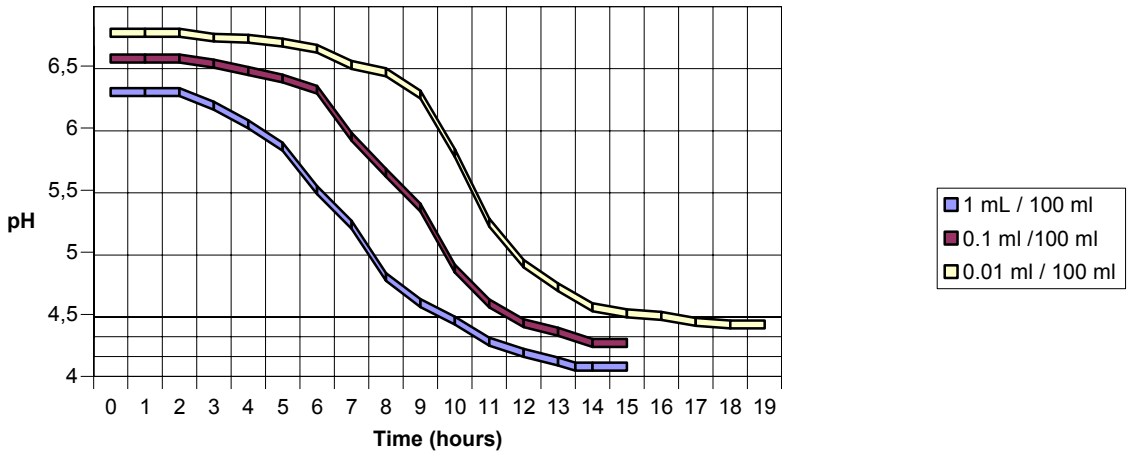


Fig. 5.16: Influence of inoculum amount on the pH value of MRS broth during the fermentation with free *L. acidophilus*

5.5 Viability of microencapsulated *L. acidophilus* during storage

In the present study, the microencapsulated *L. acidophilus* have been divided according to the main used capsule material(s) into the following 10 groups: gum arabic (G.A), soy protein, whey protein, soy milk, G.A+soy protein, G.A+whey protein, G.A+soy milk, G.A+proteins, gelatin, and gelatin+proteins or soy milk.

Figs. 5.17-5.31 describe the viability of microencapsulated cells during refrigerated storage. In general, microencapsulated cells varied in their viability under refrigeration at 5°C for weeks.The initial counts ranged from $8.7x10^9$ to $3.2x10^7$ cfu/g.

Therapeutic benefits indicate the minimum level of probiotic bacteria has been suggested to be 10^5 to 10^6 viable cells per ml or g of product (Robinson, 1987). As shown in Figs. 5.17-5.20 the cell counts using gum arabic as a main capsule material were about 10^5 to 10^6 cfu/g (recommended therapeutic-minimum counts) after 2 weeks for, G.A, G.A+ gum guar, and G.A+ gum karaya; after 3 weeks for, G.A+mannitol, G.A+maltodextrin, G.A+skim milk, G.A+carrageenan, G.A+glycerol, G.A+tween 20, and G.A+lecithin; after 4 weeks for, G.A+pectin, G.A+alginate, G.A+tomato juice, and G.A+ soy bean flour; and after 5 weeks for G.A+gelatin. The viable cells declined to between 10^2 and 10^4 cfu/g at the end-points of the storage which were after, 4 weeks for, G.A, G.A+gum guar, G.A+gum karaya, and

G.A+carrageenan; after 5 weeks for, G.A+mannitol, G.A+maltodextrin, G.A+glycerol, G.A+tomato juice, G.A+tween 20, and G.A+lecithin; after 6 weeks for, G.A+pectin, and G.A+skim milk; after 7 weeks for, G.A+alginate, and G.A+soybean flour; and after 8 weeks for G.A+gelatin.

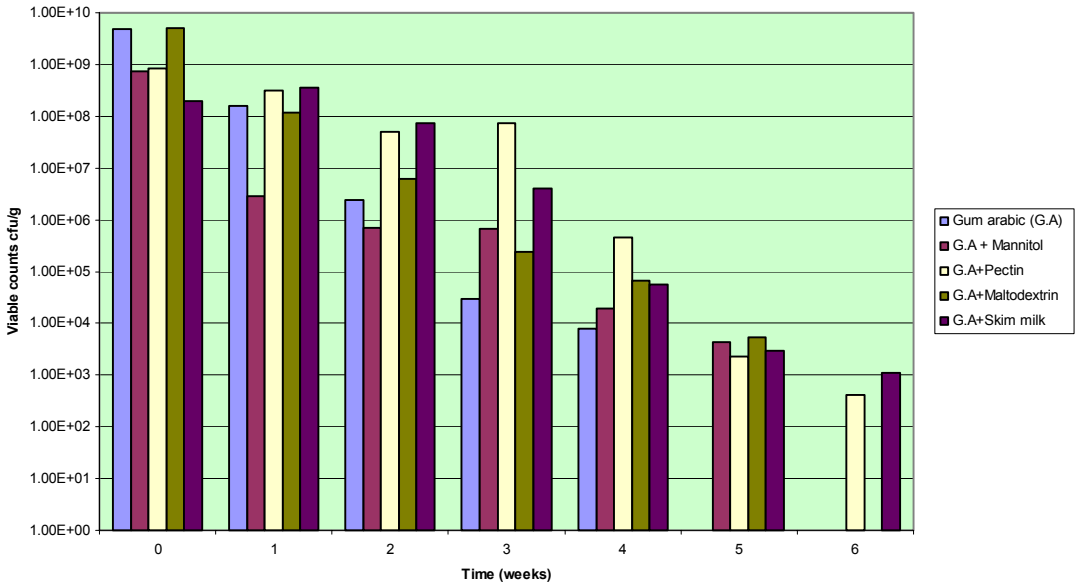


Fig. 5.17: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic as a main capsule material

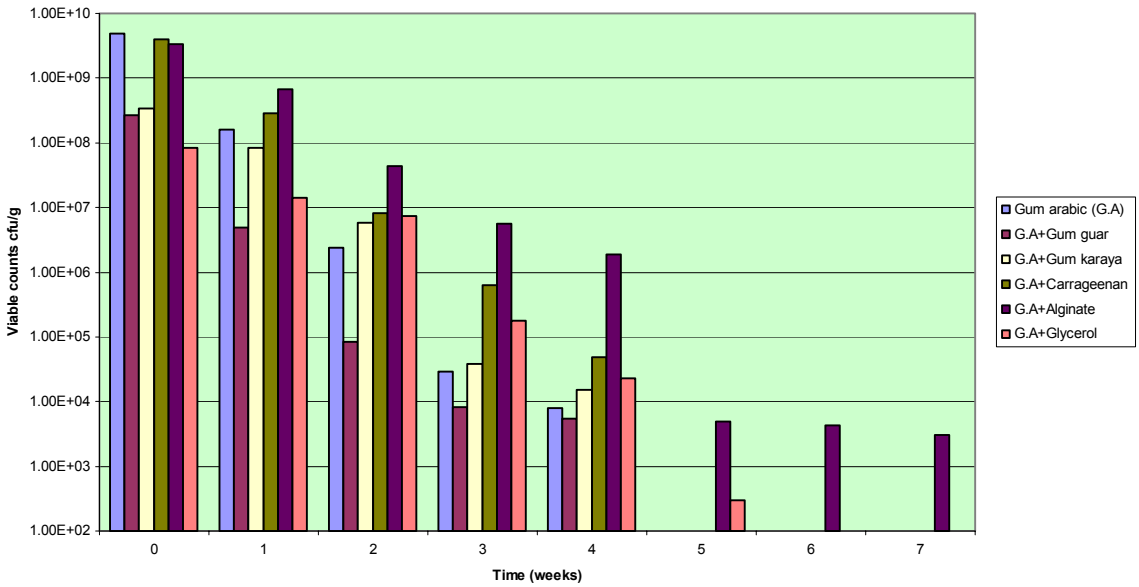


Fig. 5.18 Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic as a main capsule material

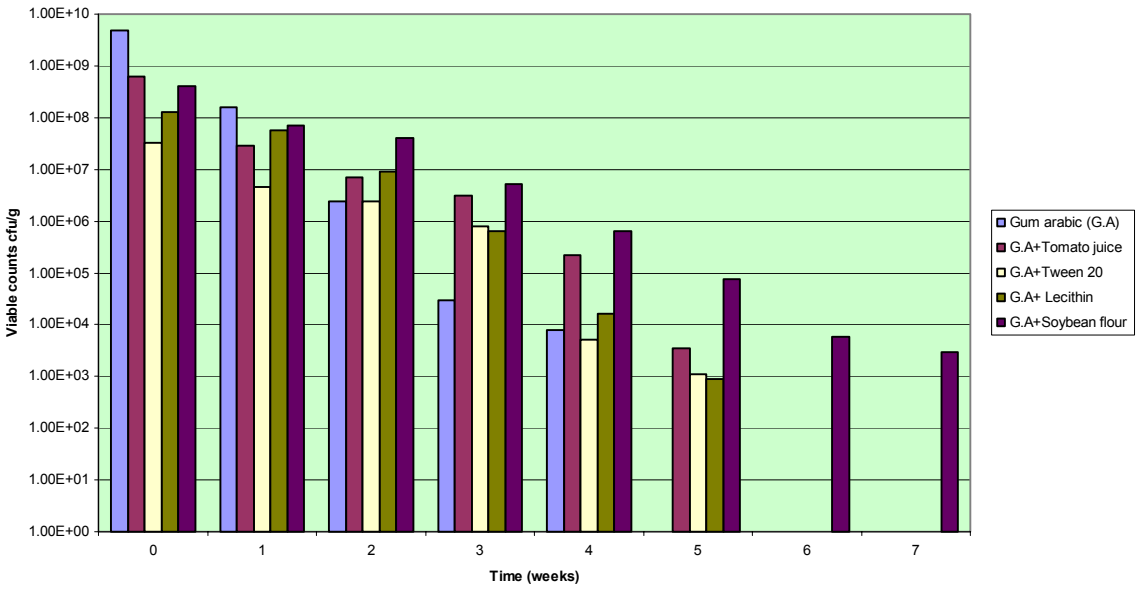


Fig. 5.19: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic as a main capsule material

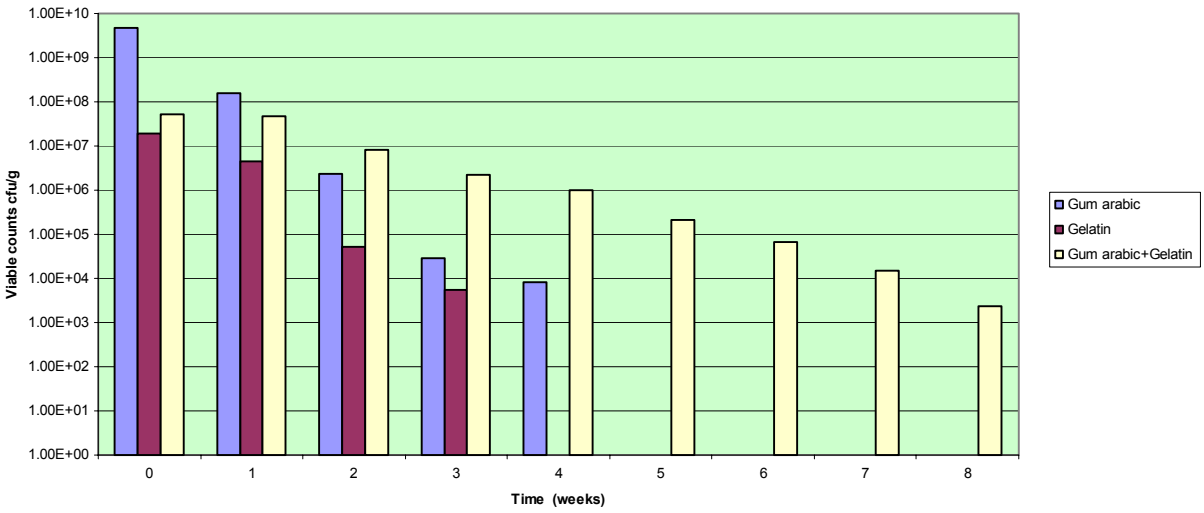


Fig. 5.20: Viabe counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic and gelatin as main capsule materials

The viable counts using soy protein as a main capsule material are presented in Fig. 5.21. The numbers decreased to $1.6x10^5$, $4.3x10^5$, and $3.7x10^6$ cfu/g after 8, 9, and 9 weeks; and decreased to $2.8x10^4$, $5.7x10^3$, and $1.1x10^3$ cfu/g as storage end-points after 9, 12, and 12 weeks for soy protein, soy protein+pectin, and soy protein+alginate respectively.

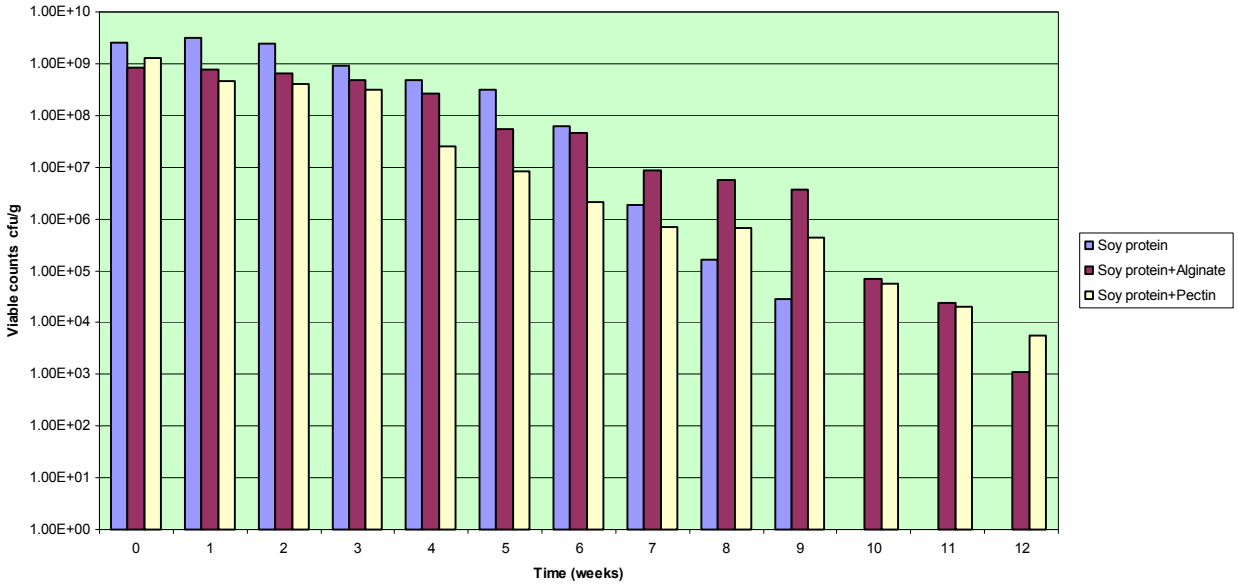


Fig. 5.21: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using soy protein as a main capsule material

For whey protein group (Fig. 5.22), cell numbers declined to $3.6x10^5$ and $5.1x10^3$ cfu/g after 7 and 9 weeks for whey protein; $2.4x10^5$ and $5x10^2$ cfu/g after 8 and 10 weeks for whey protein+alginate; and $6.6x10^5$ and $4.9x10^3$ after 9 and 10 weeks for whey protein+pectin.

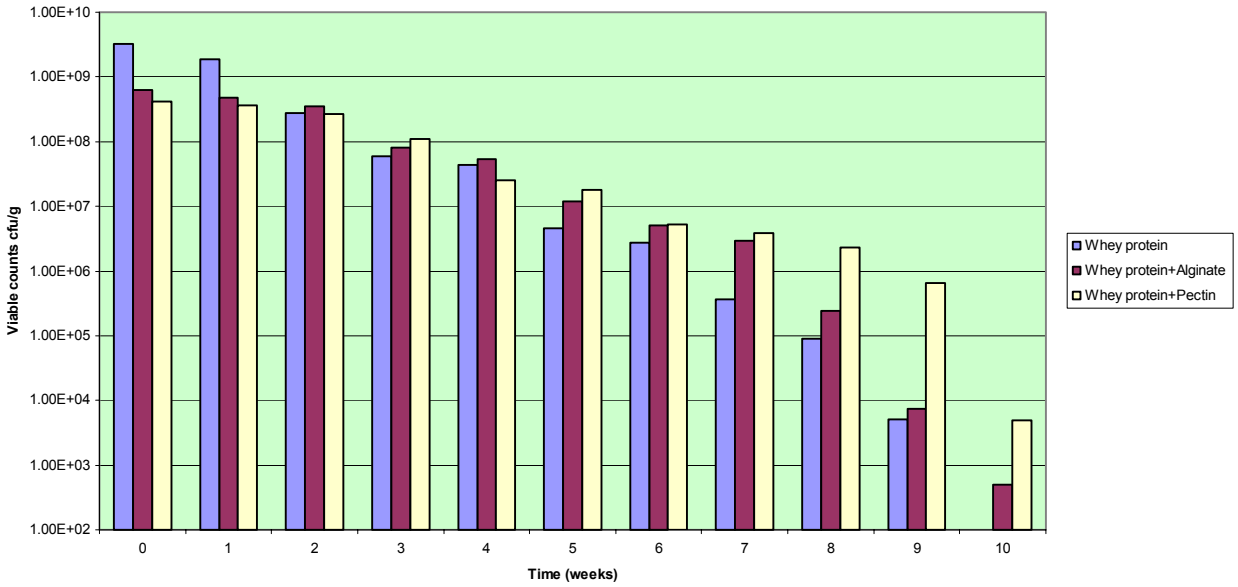


Fig. 5.22: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using whey protein as a main capsule material

Fig. 5.23 shows viable counts of microencapsulated cells during storage using soy milk as a main capsule material. Its viability reached $6x10^5$, $1.8x10^5$ and $2.1x10^5$ cfu/g after 7, 8, and 10 weeks for soy milk, soy milk+pectin, and soy milk+alginate respectively, while it reached $1.4x10^4$, $3.2x10^3$, and $4x10^3$ cfu/g after 9, 10, and 12 weeks for soy milk, soy milk+pectin, and soy milk+alginate respectively.

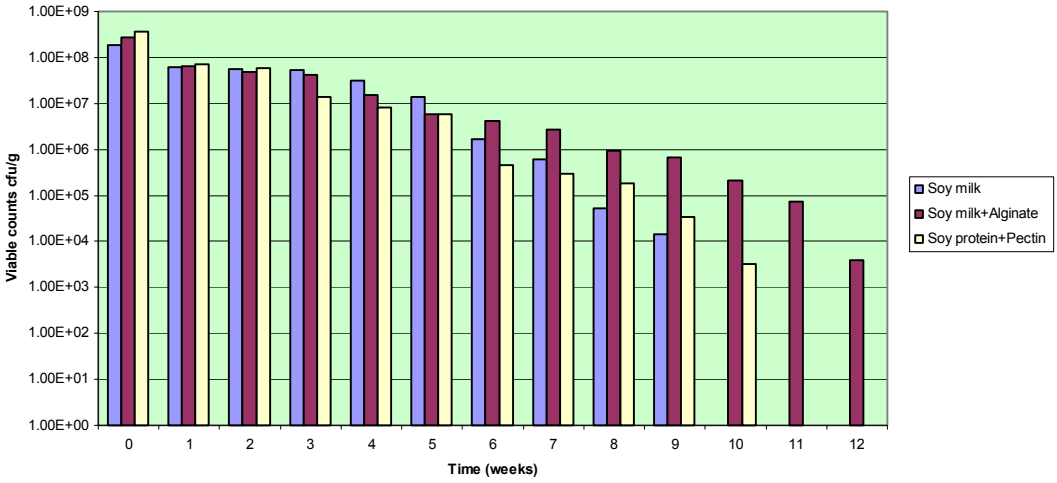


Fig. 5.23: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using soy milk powder as a main capsule material

In the case of using G.A+soy protein as main capsule materials (Fig. 5.24), viable counts reduced to $3.8x10^5$ and $6.1x10^2$ cfu/g after 10 and12 weeks for G.A+soy protein; $4.5x10^5$ and $2.4x10^3$ cfu/g after 10 and 12 weeks for G.A+soy protein+pectin; and $4.9x10^5$ and $2.2x10^3$ cfu/g after 11 and 13 weeks for G.A+soy protein+alginate.

Microencapsulated cells with G.A+whey protein group are illustrated in Fig. 5.25. Viable counts reduced to advised therapeutic-minimum counts after 10, 11, and 11 weeks for G.A+ whey protein, G.A+whey protein+pectin, and G.A+whey protein+alginate respectively; and reached the end-points of storage after 14, 14, and 15 weeks for G.A+whey protein, G.A+whey protein+pectin, and G.A+whey protein+alginate respectively.

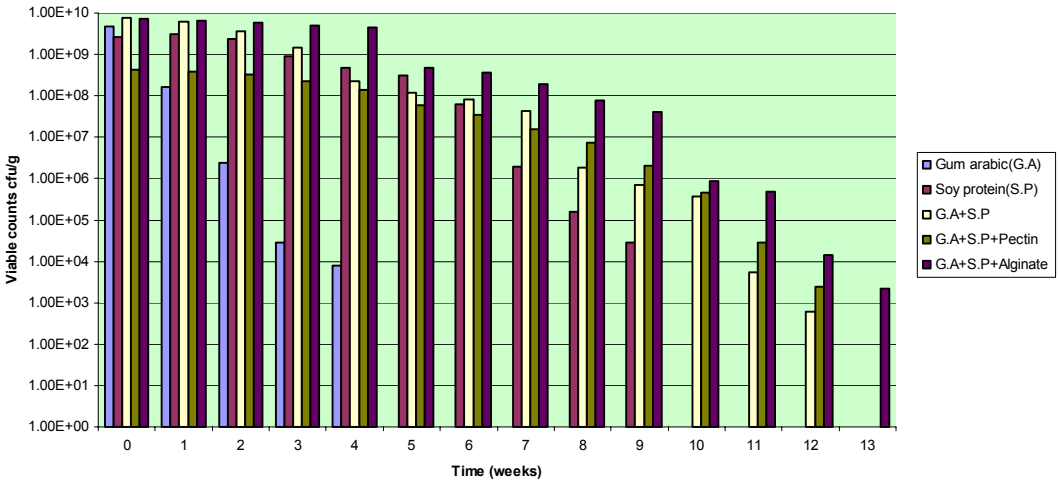


Fig. 5.24: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic and soy protein as main capsule materials

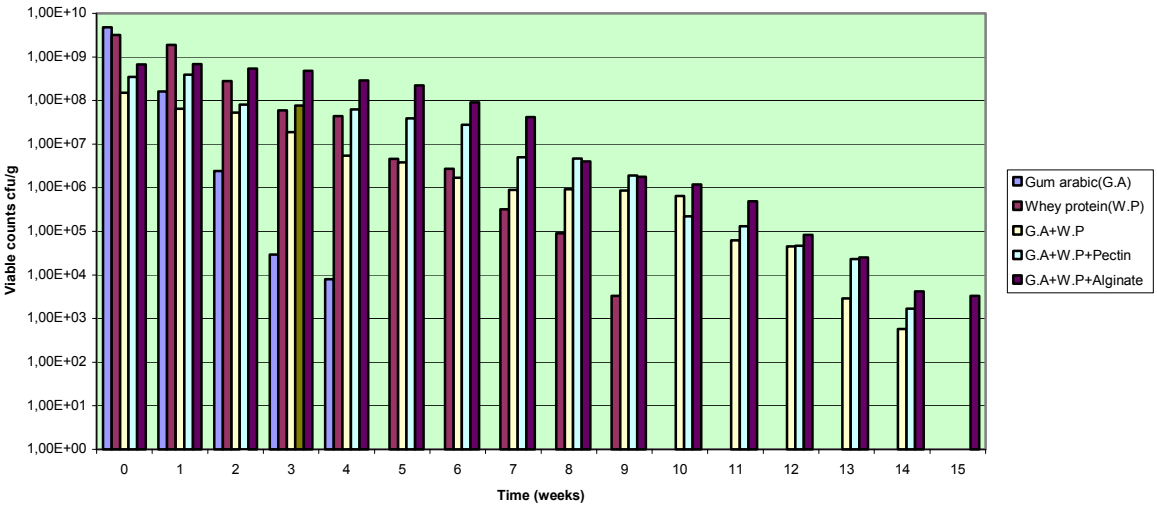


Fig. 5.25: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic and whey protein as main capsule materials

Fig. 5.26 shows the viable counts of G.A+soy milk group. Viable counts arrived to $2.7x10^5$ and $5.3x10^2$ cfu/g after 11 and 13 weeks for G.A+soy milk; $1.4x10^5$ and $5.4x10^3$ cfu/g after12

and 14 weeks for G.A+soy milk+pectin; and $2.6x10^5$ and $3.1x10^2$ cfu/g after 11 and 14 weeks for G.A+soy milk+alginate.

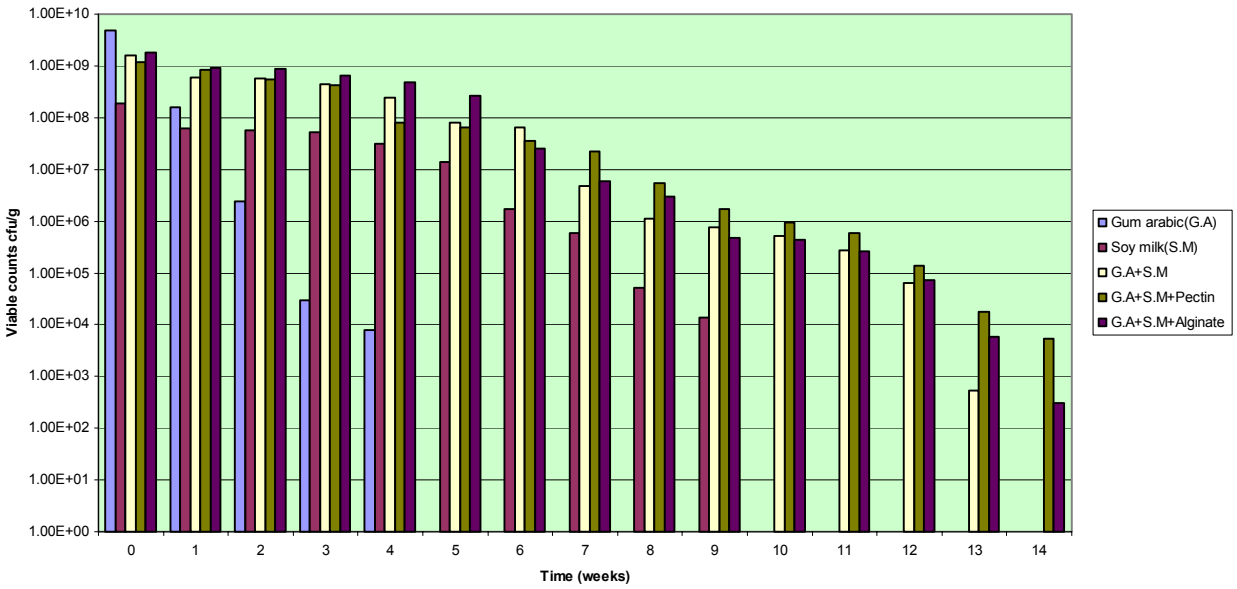


Fig. 5.26: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic and soy milk as main capsule materials

The viable counts remained at $1.6x10^5$ and $7.2x10^2$ cfu/g after 11 and 14 weeks for G.A+whey protein+soy protein (Fig. 5.27).

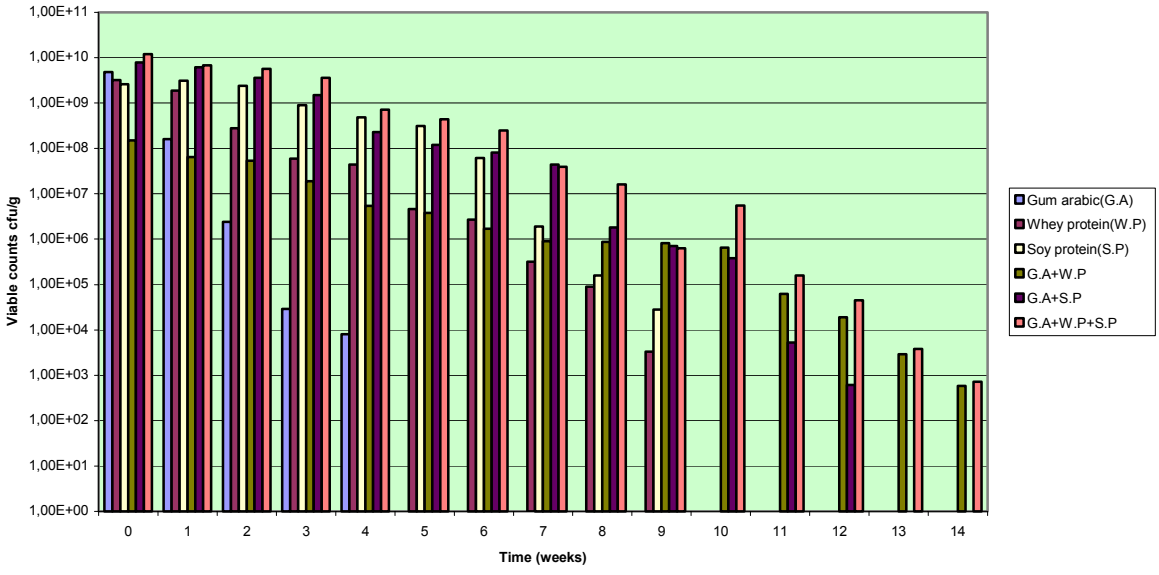


Fig. 5.27: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gum arabic and proteins as main capsule materials

When gelatin was used as a main capsule material (Figs. 5.28-5.30) the viable counts remained at recommended therapeutic-minimum counts after: 2 weeks for gelatin, gelatin+lecithin, and gelatin+glycerol; 3 weeks for gelatin+mannitol, gelatin+maltodextrin, gelatin+alginate, and gelatin+tomato juice; 4 weeks for gelatin+pectin; and 5 weeks for gelatin+skim milk, and gelatin+soybean flour. Viable counts reached the end-points after: 4 weeks for gelatin, gelatin+lecithin, and gelatin+glycerol; after 5 weeks for gelatin+alginate, gelatin+mannitol, gelatin+pectin, and gelatin+maltodextrin; and after 7 weeks for gelatin+skim milk, and gelatin+soybean flour.

For the group of gelatin+ proteins or soy milk (Fig. 5.31), the viable counts remained at advised therapeutic-minimum counts after 8 weeks, and reached the end-points after 11 weeks for gelatin+whey protein, gelatin+soy protein, and gelatin+soy milk.

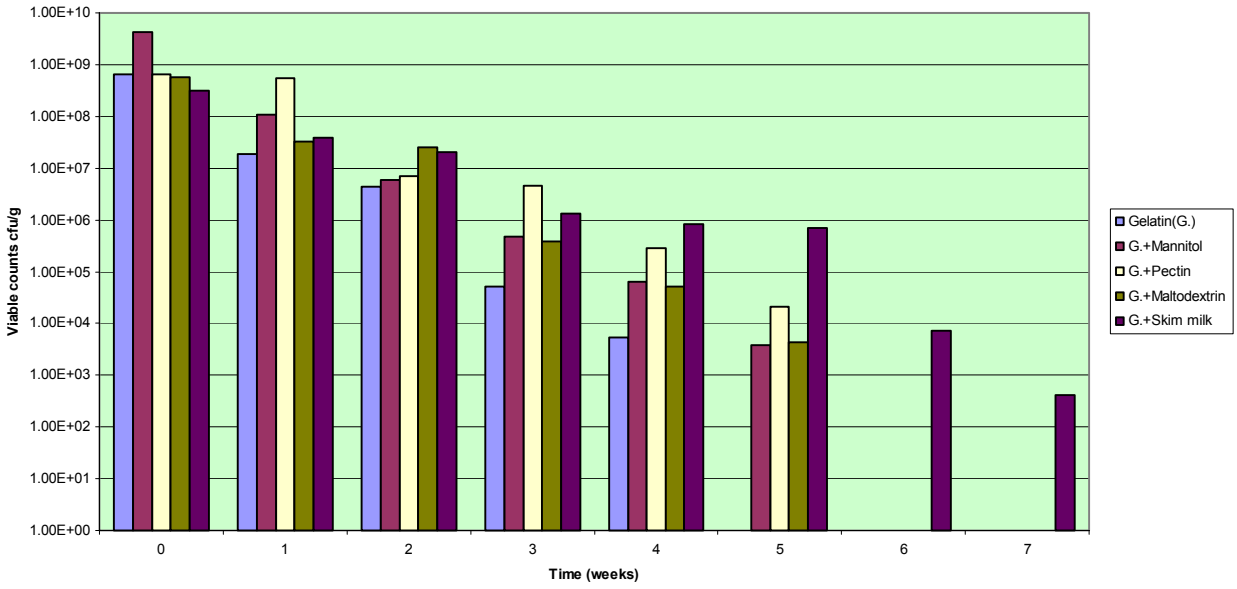


Fig. 5.28: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gelatin as a main capsule material

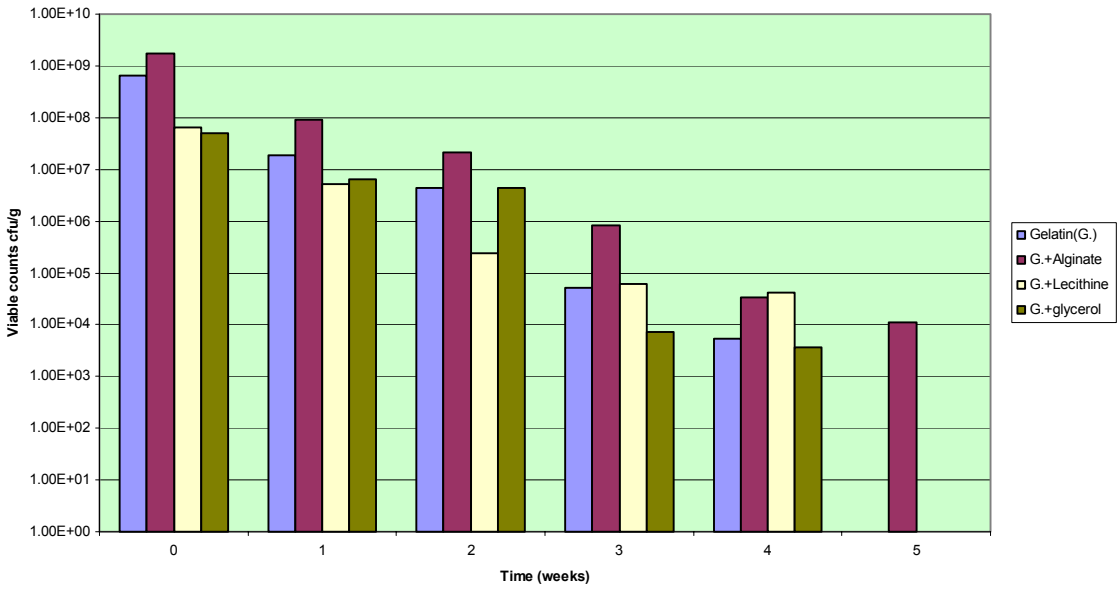


Fig. 5.29: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gelatin as a main capsulematerial

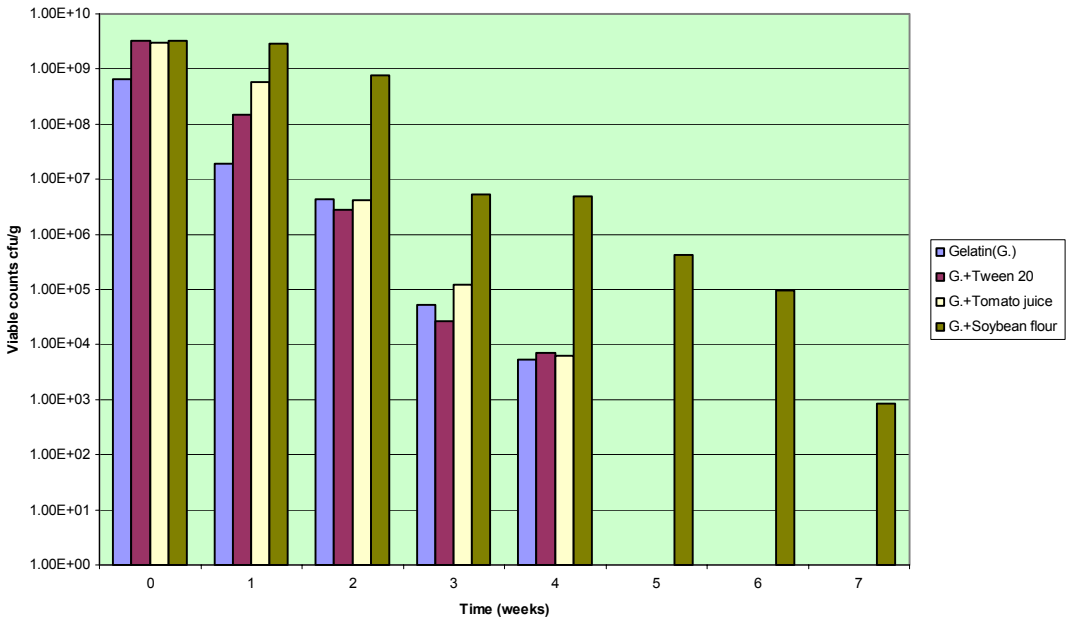


Fig. 5.30: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gelatin as a main capsule material

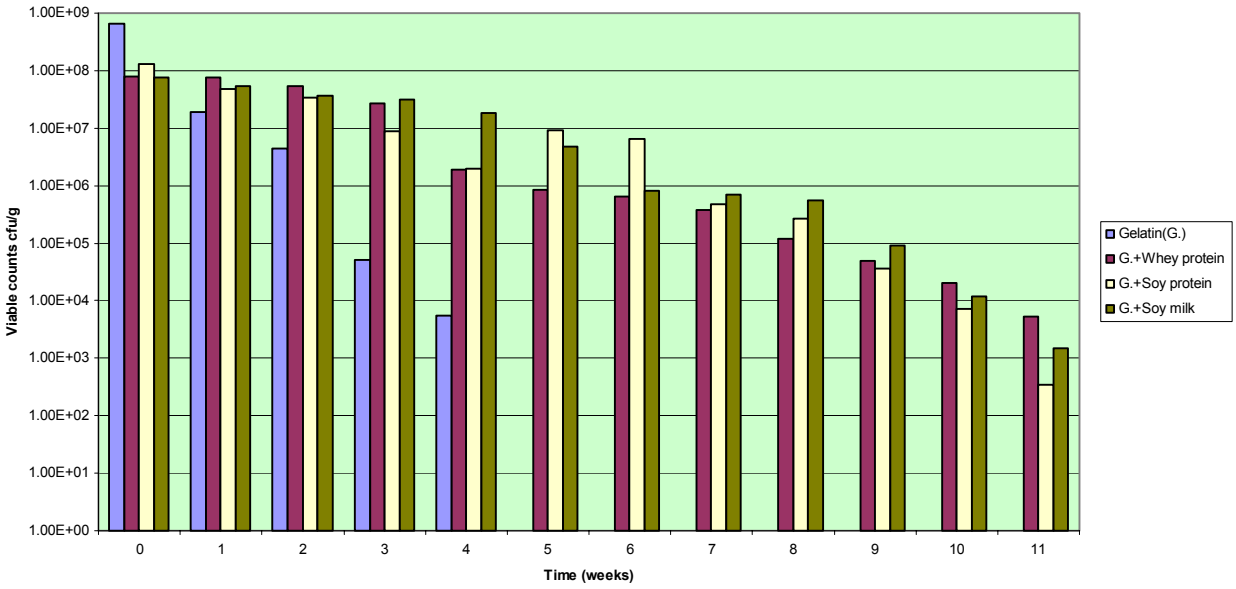


Fig. 5.31: Viable counts of *L. acidophilus* after spray drying and during storage at 5°C using gelatin and proteins or soy milk as main capsule materials

5.6 Influence of heat treatment on *L. acidophilus*

To determine the effect of different temperatures, *L. acidophilus* was exposured to 37, 40, 45, 50, 55, and 60°C for 30 minutes and the cfu at each temperature was determined (Fig. 5.32). The cfu increased at 37 and 40°C; remained the same at 45°C; decreased slightly at 50°C; and decreased noteworthly at 55 and 60°C.Therefore, 60°C was chosen as the lethal temperature, due to the extreme reduction of the cfu at this temperature, but no all cells were killed at this temperature.

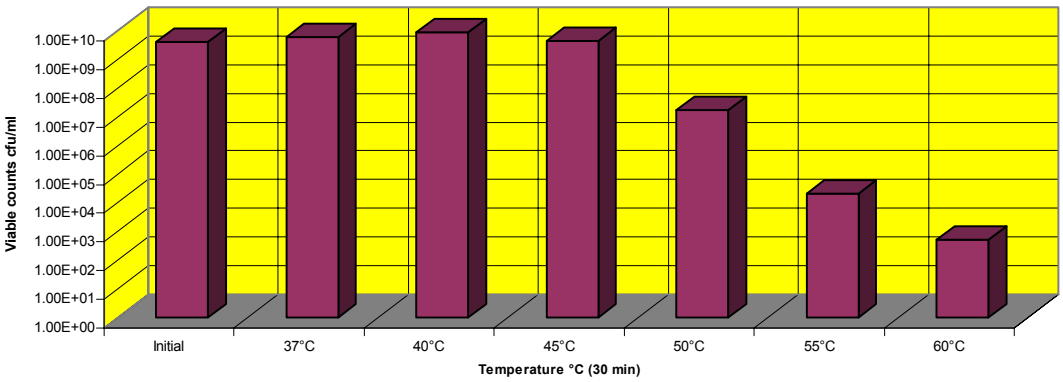


Fig. 5.32: Numbers of cfu of free *L. acidophilus* following exposure to different temperatures

Microencapsulated *L. acidophilus* in different capsule materials was subjected to different temperatures initiating with 60°C (lethal temperature for free cells). Microencapsulated cells exposed to 60°C/30 min; 63°C/30 min; 65°C/15 min; and 65°C/30 min. At 60°C (Fig. 5.33) the viable counts were $2.9x10^3$, $6.2x10^3$, $5.5x10^4$, $7.8x10^5$, and $5.6x10^4$ cfu/g for gum arabic, gelatin, soy protein+G.A, whey protein+G.A, and soy milk+G.A respectively.

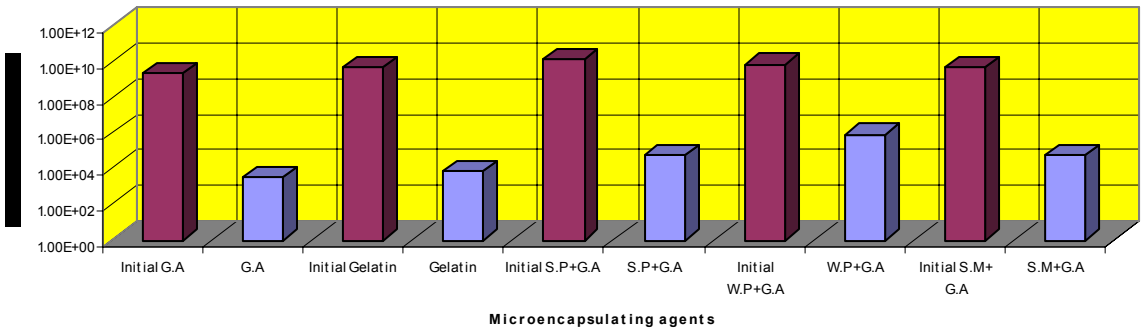


Fig. 5.33: Numbers of cfu of microencapsulated *L. acidophilus* following exposure to 60°C for 30 min

At 63°C (Fig. 5.34), the cells were less thermotolerant than at 60°C as, $7.2x10^4$, $5x10^5$, $3.6x10^5$ cfu/g were recorded for soy protein+G.A, whey protein+G.A, and soy milk+G.A respectively, while undetectable counts were found for gum arabic and gelatin.

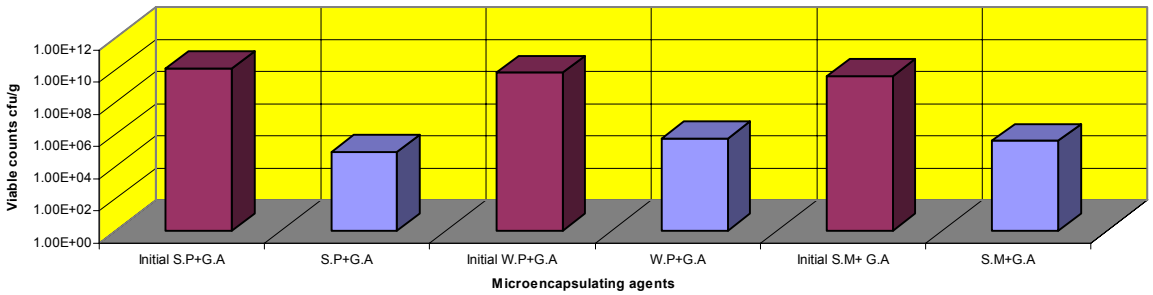


Fig. 5.34: Numbers of cfu of microencapsulated *L. acidophilus* following exposure to 63°C for 30 min

At 65°C/15 min (Fig. 5.35), the viable counts declined substantially to $6.3x10^3$, $1.7x10^4$, and $8.2x10^3$ cfu/g for soy protein+G.A, whey protein+G.A, and soy milk+G.A respectively.

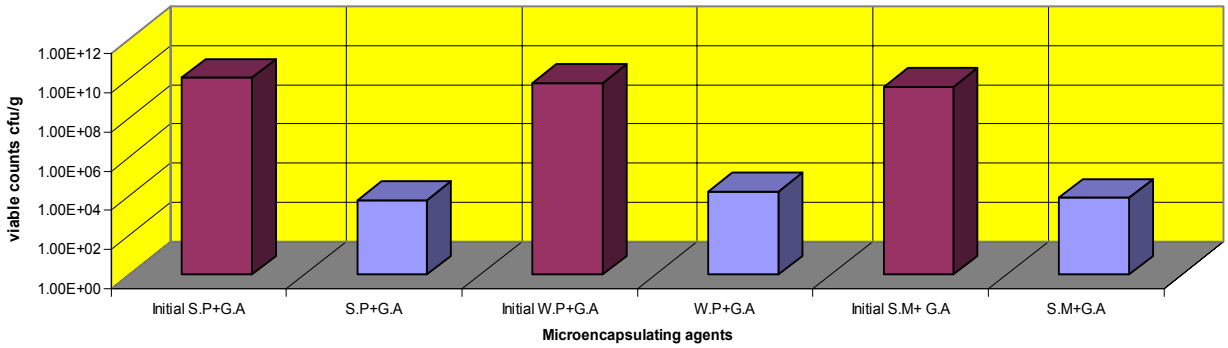


Fig. 5.35: Numbers of cfu of microencapsulating *L. acidophilus* following exposure to 65°C for 15 min

At 65°C/30 min (Fig. 5.36) no colony forming units could be observed, except for whey protein +G.A, which was still more than 10^2 cfu/g.

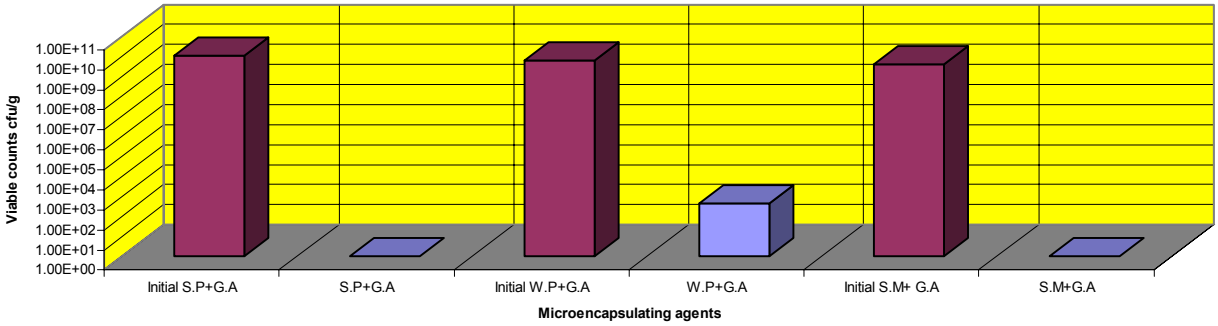


Fig. 5.36: Numbers of cfu of microencapsulated *L. acidophilus* following exposure to 65°C for 30 min

5.7 Viability of microencapsulated and non-microencapsulated cells in high sucrose concentrations

The results in Figs. 5.37-5.42 indicated the viability of free and microencapsulated *L. acidophilus* in high sucrose concentrations (5, 10, 15, 20, and 25%) during storage at 5°C. From the results in figure 5.37, free cells showed little viability differences which could be observed in sucrose concentrations of 5 to 15% as compared to the control (distilled water). The numbers decreased approximately from 4.2x109 cfu/ml (initial count) to 6.7x106 – 5.1x105 cfu/ml after 2 weeks; and declined to 2.7x103 – 4.8x103 cfu/ml after 3 weeks. Free cells were more sensitive to 20 and 25% sucrose concentrations reached 7.1x103 and 4.8x102 cfu/ml for 20% sucrose after 2 and 3 weeks respectively; and reached 5.4x106 cfu/ml after 1 week, and 8.9x102 cfu/ml after 3 weeks for 25% sucrose concentration.

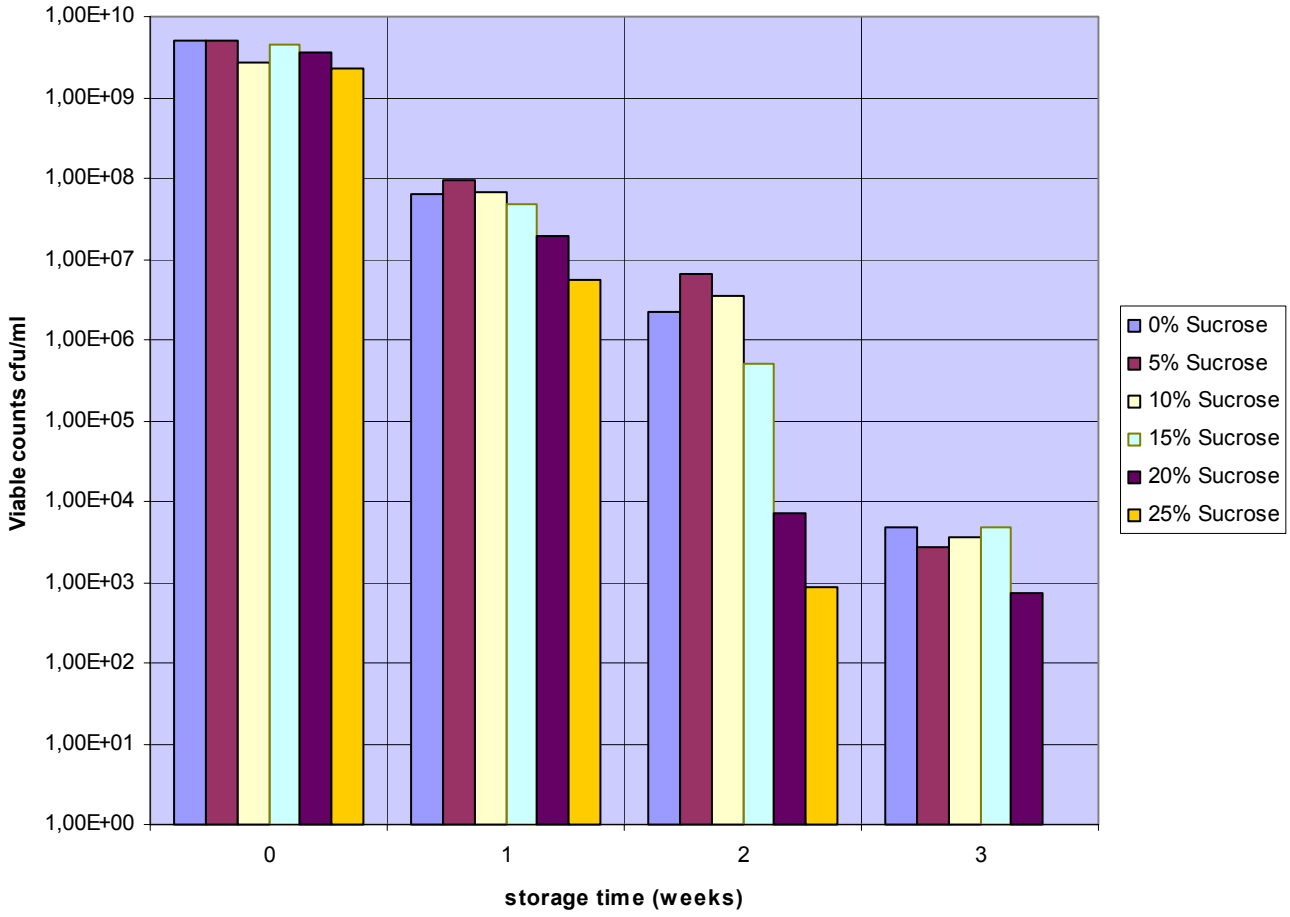


Fig. 5.37: Survival of free *L. acidophilus* in different sucrose solutions during storage at 5°C

When gelatin was used as a main capsule material (Fig. 5.38), the total viable counts showed more unalterability at 5, 10, and 15% sucrose concentrations than the free cells reached $6.9x10^7$, $4.3x10^7$, and $7.4x10^6$ cfu/g after 3 weeks at 5, 10, and 15% sucrose concentrations respectively. After 3 weeks storage at 20 and 25% sucrose concentrations, the numbers reached $3.4x10^5$ and $5.8x10^4$ cfu/g respectively, corresponding to $2.6x10^5$ cfu/g for the control (0% sucrose).

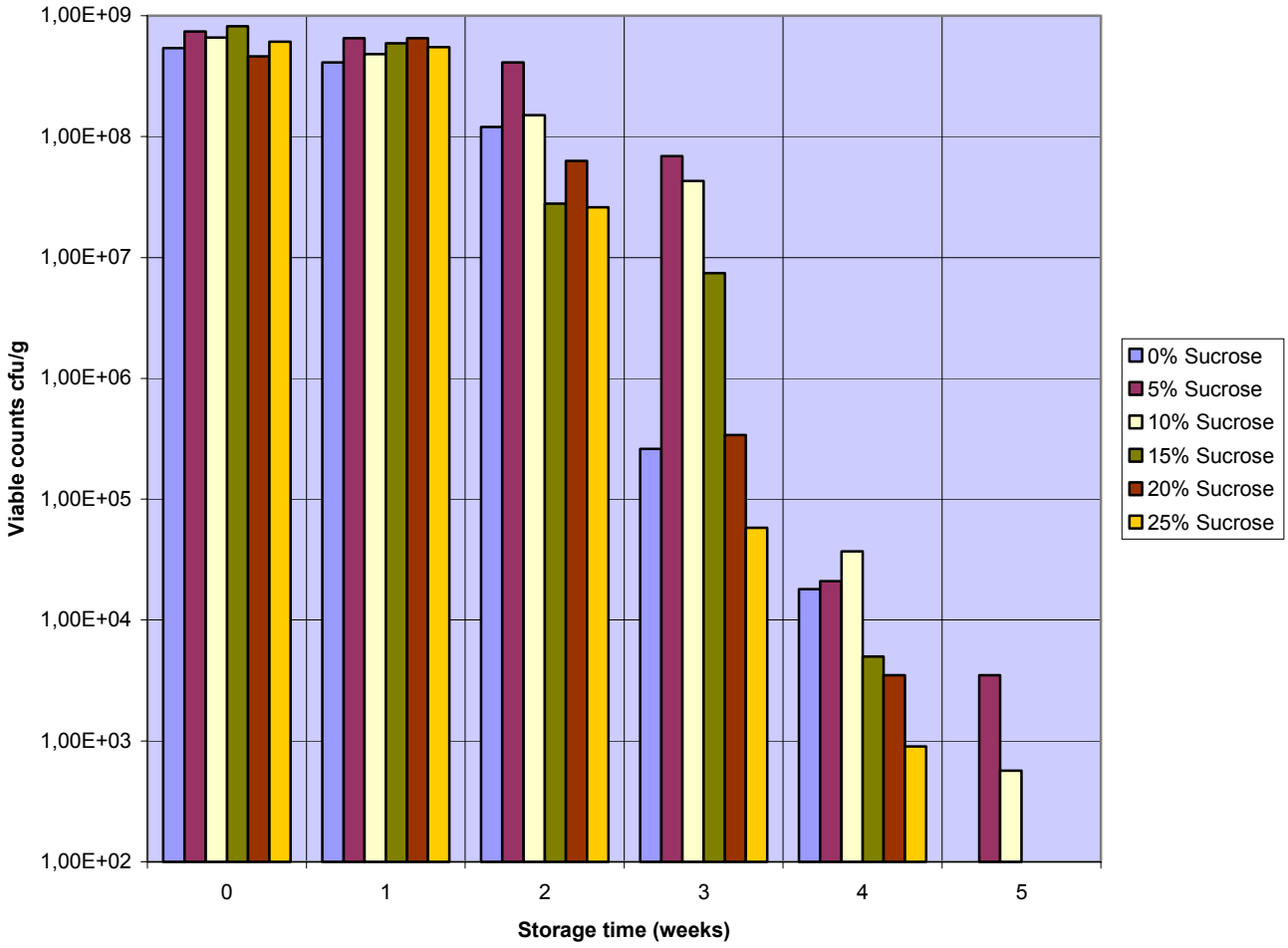


Fig. 5.38: Survival of microencapsulated *L. acidophilus* with gelatin in different sucrose solutions during storage at 5°C

The data for gum arabic are illustrated in Fig. 5.39. For all the tested sucrose concentrations, the control (0% sucrose) showed the best viability being $6.1x10^7$ cfu/g after 3 weeks. On the other hand, the numbers decreased to $8.3x10^6$, $2.4x10^6$, $2.9x10^6$, $1.5x10^7$, and $2.6x10^6$ cfu/g after 2 weeks for 5, 10, 15, 20, and 25% sucrose concentrations respectively.

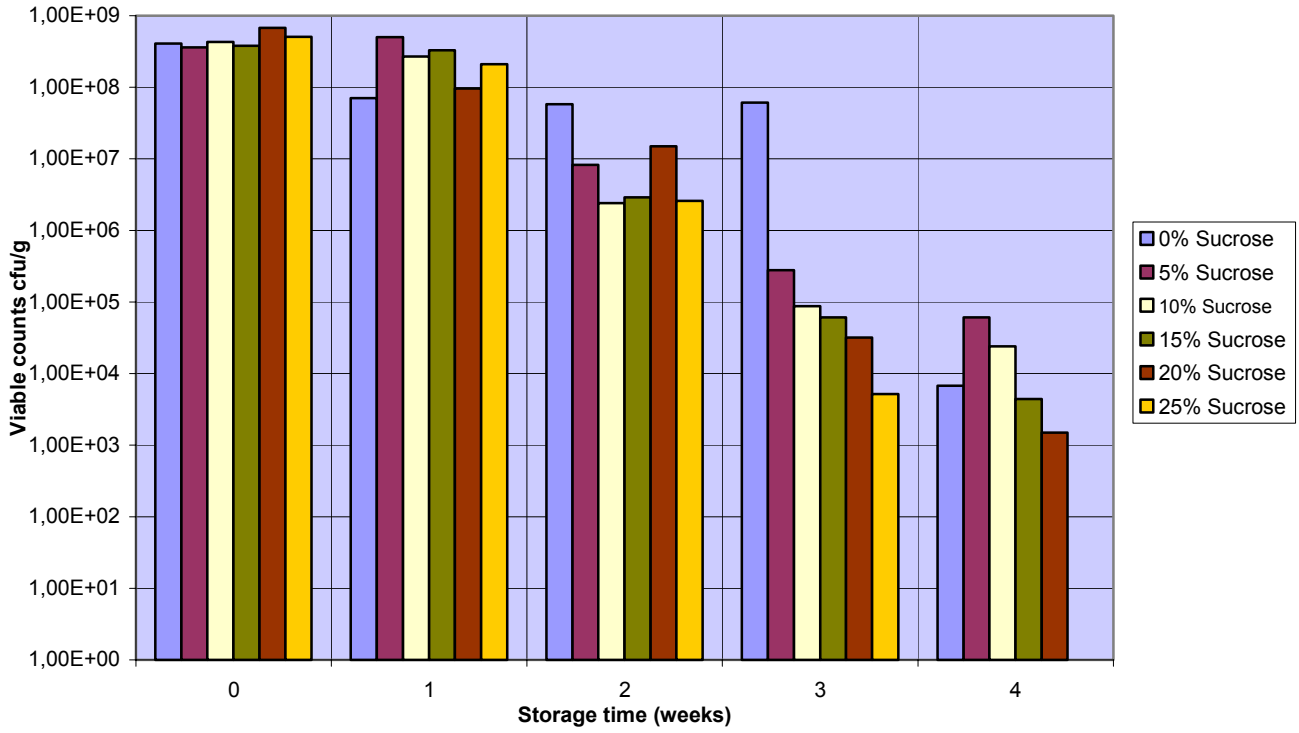


Fig. 5.39: Survival of microencapsulated *L. acidophilus* with gum arabic in different sucrose solutions during storage at 5°C

In the case of soy milk+G.A, soy protein+G.A, whey protein+G.A (Figs. 5.40-5.42) little variability was observed between the different tested sucrose concentrations, showed the best viability. They retained the recommended therapeutic-minimum numbers after 5 –6 weeks in exception that this value was reached after 4 –5 weeks for the higher sucrose concentration of 25%.

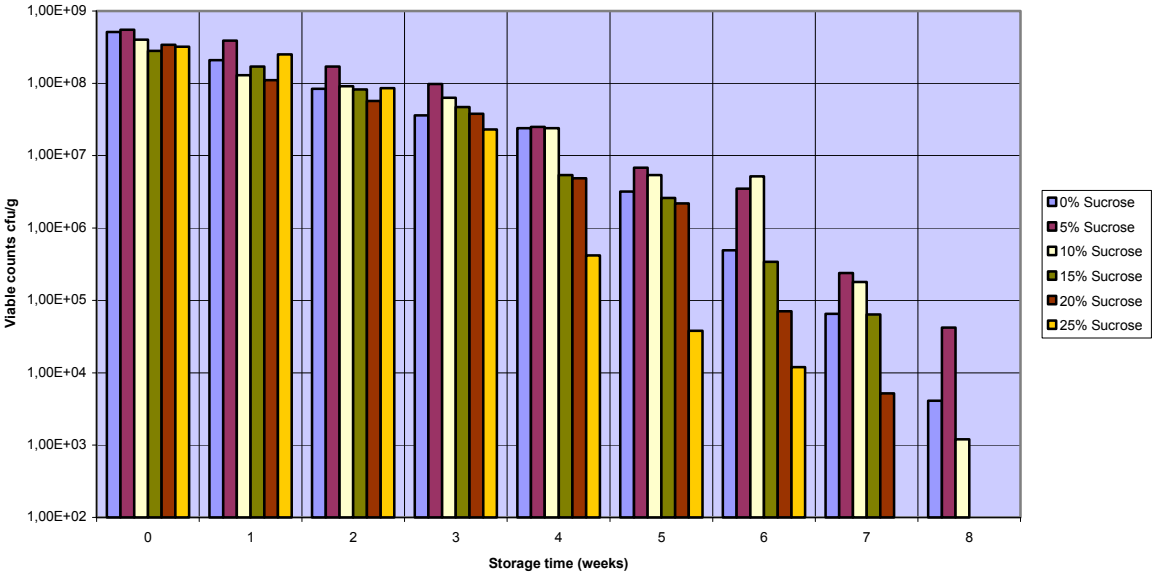


Fig. 5.40: Survival of microencapsulated *L. acidophilus* with soy milk+gum arabic in different sucrose solutions during storage at 5°C

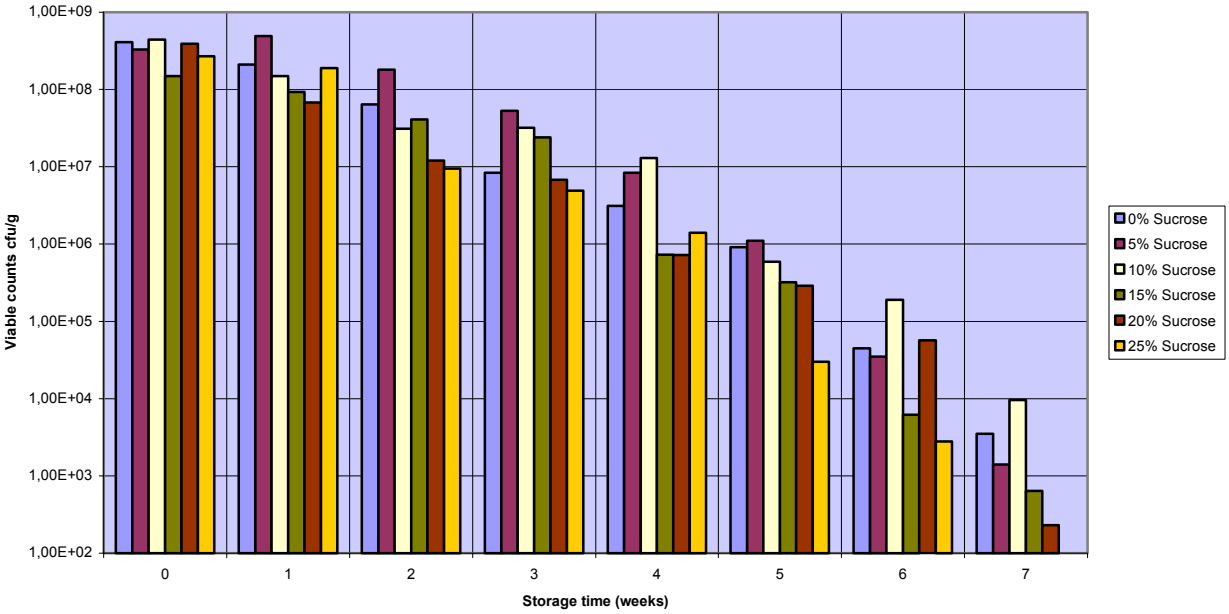


Fig. 5.41: Survival of microencapsulated *L. acidophilus* with soy protein+gum arabic in different sucrose solutions during storage at 5°C

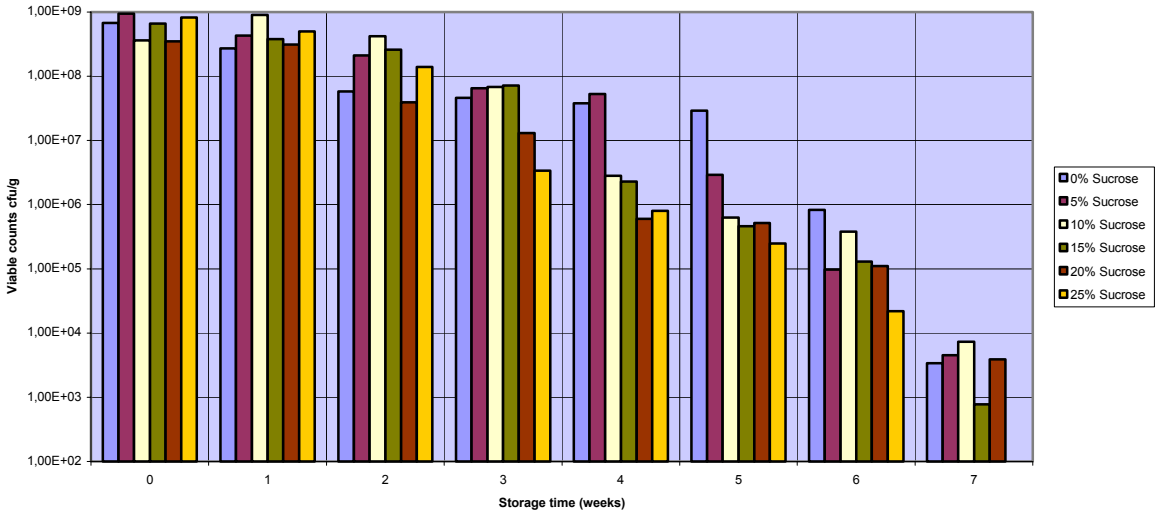


Fig. 5.42: Survival of microencapsulated *L. acidophilus* with whey protein+gum arabic in different sucrose solutions during storage at 5°C

5.8 Viability of microencapsulated and non-microencapsulated cells in different NaCl solutions

The determination of the viability of *L. acidophilus* in different sodium chloride concentrations (1, 2, 3, 4, and 5%) during storage at 5°C, was made weekly through cfu at each salt concentration.

The cfu of free cells (Fig.5.43) decreased steadily during the storage in 1, 2, and 3% NaCl concentrations after 3 weeks and reached $1.3x10^4$, $2.1x10^3$, and $3.4x10^3$ cfu/ml for 1, 2, and 3% NaCl concentrations respectively; declined rapidly in 4 and 5% NaCl concentrations. The numbers decreased by 7 log cycles compared to the initial count ($4.2x10^9$ cfu/ml) after 2 weeks in 4%, and 6 log cycles after 1 week for 5% NaCl. Similar results were observed for gum arabic (Fig. 5.44).

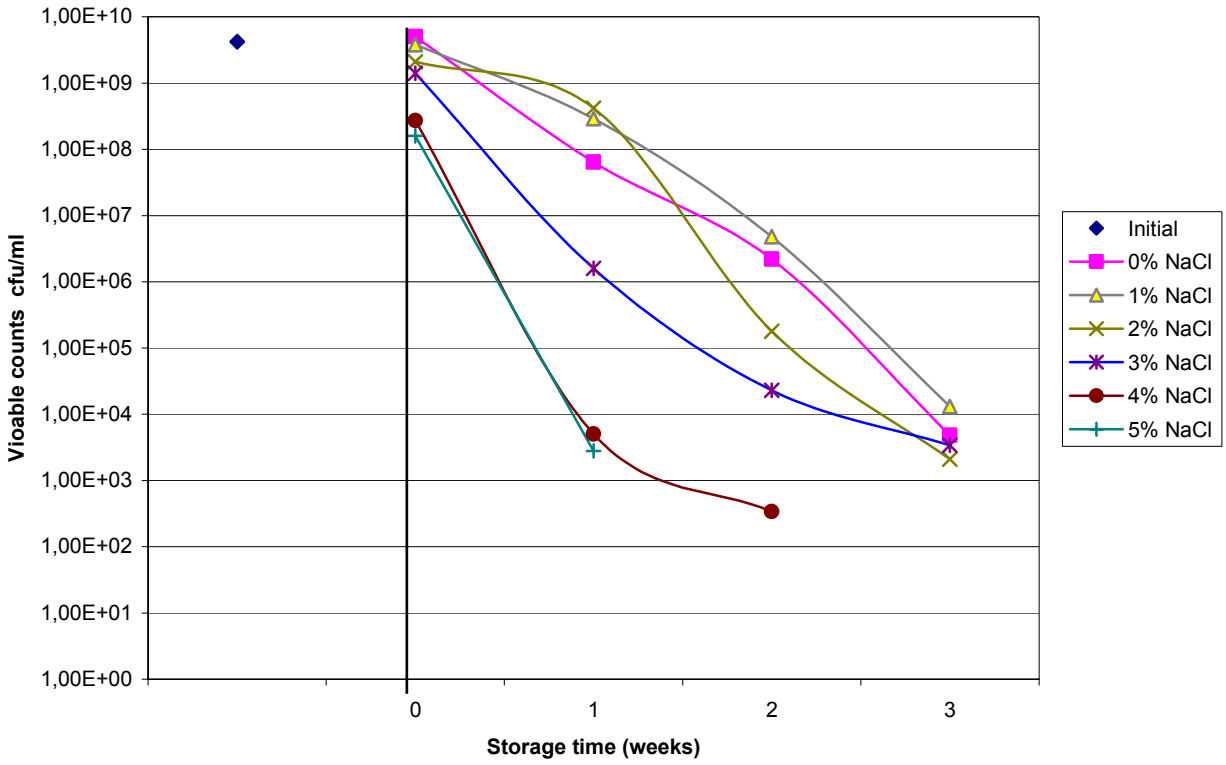


Fig. 5.43: Effect of different NaCl concentrations on the survivability of free *L. acidophilus* during storage at 5°C

For gelatin (Fig. 5.45), the numbers decreased slower and more steadily (from initial $6.8x10^8$ cfu/g) reached $1.9x10^4$, $6x10^3$ cfu/g after 4 weeks for 1 and 2% NaCl concentrations respectively; $5.9x10^3$ and $4.8x10^3$ cfu/g after 3 weeks for 3 and 4% NaCl respectively; and $7.5x10^3$ cfu/g after 2 weeks for 5% NaCl concentration.

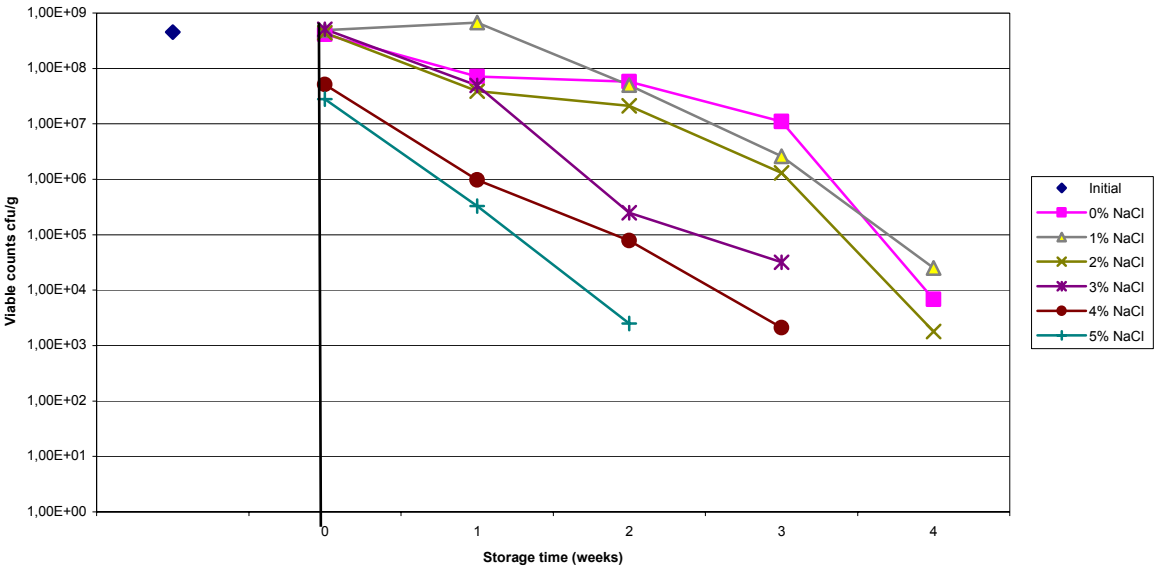


Fig. 5.44: Effect of different NaCl concentrations on the survivability of microencapsulated *L. acidophilus* with gum arabic during storage at 5°C

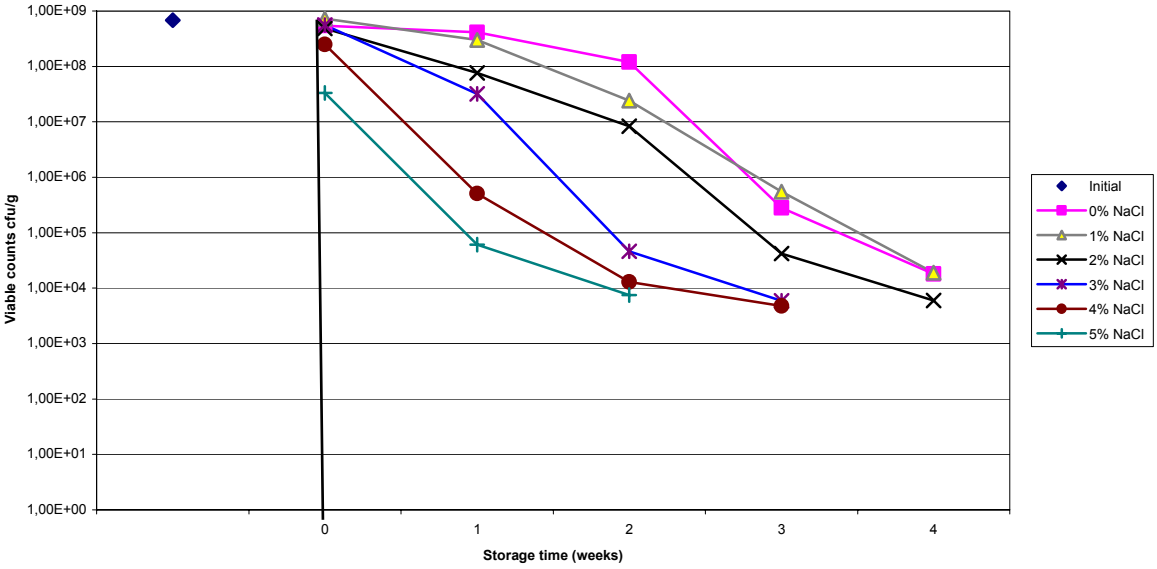


Fig. 5.45: Effect of different NaCl concentrations on the survivability of microencapsulated *L. acidophilus* with gelatin during storage at 5°C

In the case of of soy protein+G.A (Fig. 5.46), the viability decreased from $3.8x10^8$ to $6.3x10^5$ and $4.7x10^5$ cfu/g after 5 weeks for 1 and 2% NaCl concentrations respectively; $2.6x10^5$ cfu/g

for 3% NaCl after 4 weeks; $1.2x10^5$ cfu/g after 2 weeks for 4% NaCl; and $2.9x10^5$ cfu/g after 1 week for 5% NaCl during storage.

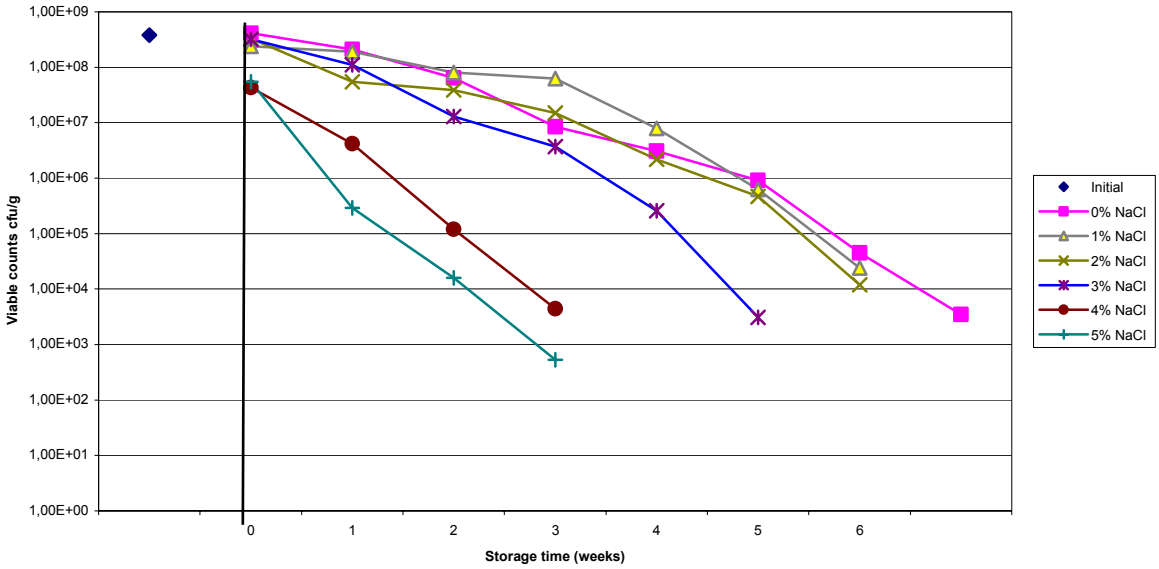


Fig. 5.46: Effect of different NaCl concentrations on the survivabilty of microencapsulated *L. acidophilus* with soy protein and gum arabic during storage at 5°C

Storage of microencapsulated cells with whey protein+G.A in different sodium chloride solutions (Fig. 5.47), showed the followingcfu: $3.4x10^6$ cfu/g after 6 weeks in 1% NaCl; $6.2x10^6$ cfu/g after 5 weeks in 2% NaCl; $3.7x10^6$ cfu/g after 3 weeks in 3% NaCl; $2.9x10^5$ cfu/g after 2 weeks in 4% NaCl; and $5.7x10^6$ cfu/g after 1 week in 5% NaCl (from initial $8.5x10^8$ cfu/g) during storage.

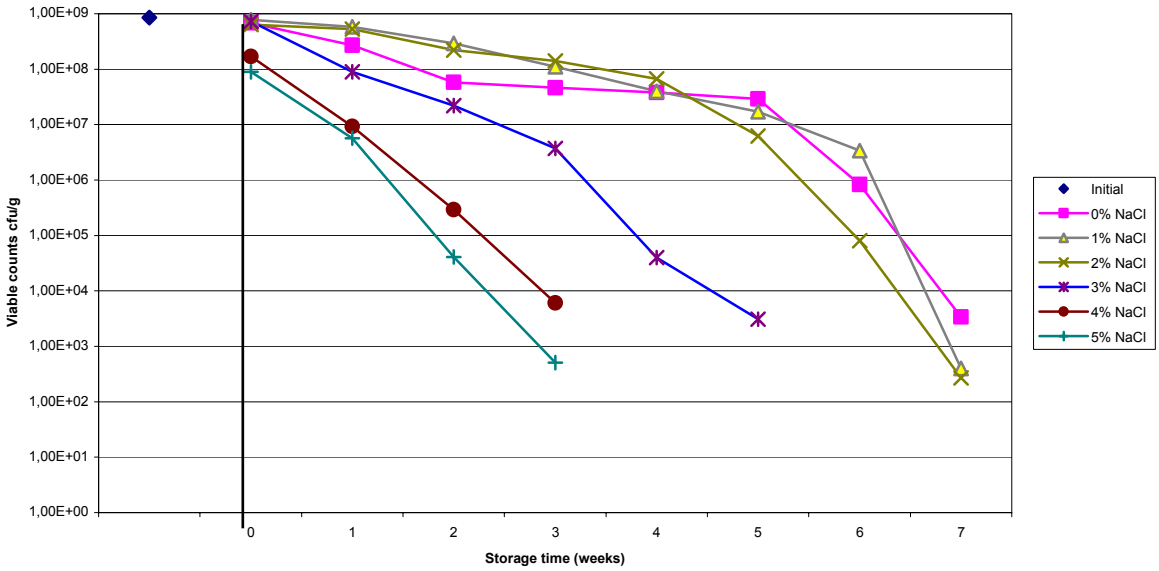


Fig.5.47: Effect of different NaCl concentrations on the survivability of microencapsulated *L. acidophilus* with whey protein and gum arabic during storage at 5°C

Microencapsulated cells with soy milk+G.A showed more stability to salt compared to the other capsule materials. However, as shown in Fig. 5.48, the viable counts reduced from $4.7x10^8$ to $1.9x10^6$ and $3.6x10^5$ cfu/g after 6 weeks in 1 and 2% NaCl respectively; $6.2x10^5$ cfu/g after 5 weeks in 3% NaCl; and $2.1x10^5$ and $1.3x10^5$ cfu/g after 2 weeks in 4 and 5% NaCl respectively during storage.

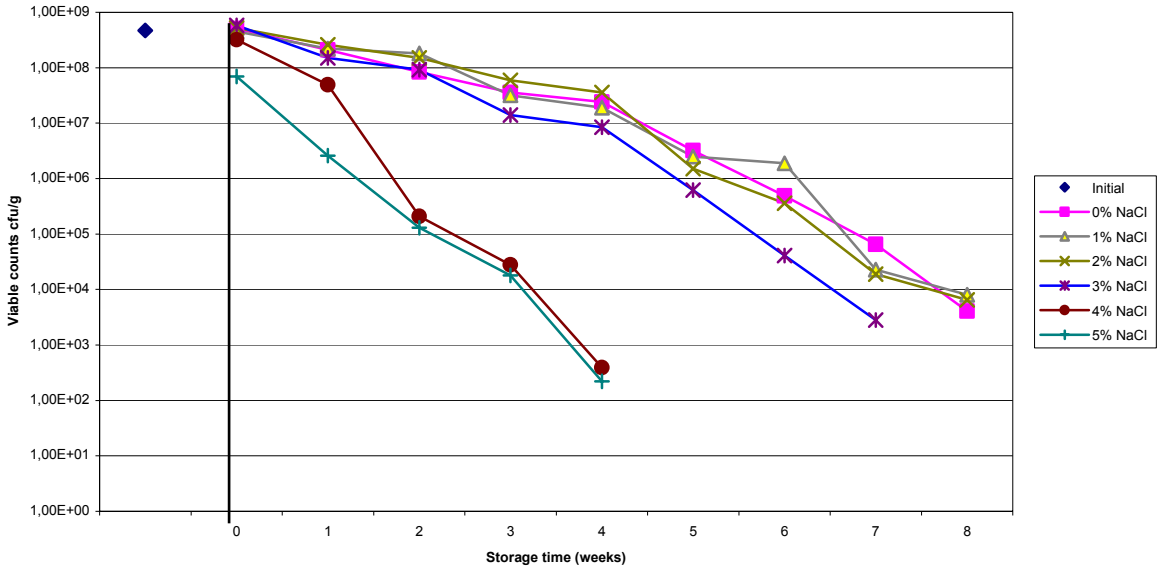


Fig. 5.48: Effect of different NaCl concentrations on the survivability of microencapsulated *L. acidophilus* with soy milk and gum arabic during storage at 5°C

5.9 Viability of free and microencapsulated *L. acidophilus* in the presence of organic acids

Figs. 5.49-5.54 contain data obtained with free and microencapsulated *L. acidophilus* in different lactic acid solutions during storage at 5°C with different pHs (3, 4, and 5) and distilled water (pH 6.4) as a control.

In general, the total viable counts decreased in all tested pHs with the time progress during storage.The most observed decrease was at pH 3.0 followed by pH 4.0 and pH 5.0 respectively as compared to the control (pH 6.4).

It should be mentioned also that the decline was more rapid for free cells than for microencapsulated cells.

At pH 3.0, the numbers remained at $6.8x10^5$ cfu/ml, $1.5x10^6$ cfu/g, and $2x10^6$ cfu/g after 1 week storage for free cells, gelatin, and gum arabic respectively; the numbers remained at $9.2x10^5$ cfu/g after 2 weeks for whey protein+G.A; and $2.4x10^5$ and $6.2x10^5$ cfu/g after 3 weeks for soy protein+G.A and soy milk+G.A respectively.

At pH 4.0, the numbers remained at $1.3x10^6$ cfu/ml after 1 week for free cells; $2.7x10^5$ and $4.5x10^5$ cfu/g after 2 weeks for gelatin and gum arabic respectively; $1.2x10^5$ and $9.1x10^5$ cfu/g after 4 weeks for soy protein+G.A and soy milk+G.A respectively; and $3.4x10^5$ cfu/g after 5 weeks for whey protein+G.A.

At pH 5.0, the numbers remained at $2.4x10^6$ cfu/ml, $7.7x10^5$ cfu/g, and $2.9x10^5$ cfu/g after 2 weeks for free cells, gelatin, and gum arabic respectively; $8x10^5$, $2x10^5$, and $1.6x10^5$ cfu/g after 5 weeks for whey protein+G.A, soy protein+G.A, and soy milk+G.A respectively.

Approximately similar results were observed for acetic (Figs. 5.55-5.60) and citric acids (Figs. 5.61-5.66).

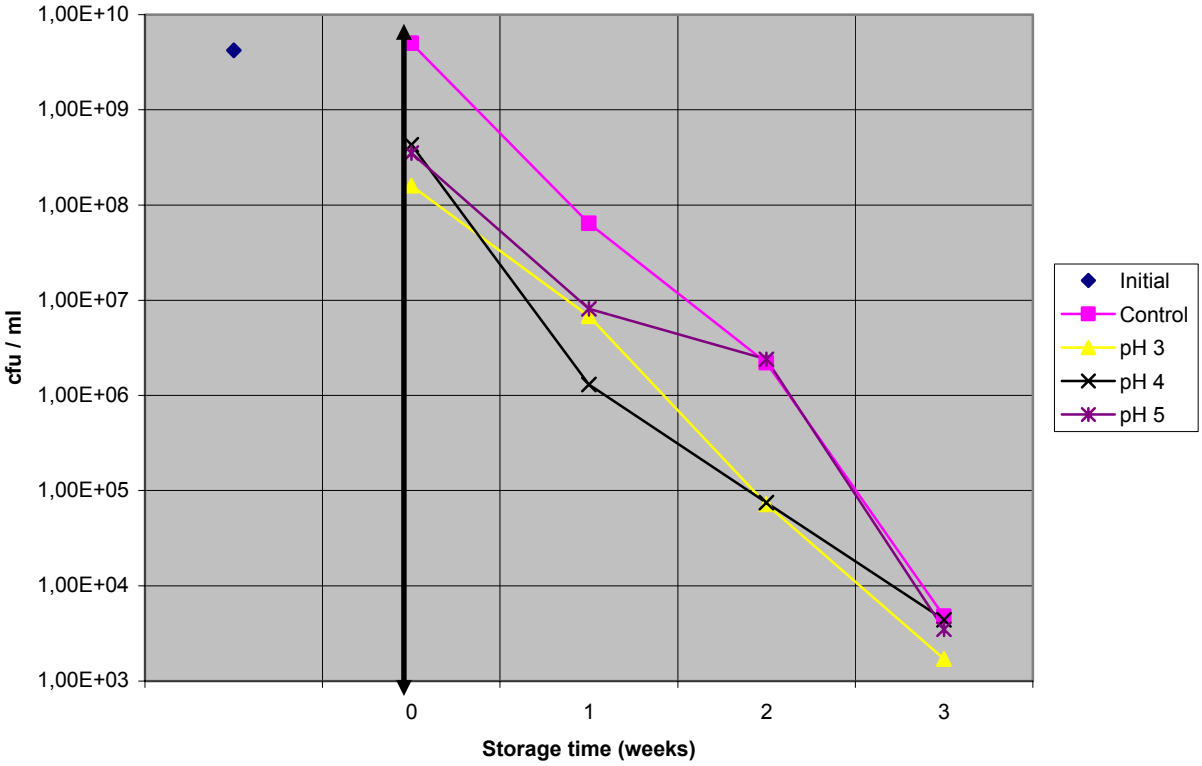


Fig. 5.49: Effect of pH values on the viability of free *L. acidophilus* in different lactic acid solutions during storage at 5°C

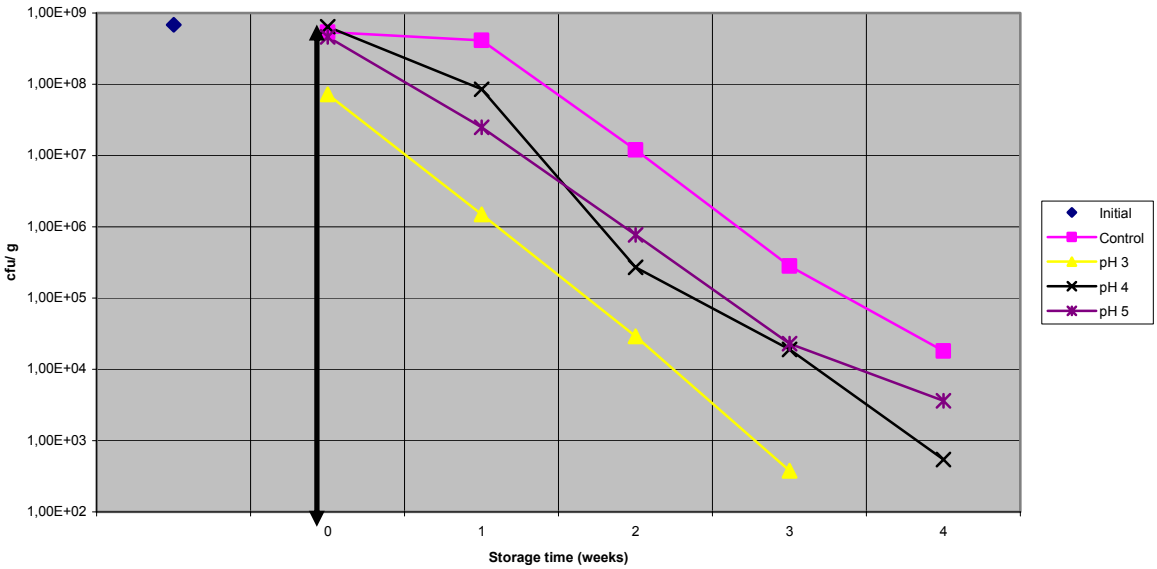


Fig. 5.50: Effect of pH values on the viabilty of microencapsulated *L. acidophilus* with gelatin in different lactic acid solutions during storage at 5°C

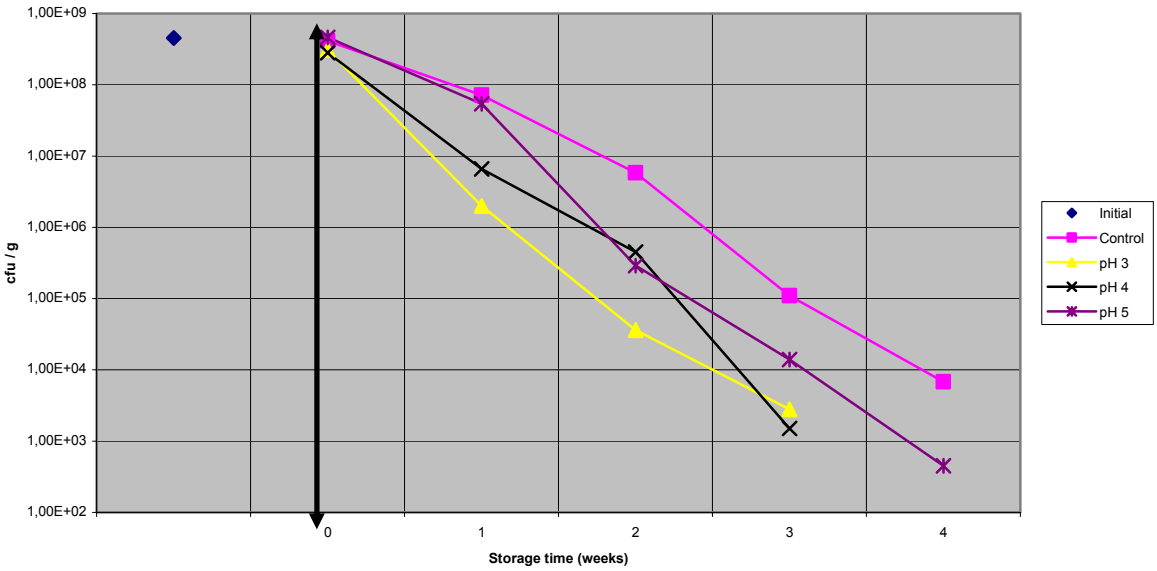


Fig. 5.51: Effect of pH values on the viability of microencapsulated *L. acidophilus* with gum arabic in different lactic acid solutions during storage at 5°C

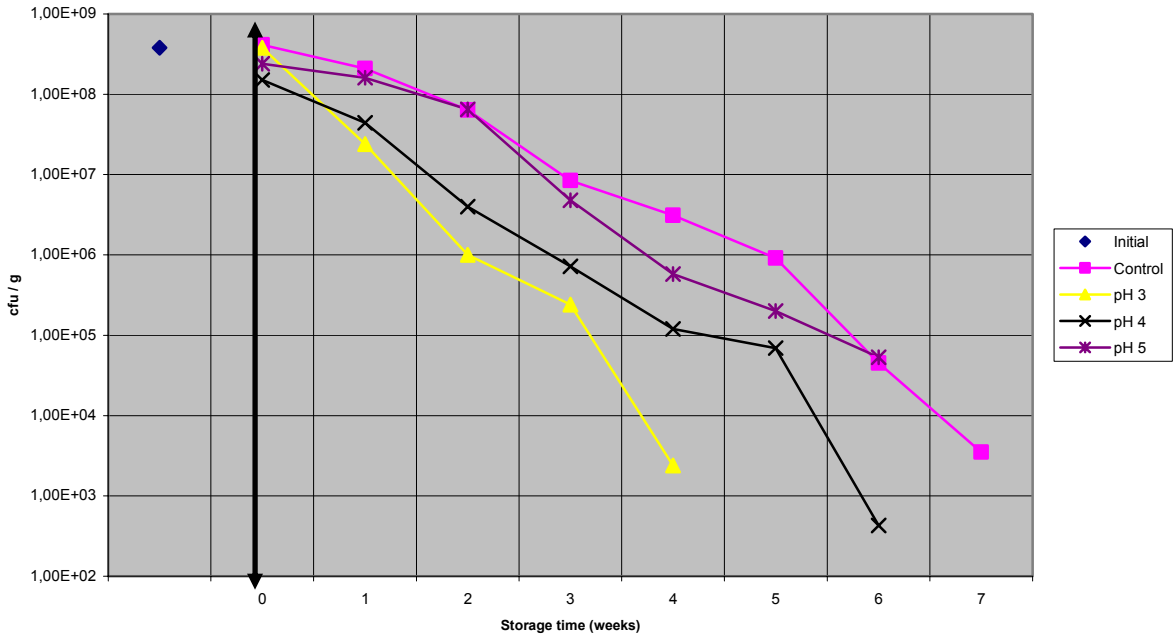


Fig. 5.52: Effect of pH values on the viability of microencapsulated *L. acidophilus* with soy protein and gum arabic in different lactic acid solutions during storage at 5°C

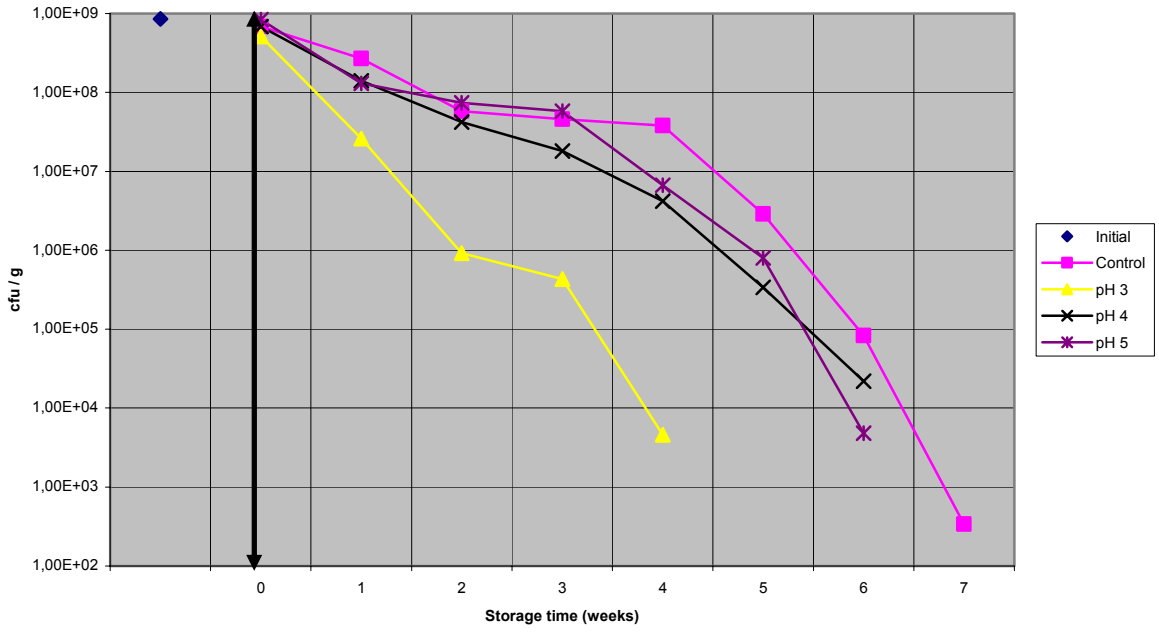


Fig. 5.53: Effect of pH values on the viability of microencapsulated *L. acidophilus* with whey protein and gum arabic in different lactic acid solutions during storage at 5°C

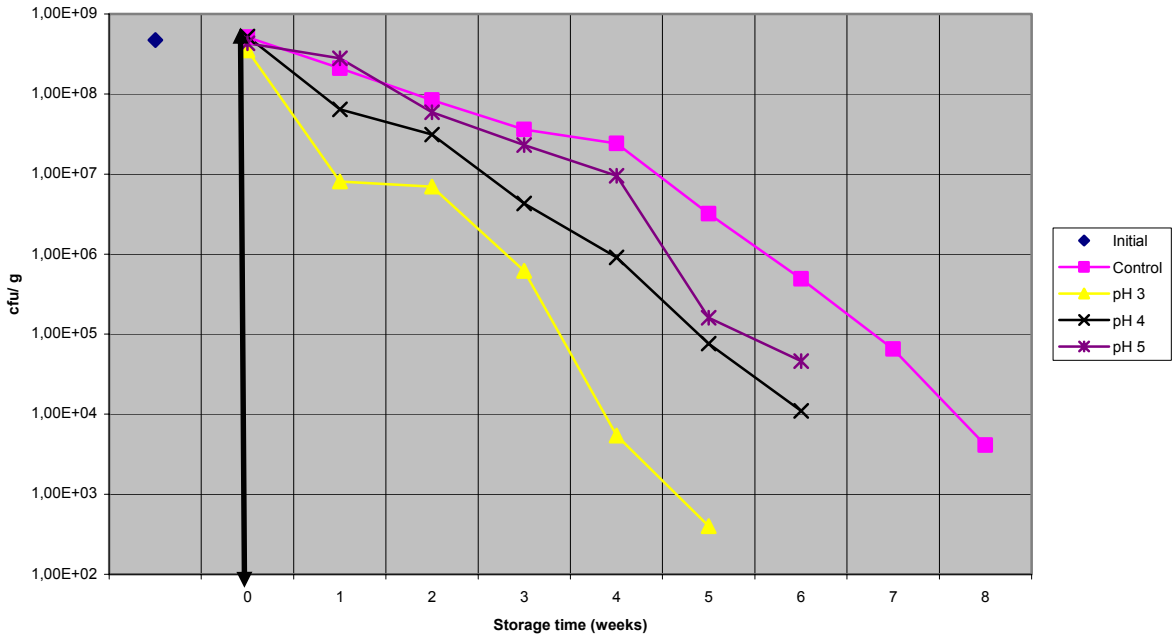


Fig. 5.54: Effect of pH values on the viability of microencapsulated *L. acidophilus* with soy milk and gum arabic in different lactic acid solutions during storage at 5°C

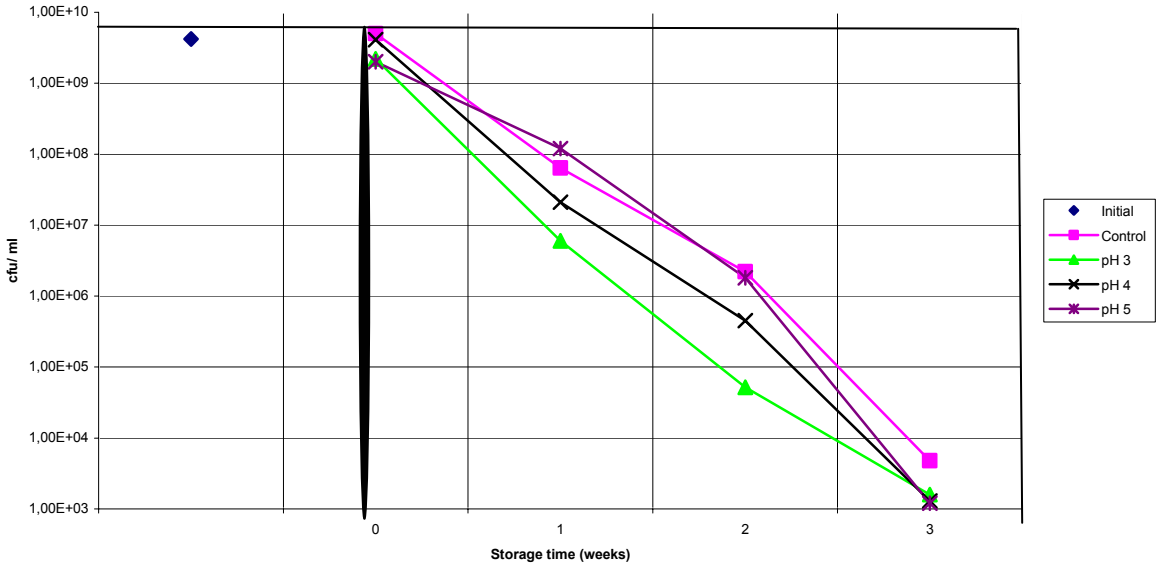


Fig. 5.55: Effect of pH values on the viability of free *L. acidophilus* in different acetic acid solutions during storage at 5°C

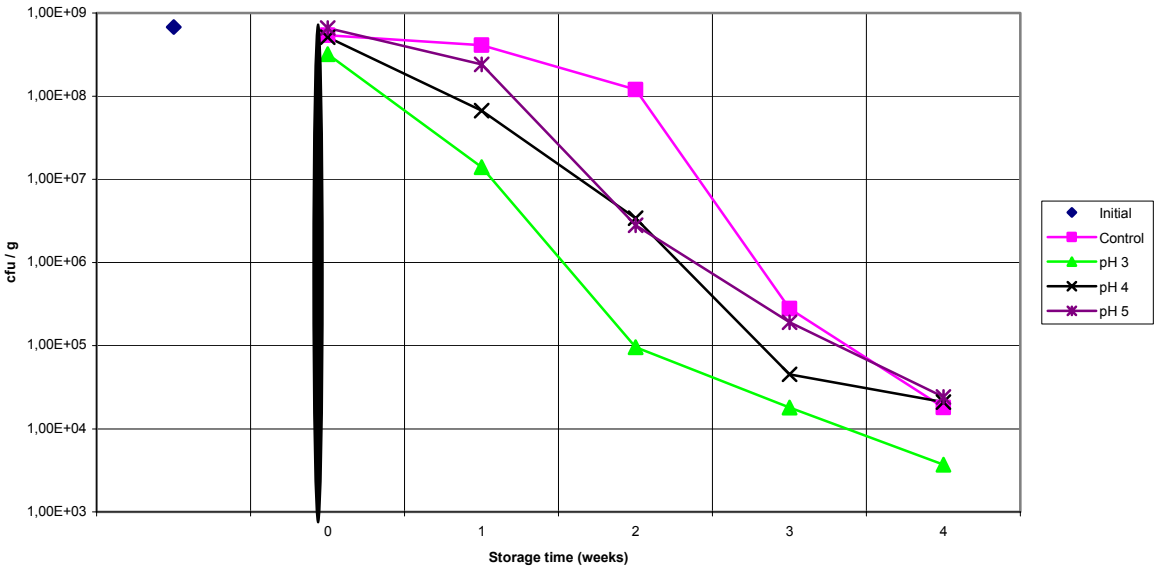


Fig. 5.56: Effect of pH values on the viability of microencapsulated *L. acidophilus* with gelatin in different acetic acid solutions during storage at 5°C

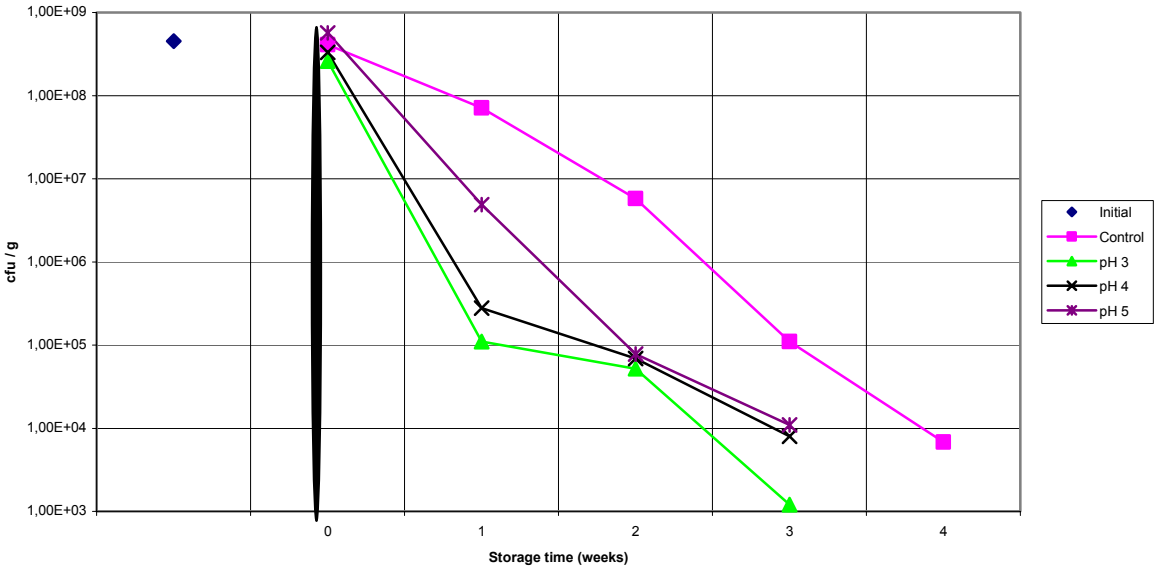


Fig. 5.57: Effect of pH values on the viability of microencapsulated *L. acidophilus* with gum arabic in different acetic acid solutions during storage at 5°C

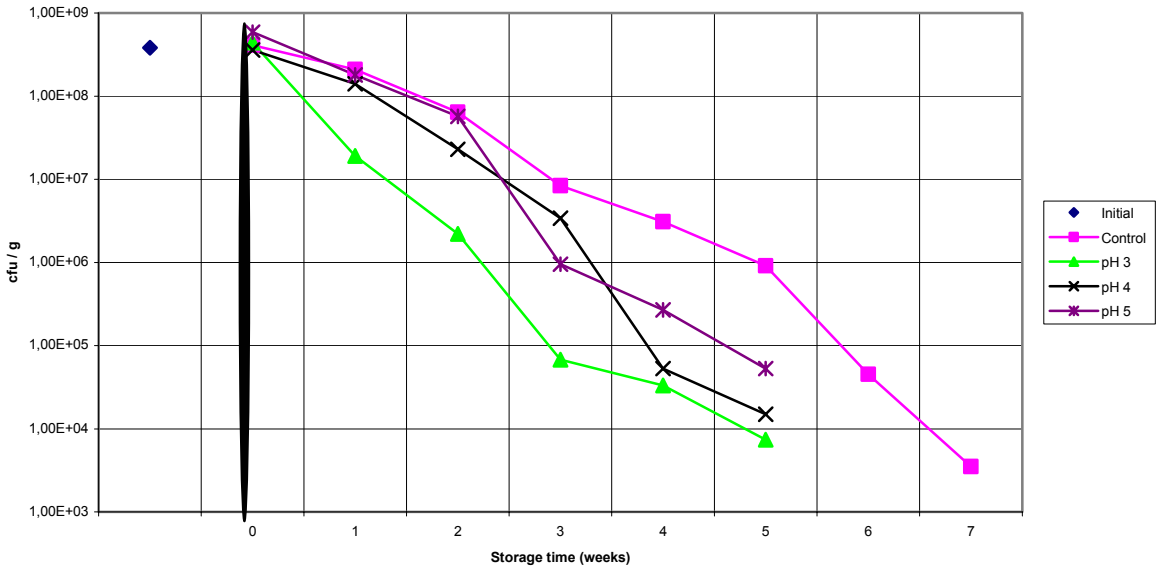


Fig. 5.58: Effect of pH values on the viability of microencapsulated *L. acidophilus* with soy protein and gum arabic in different acetic acid solutions during storage at 5°C

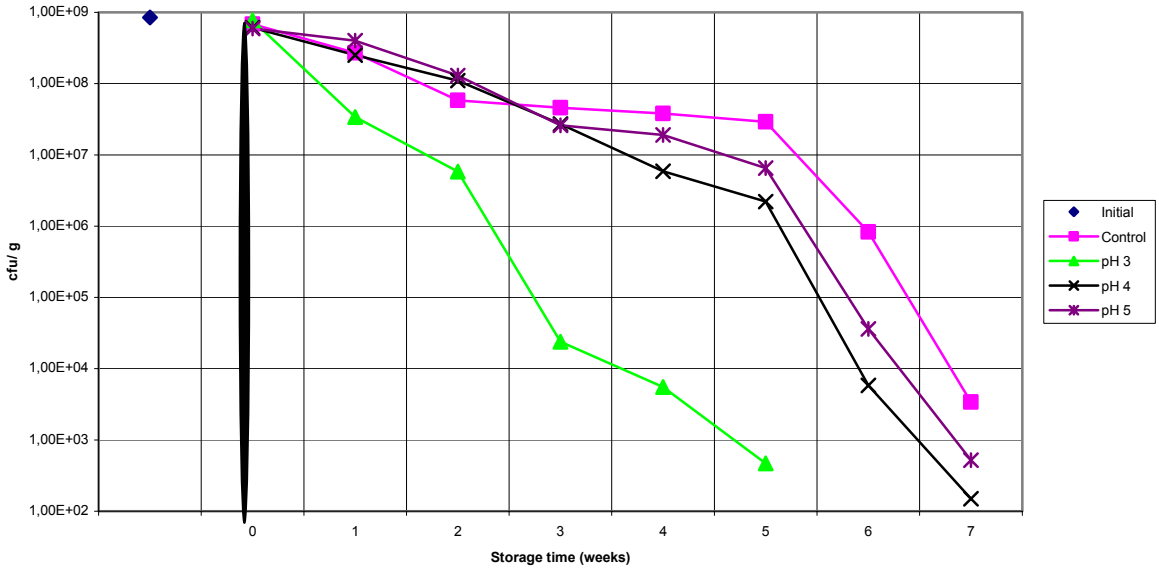


Fig. 5.59: Effect of pH values on the viability of microencapsulated *L. acidophilus* with whey protein and gum arabic in different acetic acid solutions during storage at 5°C

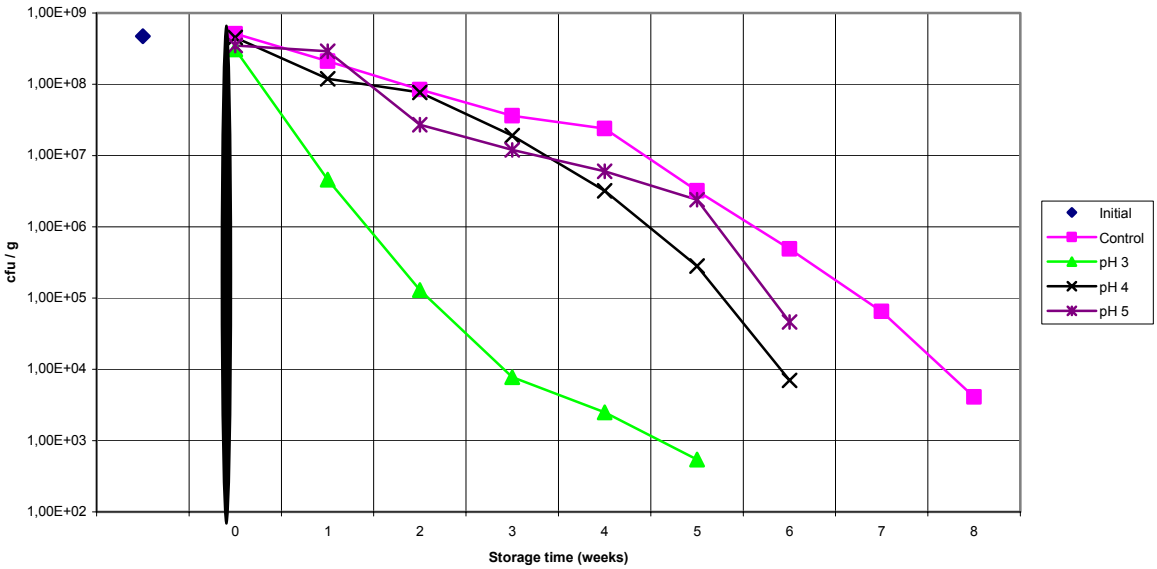


Fig. 5.60: Effect of pH values on the viability of microencapsulated *L. acidophilus* with soy milk and gum arabic in different acetic acid solutions during storage at 5°C

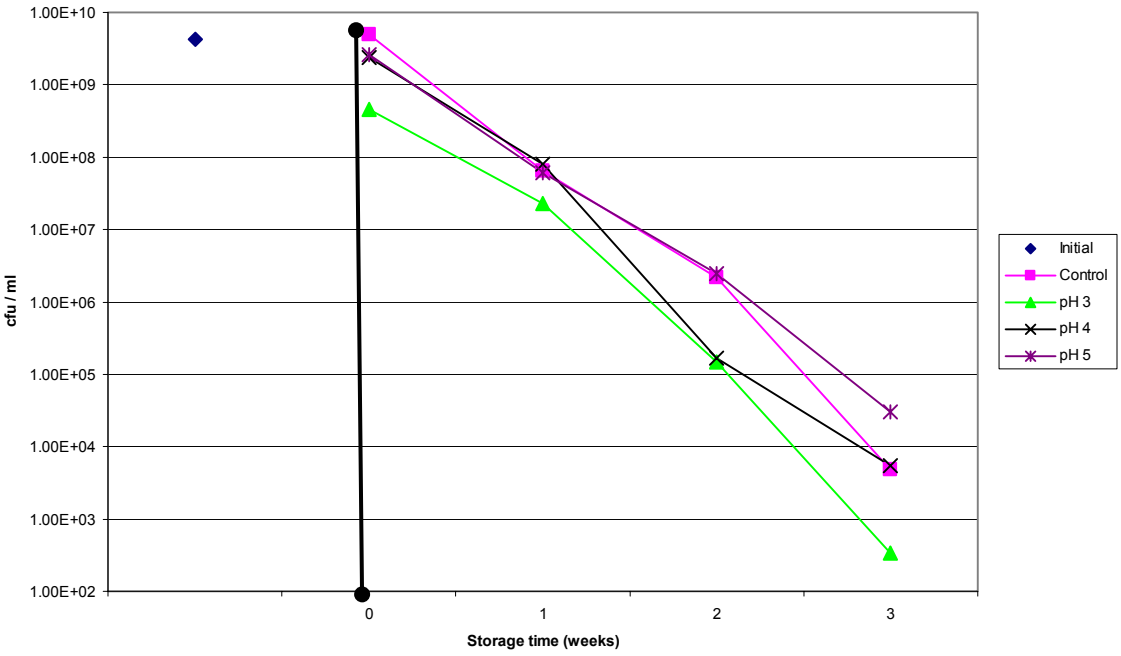


Fig. 5.61: Effect of pH values on the viability of free *L. acidophilus* in citric acid solutions during storage at 5°C

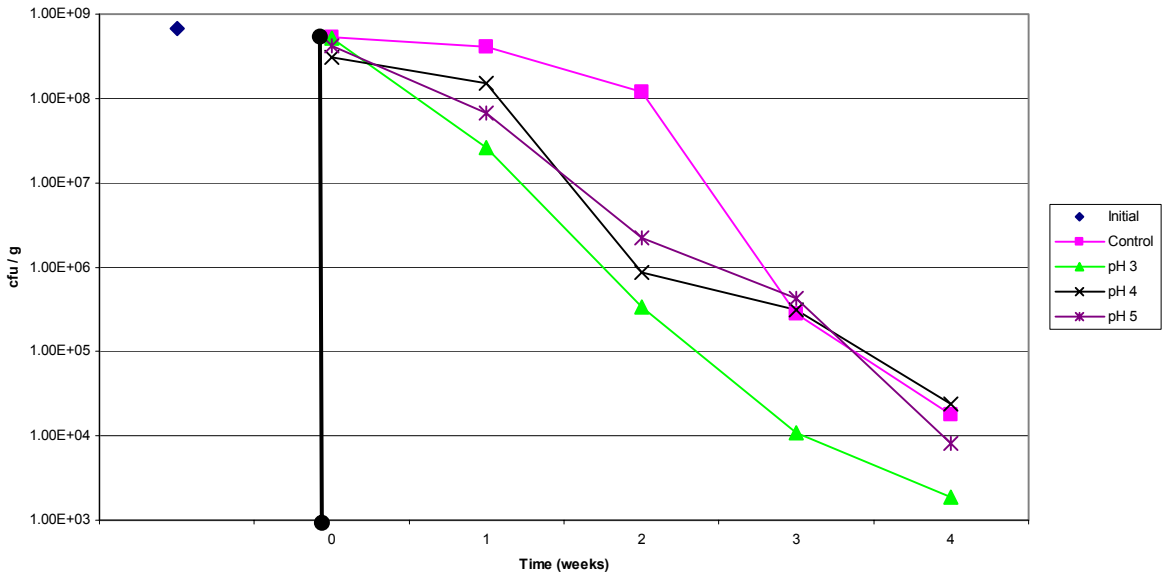


Fig. 5.62: Effect of pH values on the viability of microencapsulated *L. acidophilus* with gelatin in different citric acid solutions during storage at 5°C

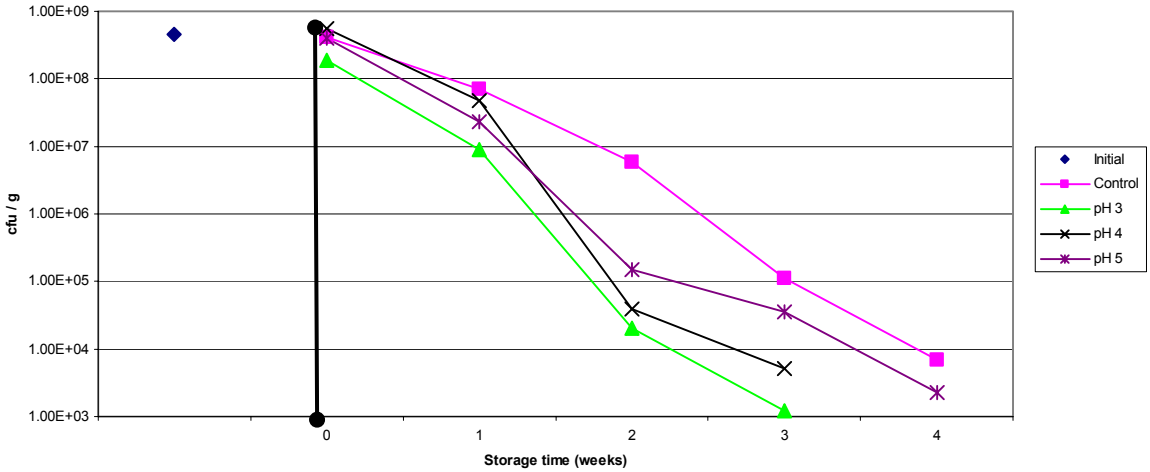


Fig. 5.63: Effect of pH values on the viability of microencapsulated *L. acidophilus* with gum arabic in different citric acid solutions during storage at 5°C

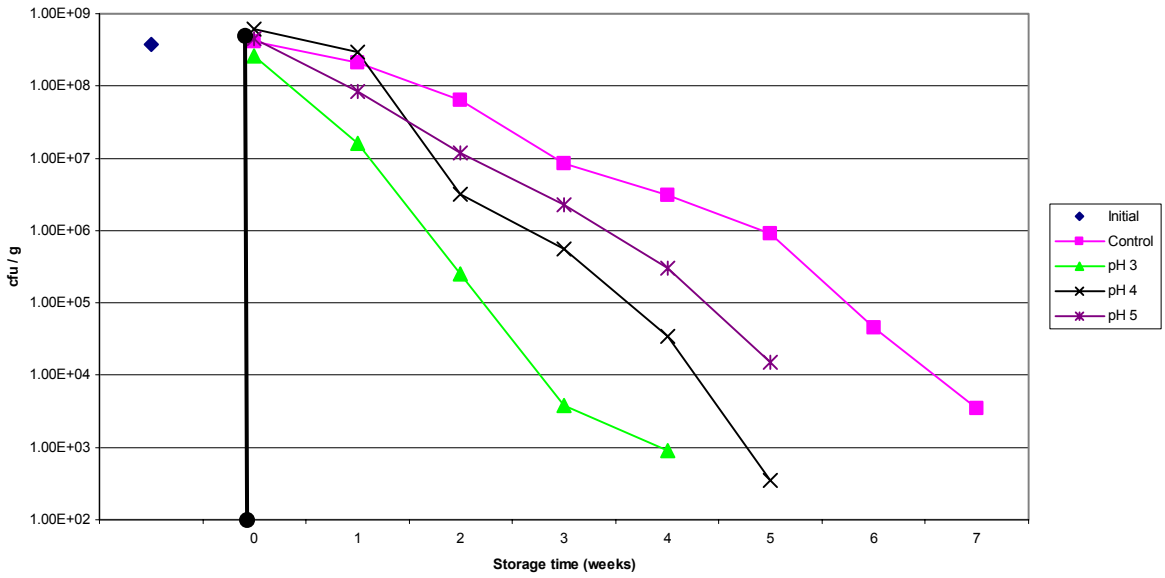


Fig. 5.64: Effect of pH values on the viability of microencapsulated *L. acidophilus* with soy protein and gum arabic in different citric acid solutions during storage at 5°C

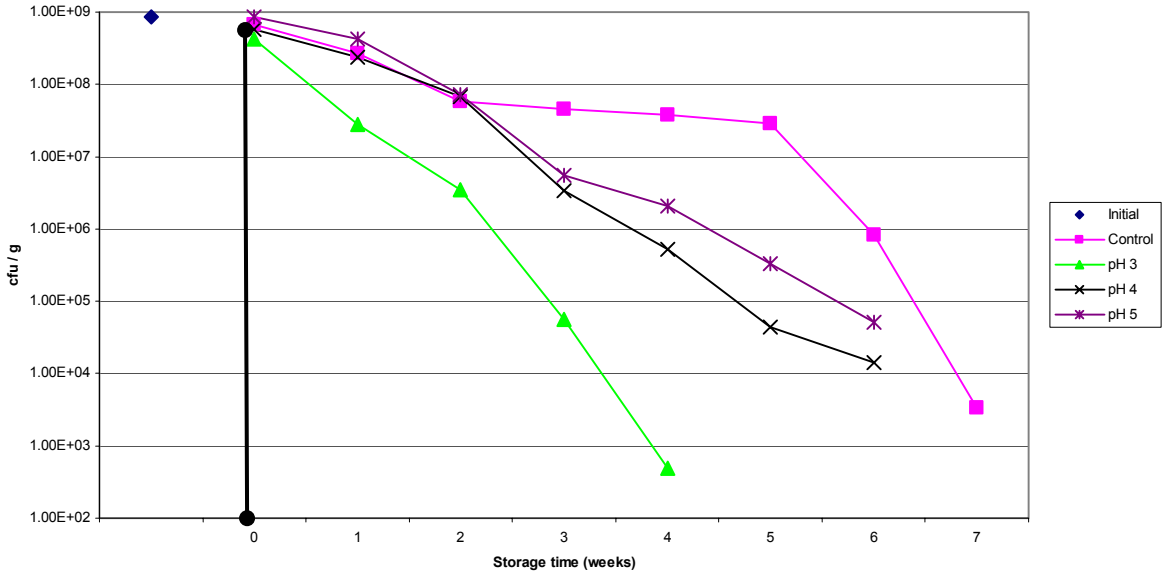


Fig. 5.65: Effect of pH values on the viability of microencapsulated *L. acidophilus* with whey protein and gum arabic in different solutions of citric acid during storage at 5°C

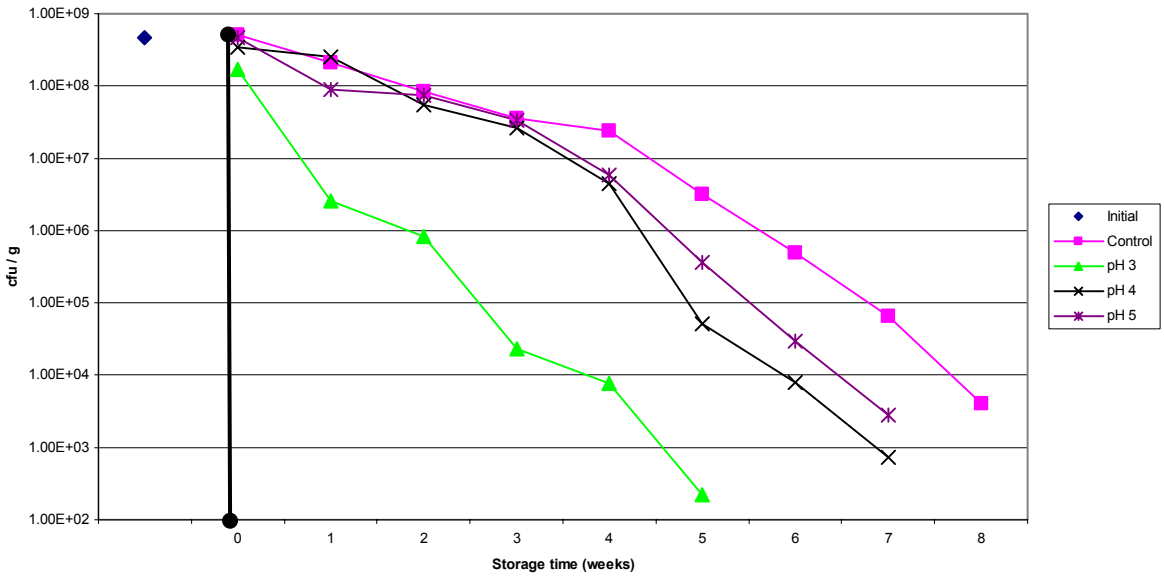


Fig. 5.66: Effect of pH values on the viability of microencapsulated *L. acidophilus* with soy milk and gum arabic in different citric acid solutions during storage at 5°C

5.10 Stability of free and microencapsulated *L. acidophilus* to simulated pH of human stomachs

The survival of free cells and microencapsulated *L. acidophilus* in: gum arabic; gelatin; whey protein+G.A; soy protein+G.A; and soy milk+G.A, under acidic conditions in HCl solutions (pH 1.0, 2.0, and 3.0) and distilled water (pH 6.4) as a control during 4 hours incubation at 37°C is illustrated in Figs. 5.67-5.72.

Viable counts of free cells (Fig. 5.67) decreased noteworthly even during the time (approximately 7 min) it took to initially plate the pHs 1.0 and 2.0 with almost three log cycles decrease in numbers compared to the initial ($2x10^9$ cfu/ml). And dropped to undetectable count (< 10^1 cfu/ml) after 2 hours incubation at the same pHs. At pH 3.0, free cells showed more acid tolerance than pH 1.0 and 2.0, as there was only one log cycle decrease in viable counts at 0 time. However, as shown in Fig. 5.67, viable counts of free cells reduced by 3 and 6 log cycles after 1 and 2 hours respectively at pH 3.0. However, no colony forming units could be detected after 3 hours incubation at pH 3.0.

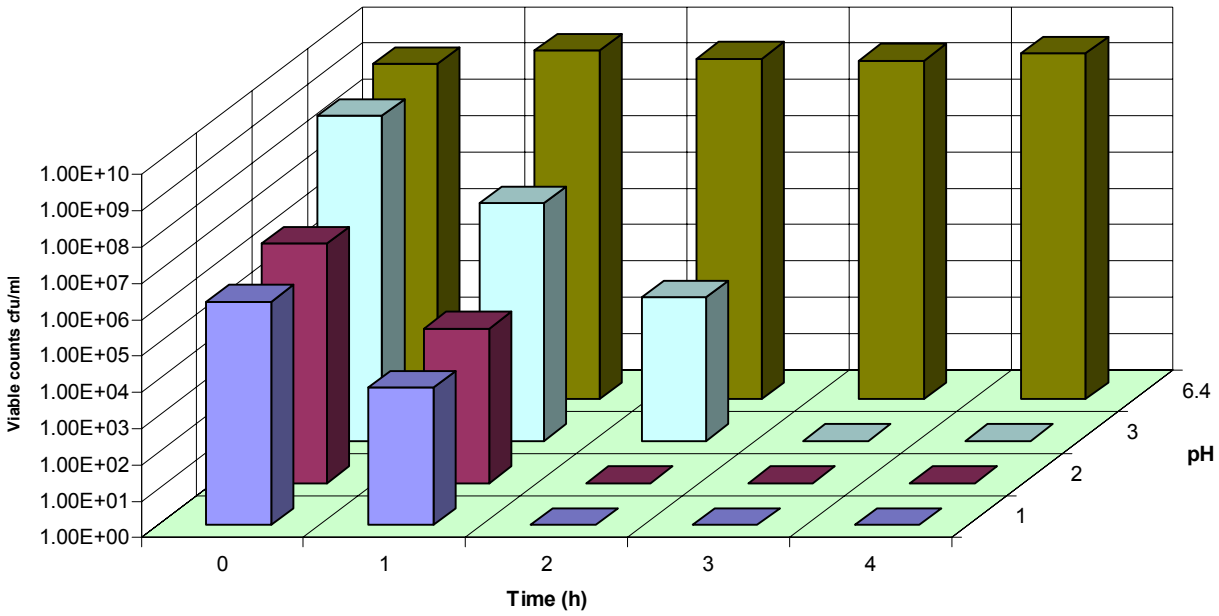


Fig. 5.67: Effect of pH on the survival of free *L. acidophilus* in HCl solutions during 4 h incubation at 37°C

On the other hand, microencapsulated cells survived obviously better than the free cells in all tested pHs. During the time it took to conduct the initial plating, the numbers for gelatin (Fig. 5.68) decreased (from $5.2x10^9$ cfu/g) by approximately 2, 2, and 1 log cycle(s) at pH 1.0, 2.0, and 3.0 respectively. Similar results were observed for gum arabic from an initial $6.4x10^9$ cfu/g (Fig. 5.69). For whey protein+G.A (Fig. 5.70), soy protein+G.A (Fig. 5.71), and soy milk+G.A (Fig. 5.72), the numbers decreased by 2 and 1 cycle(s) at pH 1.0 and 2.0 respectively from initials $6x10^8$, $2.7x10^9$, and $1.2x10^9$ cfu/g for whey protein+G.A, soy protein+G.A, and soy milk+G.A respectively.

After 1 hour incubation at pH 1.0, a considerable viability of microencapsulated cells, 1.5x105 and 3.4x105 cfu/g for soy protein+G.A and soy milk+G.A respectively were still observed. Nevertheless, microencapsulated cells in all capsule materials were found to be slightly more acid tolerant than the free cells, there were still > 102 – 103 cfu/g after 2 hours incubation at pH 1.0. While after 1 hour incubation at pH 2.0, the viable counts of microencapsulated cells were more noticeable being, 4.7x105, 2.3x105, 9x105, and 2x107 cfu/g for gum arabic, gelatin, whey protein+G.A, and soy protein+G.A respectively; and 6.1x105 cfu/g for soy milk+G.A after 2 hours incubation at the same pH.

Generally, microencapsulated cells showed gradual decrease in their viable cell numbers during 4 hours incubation at pH 3.0 and remained overall with appreciable viability in that, 3.8x104 cfu/g for gum arabic after 2 hours; 3.3x105 and 4.6x105 cfu/g for gelatin and soy protein+G.A respectively after 3 hours; and 1.2x108 and 3.4x108 cfu/g for whey protein+G.A and soy milk+G.A after 4 hours incubation at the same pH were observed.

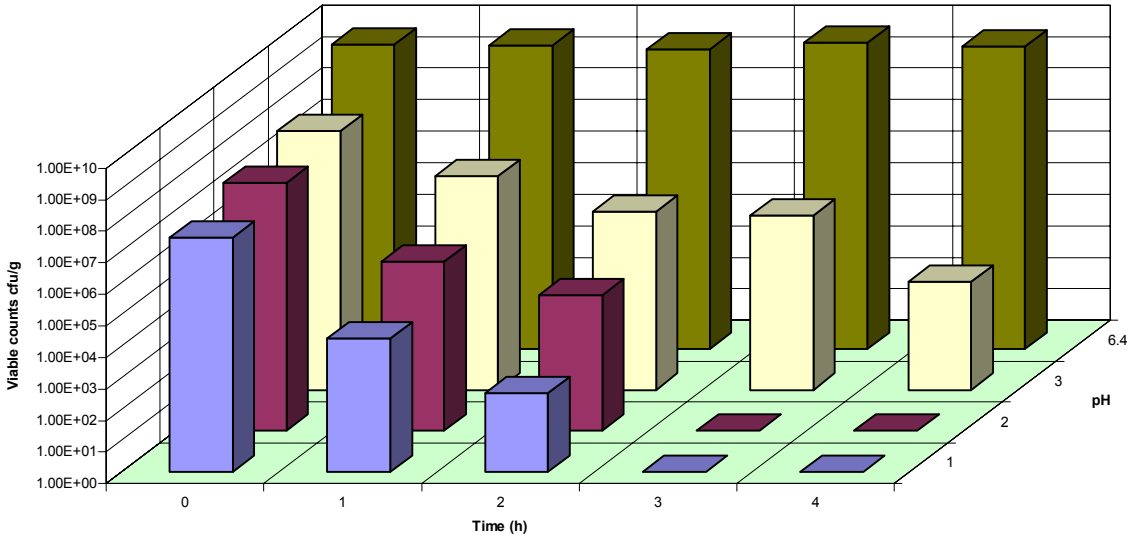


Fig. 5.68: Effect of pH on the survival of microencapsulated *L. acidophilus* with gelatin in HCl solutions during 4 h incubation at 37°C

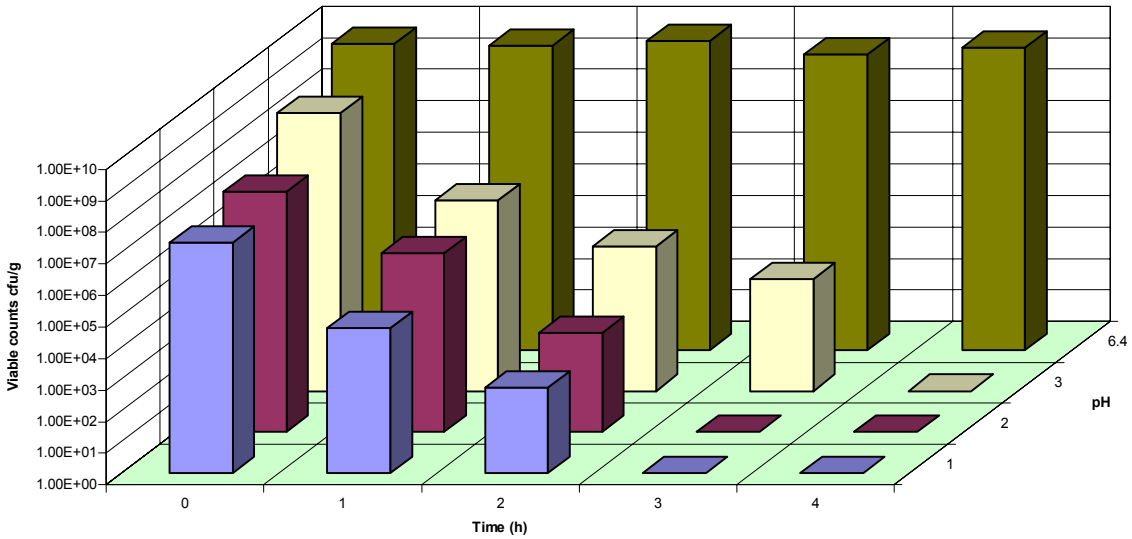


Fig. 5.69: Effect of pH on the survival of microencapsulated *L. acidophilus* with gum arabic in HCl solutions during 4 h incubation at 37°C

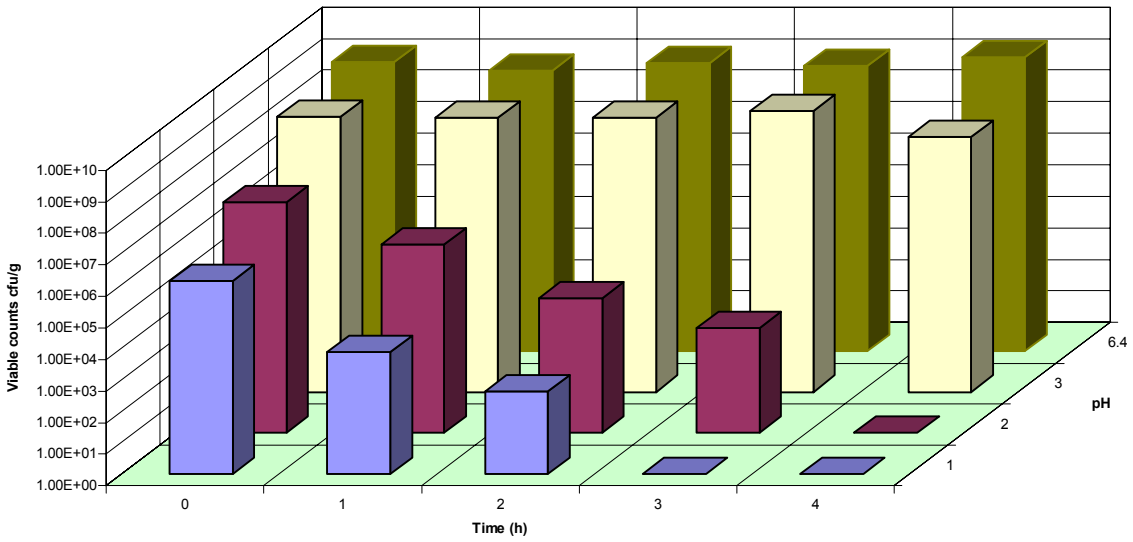


Fig. 5.70: Effect of pH on the survival of microencapsulated *L. acidophilus* with whey protein+gum arabic in HCl solutions during 4 h incubation at 37°C

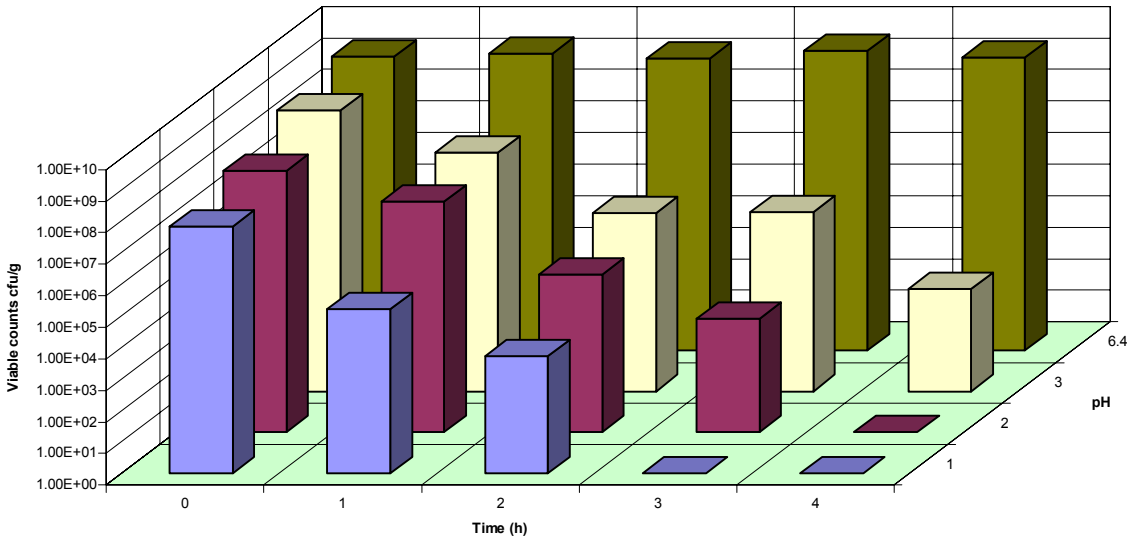


Fig. 5.71: Effect of pH on the survival of microencapsulated *L. acidophilus* with soy protein+gum arabic in HCl solutions during 4 h incubation at 37°C

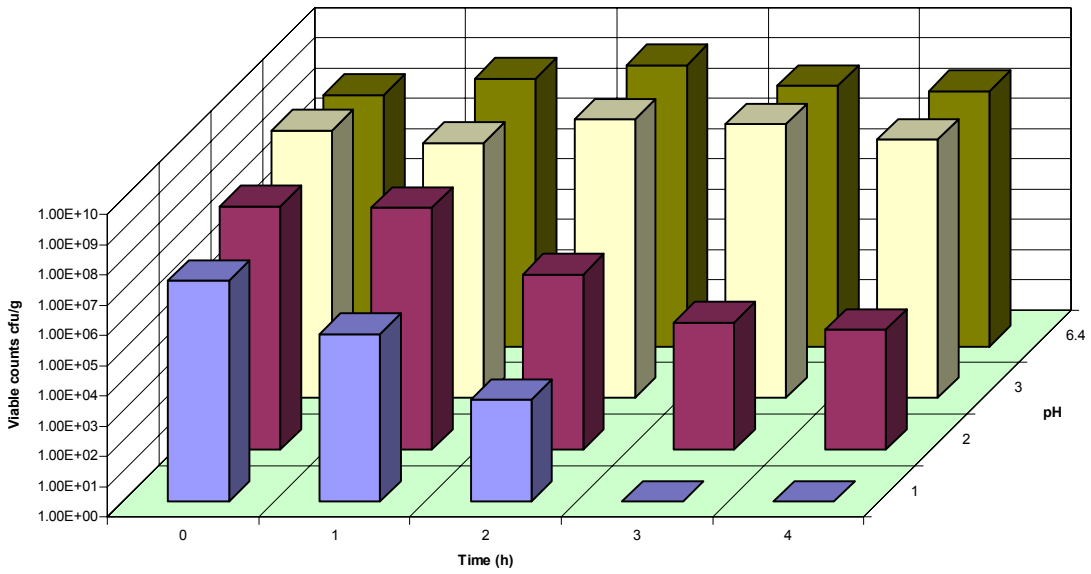


Fig. 5.72: Effect of pH on the survival of microencapsulated *L. acidophilus* with soy milk+gum arabic in HCl solutions during 4 h incubation at 37°C

5.11 Stability of free and microencapsulated *L. acidophilus* to simulated bile concentrations of human small intestines

The evaluation of *L. acidophilus* survival in bile salts, was carried out by the subjection of free and microencapsulated cells to 1%, 2% (maximum concentration found in the human small intestine), and 4% bile salts (twice the maximum concentration found in the human small intestine) (Davenport, 1977). Survivals were recorded after 1, 2, 3, and the maximum exposure up to 4 hours.

Incubation of free cells in the presence of 1% bile salts resulted in a gradually decrease of about 1, 3, 4, and 5 log cycles after 0, 1, 2, and 3 hours incubation respectively. However, no colony forming units could be detected after 4 hours incubation.

Free *L. acidophilus* declined by 2 log cycles at 0-time for both 2% and 4% bile concentrations as compared to the initial cell counts ($5.6x10^8$ cfu/ml). The viable counts decreased by 5 log cycles after 3 and 1 hour(s) incubation in 2 and 4% bile salts respectively (Fig. 5.73).

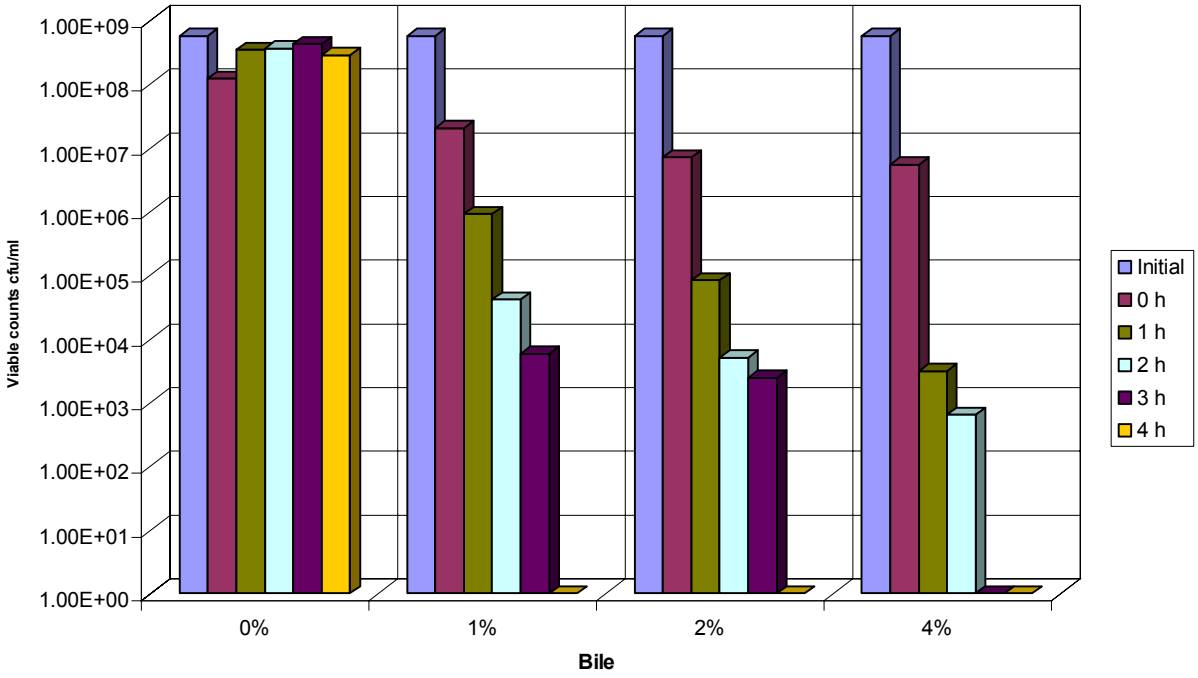


Fig. 5.73 Survival of free *L. acidophilus* in various bile concentrations during 4 h incubation at 37°C

The data of survivals of microencapsulated cells in high bile conditions are represented in Figs. 5.74- 5.78. The survivals obtained at 1% bile salts concentration during the time it took to conduct the initial plating showed a close relative numbers as compared to the initial cell count for all capsule materials. Considerable viability was observed up to 2 hours reached 2.6x105 and 1.9x105 cfu/g for gelatin and gum arabic respectively; and up to 4 hours reached 3.5x105, 1.2x105, and 9.9x106 cfu/g for whey protein+G.A, soy protein+G.A, and soy milk+G.A respectively.

In the case of 2% bile concentration, the microencapsulated cells showed only a little decrease (1 log cycle) at zero-time as compared to the initial cell counts. They could retain the recommended therapeutic-minimum numbers till:1 hour for gum arabic; 2 hours for gelatin, whey protein+G.A, and soy protein+G.A; and 3 hours for soy milk+G.A. After 4 hours incubation the numbers were, 1.8x103, 5.1x103, 4.6x103, and 1.5x104 cfu/g for gelatin, soy milk+G.A, soy protein+G.A, and whey protein+G.A respectively; and no detectable count was observed for gum arabic.

Microencapsulated cells were more impressionable to 4% bile concentration compared to 1% and 2%. Survivals at 0-time recorded decreases of 2 log cycles for gum arabic and only 1 log cycle for all other capsule materials. Then the numbers reached 2.4x103 cfu/g after 2 hours for gum arabic; and reached 3.8x102, 4.1x102, 8.1x102, 2.9x104 cfu/g after 3 hours for gelatin, whey protein+G.A, soy protein+G.A, and soy milk+G.A respectively.

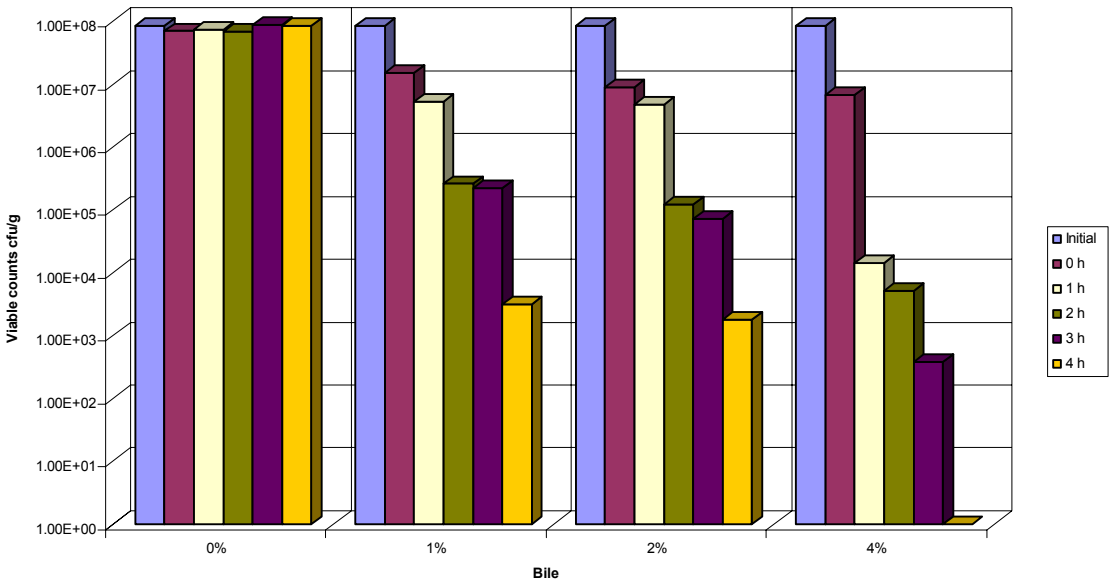


Fig. 5.74: Survival of microencapsulated *L. acidophilus* with gelatin in various bile concentrations during 4 h incubation at 37°C

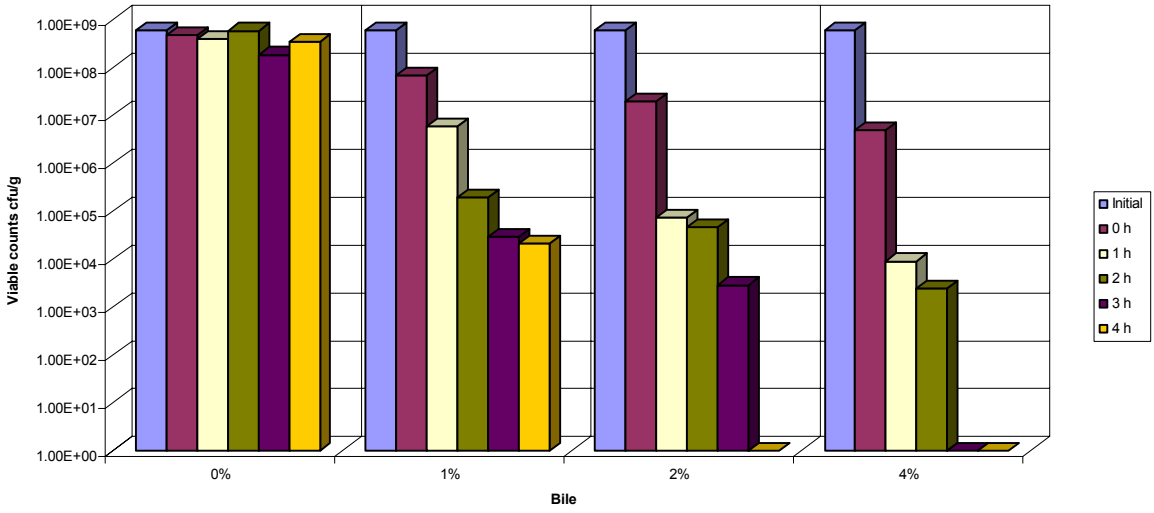


Fig. 5.75: Survival of microencapsulated *L. acidophilus* with gum arabic in various bile concentrations during 4 h incubation at 37°C

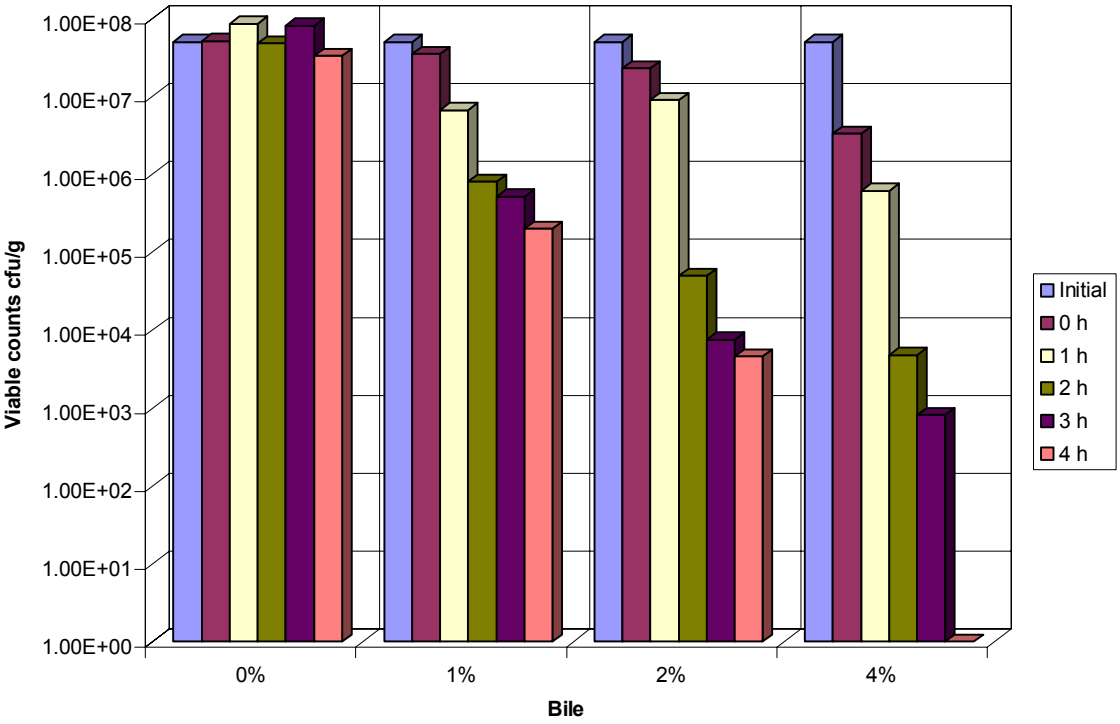


Fig. 5.76: Survival of microencapsulated *L. acidophilus* with soy protein+gum arabic in various bile concentrations during 4 h incubation at 37°C

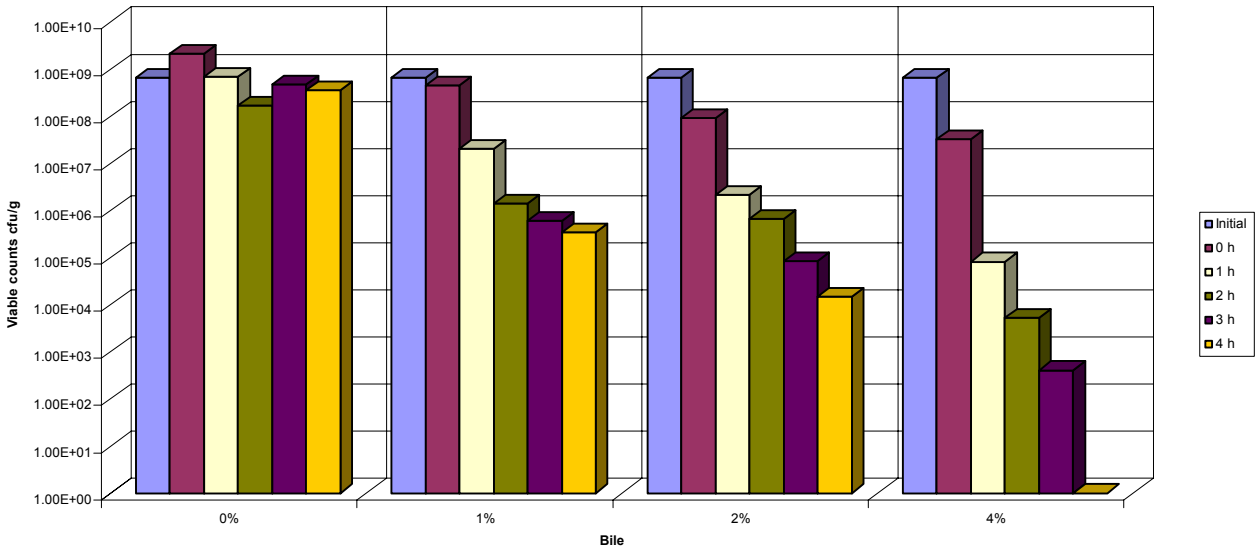


Fig. 5.77: Survival of microencapsulated *L. acidophilus* with whey protein+gum arabic in various bile concentrations during 4 h incubation at 37°C

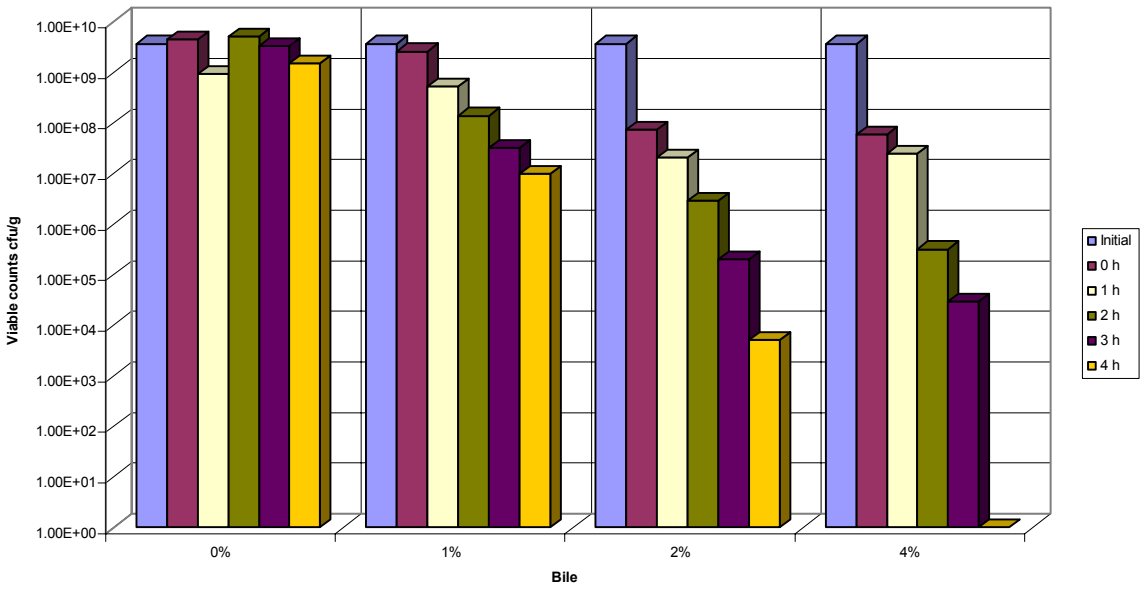


Fig. 5.78: Survival of microencapsulated *L. acidophilus* with soy milk+gum arabic in various bile concentrations during 4 h incubation at 37°C

6 Discussion

Functional foods have become more and more a daily food item. Specific mention needs to be here foods using probiotic bacteria. *L. acidophilus* and *Bifidobacterium* spp. constitute a major part of the natural microflora of the human intestine [Speck, 1978; Gilliland, 1979; Gilliland, 1989; Chitow and Trenev, 1990; Hammes and Tichaczeek, 1994] and when present in sufficient numbers create a healthy equilibrium between beneficial and potentially harmful microflora in the gut [Beck and Necheles,1961; Gilliland and Speck, 1977; Collins and Hardt, 1980; Anand *et al*., 1984]. Inclusion of live cultures of *L. acidophilus* and *Bifidobacterium* spp. in the diet produce several therapeutic benefits to the hosts [Shahani and Chandan, 1979; Gracey, 1981; Shah and Jelen, 1990; Hawkins, 1993; Hoover, 1993]. It has been suggested that to have any therapeutic effects, the minimal number of viable cells of *L. acidophilus* and *Bifidobacterium* spp. in a product should be $>10^5$/g [Speck, 1978; Kim, 1988].

Probiotic bacteria have to overcome four main different physiological and technological barriers from their fermentation to the food consumption. These are:
(1) Microencapsulation process stress,
(2) Stress caused by the storage time and conditions,
(3) Influence within the food product until consumption, and
(4) Stress within the intestinal tract.

However, it should be noted that these stress factors deteriorate the physiological activity of the living microorganism cells. Most bacteria cannot survive these stress factors. Probiotic bacteria have even shown reduced or poor viability. Further barriers can be distinguished in the intestinal tract. The most important barriers are:
(i) stomach acid,
(ii) bile salts, and
(iii) digestive enzymes.

The encapsulation process and the capsule material influence the viability as compared to when the bacteria were not encapsulated [Krückeberg et al., 2002]. In regard to the utility of microencapsulation, there are a few publications dealing with the effects of different capsule materials, especially polysaccharides (e.g., alginate) on lactic acid bacteria [Larish, 1994; Kim et al., 1996; Khalil, 1998]. However, all indications are that proteins play an important role in the stabilizing effect of encapsulated bacteria. In this study *L. acidophilus* served as a model microorganism for other probiotic bacteria to investigate the influence of different types of capsule materials in the stability of encapsulated probiotic bacteria.

6.1 Survival characterization of *L. acidophilus* after microencapsulation process

Spray drying, which has high production rate and low operation cost, is a well-known technology in the food industry. It is one of the common methods used to prepare food adjuncts which are dry, stable and occupy small volume [Potter, 1980]. In addition, spray drying is used for the preservation and concentration of microorganisms [Fu and Etzel, 1995; Teixeira et al., 1995; To and Etzel, 1997a,b]. Furthermore, the use of spray drying to prepare starter cultures which are used to prepare lactic-fermented products or used as adjuncts to enhance the flavor of cheese have been reported by various investigators [Johnson and Etzel, 1993; Johnson et al., 1995; To and Etzel, 1997a,b]. However, microorganisms are subjected to heat and dehydration damage during spray drying. Therefore, the survival of microorganisms becomes crucial if spray drying is employed for the preparation of microbial culture. To date, numerous studies concerning the survival of various lactic cultures affected by spray drying have been reported by various investigators [Espina and Packard, 1979; Kim and Bhowmik, 1990; Fu and Etzel, 1995; Johnson and Etzel, 1995; Teixeira et al., 1995; To and Etzel, 1997a].

In this study, *L. acidophilus* was subjected to spray drying with various capsule materials and the survivability after spray drying was investigated. The outlet air temperature was a major parameter affecting number of survivors. Suitable conditions were: inlet air 170°C, outlet air 55°C.

It was reported that the stage of growth affects the heat resistance of microoganisms, which are least sensitive to heat at their stationary phase [Hurst and Collins, 1974; Teixeira et al., 1994]. After subjecting *L. delbrueckii* ssp. *bulgaricus* to spray drying, Teixeira et al. [1995] recommended the drying of these cells in their stationary growth phase to obtain a high number of viable cells. Hence, in the present study, *L. acidophilus* cells in their stationary phase were collected and spray dried.

In all instances in this investigation, it was found that spray drying resulted in the reduction of viable *L. acidophilus* with only a population reduction of ca. 1.0 – 2.0 log/g dry weight under the test conditions. The powder obtained after spray drying contained *L. acidophilus* with a population of approximately 10^8 – 10^9 cfu/g dry weight, meeting the number required for use as a probiotic product [Ishibashi and Shimamura, 1993].

In addition to differences in chemical characteristics, the capsule materials possessed different physical properties such as thermal conductivity, thermal diffusivity, etc [Lian et al., 2002]. Therefore, it is reasonable to expect that these carriers tested in the present

study may exert different degrees of protective effect on the microencapsulated cells of *L. acidophilus* when subjected to heat inactivation during spray drying and, thus, lead to a different extent of the survival of *L. acidophilus.*

In the present study, 55°C outletair temperature was used and maintained during spray drying. Lethal thermal injury is the main reason for reduced cell viability [To and Etzel, 1997a]. Various investigators have reported that increasing outlet air temperature reduces the survival of microorganisms after spray drying [Lichari and Potter, 1970; Labuza et al., 1972; Espina and Packard, 1979; Kim and Bhowmik, 1990; To and Etzel, 1997a,b). Espina and Packard. [1979] spray dried *L. acidophilus* with reconstituted non-fat dry milk and observed a sharp decrease in number of survivors as the outlet air temperature was raised. To and Etzel, [1997a,b] spray dried *B. linens* with condensed skim milk and indicated that the percentage of survival of *B. linens* was halved for every 5°C increase in the outlet air temperature.

The feed concentrations in this study were maintained mostly between 6-9%. Lian et al. [2002] demonstrated that, with 10% gelatin, gum arabic and soluble starch exhibited the highest percentage of survival for *B. infantis* after spray drying. Increasing the concentration of gelatin, gum arabic, and soluble starch caused survival reduction of the test organism after spray drying. These results are comparable with the observation of Espina and Packard [1979] on *L. acidophilus*.

Several factors for and against the survival of microorganisms are interrelated during the spray drying process. First, as water activity decreases on the surface of the particle, wet bulb temperatures are exceeded. It is at this point that bacteria may be subjected to killing temperatures [Ilizondo and Labuza, 1974]. However, it is also reported that bacteria are less sensitive to effect of heat in the intermediate moisture range [Karel, 1995]. On the other hand, higher solid content in the feed solution would result in large particles, which are subjected to greater heat damage than smaller ones. Microorganisms entrapped in the large particles would also be subjected to more heat damage [Espina and Packard, 1979]. This may explain the slightly decreased survival of *L. acidophilus* observed in the present study after spray drying.

The culture purity is the most important property of the culture to be used as dietary adjuncts. Gilliland and Speck [1977] evaluated a number of products which are marketed as sources of *lactobacilli* in dietary adjuncts. They identified the cultures using methods previously described [Gilliland and Speck, 1977]. All of the products tested indicated to be sources of *L. acidophilus* (based on information supplied on the products labels). Only three of the products (one from a pharmacy and two from dairy

cases) contained organisms identified as *L. acidophilus.* One of these products which was supposed to contain only *L. acidophilus* also contained *L. casei* and *L. plantarum*. In the present study, the culture was identified by Api test using the growth characteristics (Tab. 5.1). The data were comparable with: the manufacturer's API test recommendations for *lactobacilli* strains identification; Kandler and Weiss [1986]; and Sriivas et al. [1990] as a pure *L. acidophilus* culture. The powder purity of microencapsulated *L. acidophilus* after spray drying was also evaluated by plating and counting on MRS agar, plate count agar and malt extract agar.

The present study evaluated the acidification kinetics of free and microencapsulated *L. acidophilus*. The rate of acidification of MRS broth using free and microencapsulated *L. acidophilus* was slower for microencapsulated cells than that observed for free cells which incubated under similar conditions. A similar pattern was also observed by Sultana et al. [2000]. They found that the inoculated RSM (reconstituted skim milk) broth medium with free *L. casei* and *L. acidophilus* achieved a pH of 5.0 after 6 hours, while it took more than 30 hours for the encapsulated cells to attain that same pH point. Larish et al. [1994] found that the encapsulated cells took 17% longer than free *lactobacilli* to reduce the pH of milk to 5.5. This may be due to the slower uptake of nutrients and the slower release of metabolites across the microencapsulating agents.

6.2 Stability of microencapsulated *L. acidophilus* during storage

One very important property of the culture to be used as a dietary adjunct is that the organism should remain viable during storage before consumption. However, such dietary cultures will not play an effective biological role in products unless they are present in sufficient viable numbers by the time of consumption. For this reason, changes in the population of viable bacteria during the expected shelf-life of product in question should be known to some extent and taken as a basis for selection criteria of such strains.

To have any desirable health effects. *L. acidophilus* cells must be present throughout the shelf-life of acidophilus products, and their detectable counts should be over 10^6 cfu/ ml [Kurmann, 1988; Valdez and Giori, 1993; Medina and Jordano, 1995; Nighswonger et al., 1996]. However, it was demonstrated that the ineffectiveness of acidophilus products in improving the health claims, was due to insufficient viable counts of *L. acidophilus* [Gilliland, 1989; Fernandes et al., 1992]. *L. acidophilus* may die quickly during refrigerated storage and the counts of viable bacteria sufficient to be consumed by consumers were maintained only for 1 week [Kulp, 1931; Kosikowski, 1977]. There

was much doubt about the real numbers of viable cells that reach consumers. Therefore, it is important to deal with the stability of *L. acidophilus* under refrigerated storage. One of the main aims of the present study was to check the viability of microencapsulated *L. acidophilus* over a period of time under refrigeration.

The results of this study showed a great variability ranging from 4 to 15 weeks in the survival ability of microencapsulated *L. acidophilus* during storage at 5°C. The variability is highly dependent on the kinds of capsule materials. The decline in viability during refrigerated storage was more rapid for microencapsulated cells with gum arabic or gelatin as main capsule materials alone without soy protein, whey protein or soy milk or with other capsule materials. It is interesting to note that the mixtures of capsule materials using gum arabic + proteins or gum arabic + soy milk showed the best viability and had better viability than mixtures of capsule materials using gelatin + proteins or gelatin + soy milk. It could be concluded that polysaccharide–protein mixtures have more stable microcapsules than protein–protein mixtures. This could be attributed to the behaviour of polysaccharide–protein interactions.

Several reports have shown that the decimal death rate of *L. acidophilus* depends not only on the strains used but also on the carrier food [Schuler-Malyoth, 1968; Robinson, 1987]. Peitersen [1990], observed a decline in the cell counts of *L. acidophilus* over 3 weeks storage at 5°C. Medina and Jordano [1995], showed that the viable cells of *L. acidophilus* decreased rapidly throughout the storage time under refrigeration at 7°C, and that a significant decline in viability was observed between days 10 and 17.

In any investigation of acidophilus products stored under refrigeration over a given time, one of the main focuses should be on the minimum viable level required for the bacterium to be beneficial to dietetics and health. Schuler-Malyoth [1968] recommended that 10^6 cfu/ml viable cells for *L. acidophilus* products should be treated as the "therapeutic-minimum counts", similarly, Kurmann [1983] stated that the viability of each prophylactically important species or strain should not be less than 10^8 cfu/ml after production, and 10^5 to 10^6 cfu/ml at the end of shelf life of the products. The present investigation showed that after 10-11 weeks of storage at 5°C, microencapsulated *L. acidophilus* using gum arabic + soy milk, gum arabic + whey protein, and gum arabic + soy protein can maintain their viability which corresponds to the advised therapeutic-minimum dose. However, the present results show that the use of microencapsulated *L. acidophilus* may represent another approach to reducing viability loss under refrigerated storage.

6.3 Influence of heat treatment on free and microencapsulated *L. acidophilus*

It is well known that heat treatment influences the survival and biochemical activity of lactic acid bacteria (Mabbit, 1961; Speck, 1962; Dutta et al., 1973; Singh and Khanna, 1980; Singh, 1983; Gilliland, 1985; Reinheimer et al., 1995; Jeffery et al., 1997; Kim et al., 2001).

L. acidophilus and other *lactobacilli* are utilized world-wide for the manufacture of cheeses, yoghurts and fermented foods [Kosikowski, 1982]. Despite extensive industrial application, knowledge of physiological adaptations by lactic acid bacteria to processing conditions remains limited. Better understanding of adaptive responses in lactic acid bacteria is important because processing conditions often subject these bacteria to adverse environmental conditions, including temperature extremes [Porubcan and Stellars, 1979; Tamine, 1981; Kosikowski, 1982; Mäyrä-Mäkinen and Bigret, 1993;). The most thoroughly characterized stress response in bacteria and higher cells is heat shock [Ang et al., 1991].

Lactobacilli have an optimal growth temperature of 30 – 40°C. *L. acidophilus* prefers temperatures near 40°C [Kandler and Weiss, 1986].

In the present investigation, free *L. acidophilus* was exposed to 37, 40, 45, 50, 55, and 60°C/30 min. 60°C was chosen as the lethal temperature due to the extreme reduction of the viable cells at this temperature. This observation on the survival of *L. acidophilus* at high temperatures agrees with the findings of Kim et al. [2001]. They reported 53°C as the sublethal temperature for *L. acidophilus* because cells were still growing at this temperature whilst 60°C was chosen as a lethal temperature. These findings are also in agreement with that of Reinheimer et al. [1995]. They stated that the acidifying and proteolytic activities of thermophilic lactic acid bacteria belonging to the genus *Lactobacillus* were high at temperatures ranging from 37 to 50°C and negligible at 55°C. On the other hand the results for microencapsulated cells demonstrated more thermotolerant cells at 60°C as compared to free cells. Microencapsulated *L. acidophilus* were less thermotolerant at higher temperatures (63°C and 65°C). However, microencapsulated cells with whey protein + gum arabic, soy protein + gum arabic, and soy milk + gum arabic exhibited better viability than microencapsulated cells with gum arabic and gelatin, which were approximately similar to the free cells.

Survival mechanisms exhibited by bacteria when confronted to stress are generally referred to as the stress response. One survival mechanism is the adaptive response. That is, when cells are exposed to a moderate level of stress, they acquire increased

resistance to a subsequent exposure to a more severe level of the same stress [Csonka and Hanson, 1991; Demple, 1991; Foster and Hall, 1991]. This mode of stress response allows bacteria to survive stress, which may normally be lethal. The higher viability of microencapsulated cells (exposed to spray drying temperature) towards heat treatments could be explained by this theory.

Thermotolerance experiments by Jeffery et al. [1997] showed that heat shock significantly improved the ability of *L. acidophilus*, *L. casei* and *L. helveticus* to withstand a 20 min exposure to high temperatures. Heat shock at 42, 50 and 52°C, were the most effective temperatures used. The rise in thermotolerance was about 27-fold in *L. acidophilus*, 11-fold in *L. helveticus* and 5-fold in *L. casei.*

In the present study, *L. acidophilus* cells were spray dried in their stationary phase. It was reported that the stage of growth affects the heat resistance of microoganisms, which are least sensitive to heat at their stationary phase [Hurst and Collins, 1974; Teixeira et al., 1994]. These findings confirm those reported by Kim et al. [2001]. They noted that the survival of the stationary phase cultures of *L. acidophilus* was significantly higher than that of the log phase cultures for bile and heat stresses.

It should be noted here that, it is probable that the ability of the cultures to grow at high temperatures and to produce acidic environment does not allow the presence of non-lactic microorganisms. Also, their ability to grow at high temperatures permit food-making at temperatures unfavorable to many potential mesophilic and psychrotrophic contaminants.

6.4 Survival of free and microencapsulated *L. acidophilus* in some deliberately simulated food conditions

The probiotic cultures must tolerate the manufacturing process so as to prepare a bioproduct and maintain cell viability during storage. Strain survival in the product will depend on many factors such as pH, presence of preservatives [Charteries et al., 1998] and even the occurrence of potential microbial growth inhibitors [Collins et al., 1998]. Some common additives used in the food and dairy industry are salts, sugars, organic acids etc. The effect of additives on the growth of lactic acid starter and probiotic bacteria have not been extensively studied [Samona and Robinson, 1993; Gomes et al., 1998; Rada and Dlabal, 1998; Vachon and Ustunol, 1998] and further information is still needed [Vinderola et al., 2002]. Beyond the additives used, some products of the lactic acid starter metabolism, such as mainly lactic acid, could be associated with the

loss of viability of added probiotic bacteria [Post, 1996]. One aim of this work was to determine the effects of some additives such as sugar (sucrose), salt (sodium chloride), organic acids (citric acid and acetic acid) commonly used in food industry on the growth of *L. acidophilus.* The study also assesses the influence of compounds produced by the lactic acid starter bacteria during their growth (lactic acid). However, additives used in the food industry can significantly influence the growth and cell viability of lactic acid starter and probiotic cultures used for fermented products. The tolerance of starters and probiotic bacteria to food additives should be a selection criteria in order to achieve the best application of strains for optimizing their growth and cell viability during the industrial process and storage of the product [Vinderola et al., 2002].

NaCl is widely used in the food industry as a preservative agent, to impart sensory characteristics and to satisfy the human daily requirements. Additionally, sodium chloride is important for controlling cheese ripening [Reinheimer et al., 1997]. The salt content of fermented products might jeopardize the cell viability of the probiotic cultures [Gomes et al., 1998]. In this study, *L. acidophilus* appeared to be sensitive to NaCl.

Roy [1991] and Guinee and Fox [1993] noted that, among several cheese processing factors, salt content and storage temperature are easily manipulated and are known to clearly affect the degree of survival and activity of *L. acidophilus* and bifidobacteria in cheese matrix individually.

Gomes et al. [1998] reported that survival of *L. acidophilus* decreased with increasing sodium chloride level above 0.51 mol L^{-1}. Among several possible explanations for this observation, some authors [Irvine and Price, 1961; Fernandez-Salguero et al., 1986; Krämer, 1997] have suggested that the reductions in water activity effected by the increase in sodium chloride concentration could account for the decreased resistance and survival during storage. Proof for this assertion has been provided for *lactobacilli* involved in food fermentations [Montano et al., 1993] and may easily be extended to encompass the results of the present study effort. The independent salt effect on *L. acidophilus* probably because *L. acidophilus*, like many other enteric bacteria, is sensitive to osmotic stress brought about by high salt concentrations [Jorgensen et al., 1994]. It should be noted, however, that a certain salinity of the storage medium (0.51 mol L^{-1}) is advocated as maximum for *L. acidophilus* during refrigeration, and that the increased sensitivity to cold and lower sodium chloride concentrations may be partially attributed to membrane destabilization [Mackey, 1984].

It is noteworthy that despite the lower viability of free *L. acidophilus* in NaCl solutions during storage, the numbers of microencapsulated cells with whey protein + gum arabic, soy protein + gum arabic, and soy milk + gum arabic in this experiment remained longer above the minimum number suggested for commercial application. Nahaisi [1986], Kim [1988], and Driessen and de Boer [1989] have proposed that the final product should contain not less than one million viable cells per gram (or milliliter) at the time of consumption for full probiotic functionality to be displayed.

Arihara and Itoh [2000] concluded that *L. gasseri* demonstrated satisfactory growth in meat containing 3.3% NaCl only after generated mutants of *L. gasseri* resisting sodium chloride. Microencapsulated cells may be regarded as an alternative way of supplying probiotics for light-salting products to consumers.

One aim of this study was to determine the viability of *L. acidophilus* during storage in different sucrose concentrations (5.0, 10.0, 15.0, 20.0, and 25.0%). Free cells showed little viability differences in sucrose concentrations of 5.0, 10.0, and 15.0% as compared to the control, with more sensitivity to both 20.0 and 25.0% sucrose concentrations. The stability of microencapsulated cells were weakly affected by the different sucrose concentration used. 20 and 25.0% sucrose had moderate effect on the microencapsulated cells. Microencapsulated cells with gum arabic + soy protein, gum arabic + whey protein, and gum arabic + soy milk showed the best viability in the high sucrose concentrations. They retained the recommended therapeutic-minimum numbers after 5-6 weeks as compared to 4-5 weeks for the higher sucrose concentration (25.0%). Godward et al. [2000] subjected probiotic cultures to some viability tests including high sucrose concentrations. In 25% sucrose, *B. infantis* decreased from 2.4 x 10^8 to 8.9 x 10^7 cfu/ml in 3 hours and *L. acidophilus* decreased from 1.1 x 10^{11} to 6.0 x 10^9 cfu/ml. Alamprese et al. [2002] studied the survival of *L. johnsonii* La1 and influence of its addition in retail-manufactured ice cream produced with different sugars (12 and 18% sucrose, and 3 and 4% glucose) and fat concentrations. They concluded that, regardless of formulation, no count decay of La1 cells was observed in ice cream stored for up to 8 months. Kaul and Mathur [1982] reported survival rates of *L. acidophilus* in ice cream with 15% sugar similar to the values found by Alamprese et al. [2002]. Also Hagen and Narvhus [1999] found no significant changes during storage at –20°C for a year, even though a different formulation and microorganism were used.

A wide variety of yoghurts are produced in the world and depending on the type of product, the total sugar content (mainly sucrose and lactose) ranges from 5.2% to 21% [Birollo et al., 2000]. Vinderola et al. [2002] studied the influence of compounds associated with fermented dairy products on the growth of 24 strains of lactic acid

starter bacteria (*Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis*) and 24 strains of probiotic bacteria (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus rhamnosus* and bifidobacteria). They tested 5, 15, and 20% sucrose and lactose concentrations and reported less sensitivity in probiotic bacteria in the presence of sugars than in lactic acid starter bacteria. Among the former, only some strains of bifidobacteria were inhibited by 15% and 20% sucrose or lactose. On the other hand, 15% of both sugars were inhibitory for some strains of lactic acid starter species. However, three *Lactococcus lactis* strains did not grow even in the presence of 5% lactose and no strain was inhibited by 5% sucrose.

Organic acids such as lactic ($C_3H_6O_3$), citric ($C_6H_8O_7$) and acetic ($C_2H_4O_2$) acids are commonly used by food manufacturers mainly as anti-microbial preservatives or acidulants in variety of food products [Davidson et al., 2002]. Many factors influence the effectiveness of organic acids as anti-microbials. The most important is undoubtedly the pH of the food. Many investigators [Corlett and Brown, 1980; Kashket, 1987; Beuchat and Golden, 1989; Ita and Hutkins, 1991; Anderson, 1992; Siragusa and Dikson, 1992; Buchanan et al., 1993; Doores, 1993; Kunz, 1994; Podolak et al., 1996; Conner et al., 1997; Krämer, 1997] demonstrated that the activity of organic acids was related to pH and that the undissociated form of the acid is primarily responsible for its anti-microbial activity. Other investigators concluded that the rate of inactivation is dependent not only on the pH of the environment but also on the identity and concentration of the acidulants used to modify the pH.

Survival of *L. acidophilus* is especially affected by low pH of the environment. Although *L. acidophilus* tolerates acidity, a rapid decrease in their numbers has been observed under acidic conditions both *in vitro* and *in vivo* [Conway et al., 1987; Hood and Zottola, 1988; Shah and Jelen, 1990; Lankaputhra and Shah, 1995]. The present study evaluated the stability of free and microencapsulated *L.acidophilus* during refrigerated storage in different lactic, citric, and acetic acids solutions with different pH values (3.0, 4.0, and 5.0). The total viable counts decreased over time in the order of pH 3.0, 4.0 and 5.0 compared to the control (pH 6.4). It should be mentioned also that the decline was more rapid for free cells than for microencapsulated cells. Little variability was observed between the different acids used at the same pHs. The protein- and soy milk-containing capsule materials highly protected and helped *L. acidophilus* to survive the low pHs harsh conditions better.

Traditionally, most yoghurts are manufactured by addition of cultures containing *S. salivarius* spp. *thermophilus* and *L. delbrueckii* spp. *bulgaricus*. These organisms are

active even at refrigerated temperatures and produce small amounts of lactic acid by fermentation of lactose resulting in pH decrease [Shah et al., 1995]. Organic acids (mainly lactic acid) may have negative effect on the survival of lactic acid bacteria [Kurmann, 1988; Peitersen, 1990].

Medina and Jordano [1995] studied the sensitivity of *L. acidophilus* to acidity during storage of BAT (Bifidus-Acidophilus-Thermophilus) products. The loss of viable cells of *L. acidophilus* was obviously related to the level of acidity in those products. In a study of fruit-flavored yoghurt, Con et al. [1996] observed a decrease in the numbers of lactic acid bacteria with increase in storage time. Zhang et al. [1997] have shown that the survival ability of *L. acidophilus* was significantly affected when subjected to low pHs. If the fact that *lactobacilli* showed a limited tolerance to the pH range from 3.5 is taken into consideration [Kashket, 1987], then the theory that the viable counts of *L. acidophilus* would decline from the starting-point of storage can be accepted. The present study confirms this.

A high concentration of *L. acidophilus* has been suggested to be an assurance that the final functional food product would contain an abundant number of viable cells (10^6 to 10^8 cfu/ml) [Kurmann, 1988; Medina and Jordano, 1995]. In addition to this, researchers also suggest that incubation should terminate at pH 4.9 to 5.0. A relatively high pH may be essential to assure the sufficient survival of *L. acidophilus* cultures selected throughout the whole commercial shelf-life [Medina and Jordano, 1995]. However, Kurmann [1988] stated that the selected intestinal strains to be used as dietary adjuncts should be sufficiently acid-tolerant, and especially resistant to the increasing acidity of the cultured milk products. In addition to the strict control of acidity level during the manufacturing process of the dairy products, the selection of proper *L. acidophilus* strains with high resistance to acidity conditions may be a good way to maintain the desired numbers and consequently therapeutic benefits [Zhang et al., 1998].

Nighswonger et al. [1996] indicated that the anti-microbial effects of the diacetyl, acetic acid and lactic acid present in the cultured buttermilk could be responsible for the declines of viability. According to the present study, a higher loss in cell viability was observed for free *L. acidophilus* than for microencapsulated cells in the presence of organic acids (lactic, citric and acetic) during refrigerated storage.

Frank and Hassan [1998] reported that neutralized juices inhibited neither lactic acid starter nor probiotic bacteria, indicating that acid injury was responsible for the inhibitory effect. Vinderola et al. [2002] also observed a satisfactory cell viability at pH

6.5 and 5.0 for both lactic acid starter bacteria and probiotic bacteria in milk acidified with lactic acid.

Kailasapathy and Supriadi [1996] produced yoghurt using mixed cultures with *L. acidophilus*. They determined the effect of changes in buffering capacity of yoghurt due to partial replacement of skim milk powder with whey protein concentrate on the survival and growth of *L. acidophilus* during refrigerated storage. They found that the numbers of *L. acidophilus* in the yoghurt remained at 10^8 level after the 3rd week of refrigerated storage. The results in the present investigation using proteins or soy milk in microencapsulating mixtures are in agreement with their findings, although Gilliland and Speck [1977] reported markedly decline in the numbers of viable cells of *L. acidophilus* placed in yoghurt within 7 days. This supports the theory that proteins raise the pH (in acidic environments), thereby offering a protective effect.

Vinderola et al. [2000] studied the survival of *B. bifidum* and *L. acidophilus* during refrigerated storage at 5°C in milk acidified with lactic acid at pH 6.5, 5.5, 4.5 and 3.5. They demonstrated that, *L. acidophilus* was more resistant than *B. bifidum* to lactic acid at pH 6.5 and 5.5. At lower pH values, *L. acidophilus* was more inhibited by lactic acid than *B. bifidum,* as the fall in cell counts was significant after 4 weeks (pH 4.5) and 9 days (pH 3.5). This proves that the product acidity has a major impact on the microbial viability during its shelf-life [Laroia and Martin, 1991; Lankaputhra and Shah, 1996; Lankaputhra et al., 1996b; Vinderola et al., 2000; Vinderola et al., 2002].

Among several possible explanations for the effect of organic acids on bacterial cells, various authors [Corlett and Brown, 1980; Salmon et al., 1984; Booth, 1985; Kashket, 1987; Beuchat, and Golden 1989; Ita and Hutkins, 1991; Davidson et al., 2002] have concluded that the inhibitory effects of organic acids can be correlated with their dissociation constants or pK_a values. In general, weak acids having higher pK_a values are thought to be more inhibitory, at a given pH, than strong acids, at the same pH. Many weak acids, in their undissociated or protonated form have the ability to penetrate the cell membrane and accumulate within the cell cytoplasm. If the interior of the cell is more alkaline than the pK_a of the acid, more of the acid will dissociate, releasing a proton and acidifying the cytoplasm of the cell. These events could result in a variety of detrimental effects. As a defence, many bacteria possess proton pumps or proton/cation exchange systems to deal with the influx of protons and to maintain the cytoplasm near neutral. However, if these pH regulatory systems are unable to function sufficiently (i.e., if the proton concentration is too great), then the pH gradient (the difference between the intracellular and the extraecllular pH) will collapse. Intracellular acidification will then result in the loss of cell viability or cell destruction. It has been

further suggested that the internal or cytoplasmic pH is the relevant pH which ultimately affects the cell's metabolic activities. Simply, Krämer [1997] summarized the effect of organic acids on bacteria and proposed that weak acids, such as common acid preservatives, inhibit bacteria via (i) a generalized intracellular acidification and (ii) a specific effect of the undissociated acid on metabolic activities. He considered the latter mechanism to be the more potent inhibitor. Generally, lowering the pH of food increases the proportion of molecules of the organic acids that are undissociated and thus increases its effectiveness as an anti-microbial agent.

6.5 Survival of free and microencapsulated *L. acidophilus* in simulated gastrointestinal conditions

Acid and bile resistance are important characteristics to be considered when selecting a culture, which should be used as a dietary adjunct [Bolin et al., 1997]. Cellular stress begins in the stomach, which has a very low pH. After the bacteria have passed through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. Thus, strains selected to be used as probiotic bacteria should be able to tolerate stomach acid and bile salts, to adhere to the epithelium, and to grow in the lower intestinal tract, in order to provide real health benefits [Chou and Weimer, 1999; Alamprese et al., 2002].

Different regions of the gastrointestinal tract have varying acid levels. The stomach and the regions immediately following have the highest acidity and the pH of these areas may fall to as low as 1.5. In order to be used as dietary adjuncts, *L. acidophilus* must be able to survive these harsh conditions and colonize in the gut [Lankaputhra and Shah, 1995].

The present study evaluated the survival of free and microencapsulated *L. acidophilus* under acidic conditions in HCl solutions (pH 1.0, 2.0, and 3.0) during 4 hours incubation as 3 hours is the maximum time for gastric emptying [Xanthopouls et al., 2000].

Free *L. acidophilus* showed intolerance to pH 1.0 by rapid and immediate reduction right from the moment of exposure. Similar results have been observed at pH 2.0 but showed more acid tolerance at pH 3.0.

Nevertheless, microencapsulated cells in all capsule materials were found to be slightly more acid tolerant at pH 1.0, and obviously more acid tolerant than free cells at pH 2.0 and 3.0 especially for protein-containing capsule materials.

The results in this experiment support the findings of Conway et al. [1987], in which the populations of 2 strains of *L. acidophilus* were reduced by 6 logarithmic cycles after 1 hour incubation in phosphate buffer at pH 1.0, and were also affected, although less intensely by pH values of 3.0 and 5.0. Hood and Zottola [1988] studied the survival of *L. acidophilus* in a pH range of 2.0 to 4.0 and observed a rapid decline in their numbers at pH 2.0. However, there was no decrease in the number of viable cells at pH 4.0. On the other hand, Marteau et al. [1997], reported the survival of more than 40% of the population of *L. acidophilus* and *B. bifidum* in a system similar to that in the gastric compartment after 120 minutes of incubation. According to Lankaputhra and Shah [1995] contradictory results can be explained for both *L. acidophilus* and *Bifidabacterium* spp. since the resistance to acid pH values and to the presence of bile can greatly vary between different strains of the same species. This confirms the need to identify those strains of probiotic organisms tolerant to gastrointestinal secretions, which therefore could be used as dietary aids.

In contrast to this study, Sultana et al. [2000], reported that microencapsulation of *L. acidophilus* and *Bifidabacterium* spp. with alginate does not enhance the bacterial survivals under acid and bile conditions. Results from their study were not conclusive about the rational of protection offered by encapsulation to the bacteria in the gastrointestinal tract. Another study by Lee and Heo [2000] indicated that the survival of alginate-immobilized bacteria may be dependent on the gel concentration and bead size. Sun and Griffiths [2000], reported that the immobilization of bifidobacteria in beads comprising of gellan-xanthan gum mixtures increases their tolerance of high acid environments. It may also be noted that probiotic bacteria have a different response to the gastrointestinal conditions [Mituoka, 1992].

In the present study, *L. acidophilus* is likely to be buffered by the protein carrier matrixes and is thus not likely to be exposed to the extremes of pH in the stomach.

Gastrointestinal system has varying concentrations of bile. The rate of secretion of bile and the concentration of bile in different regions of the intestine vary, depending mainly on the type of food consumed and it may not be possible to predict the bile concentration in the intestine at any given moment [Lankaputhra and Shah, 1995]. Concentrations ranging from 0.5% to 2.0% have been used in several microbiological media for selective isolation of bile tolerant bacteria from mixed cultures.

Several studies have documented the tolerance of lactic acid bacteria to bile [Conway et al., 1987; Berrada et al., 1991; Holcumb et al., 1991; Pochart et al., 1992; Ibrahim and Bezkorovainy, 1993; Clark and Martin, 1994]. Most of the results indicate the necessity of identifying bile tolerant strains of probiotics if they are to be used as dietary adjuncts.

In this investigation, subjection of free and microencapsulated *L. acidophilus* to 1%, 2% (maximum concentration found in the human small intestine), and 4% bile salts (twice the maximum concentration found in the human small intestine) [Davenpot, 1977] for 4 hours incubation at 37°C, showed that the number of free cells decreased gradually in the presence of 1% bile. However, no colony forming units could be detected after 4 hours incubation. Free cells were very sensitive to bile concentrations of 2% and above. On the other hand, microencapsulated cells were more bile tolerant for all tested bile concentrations. Among five used capsule materials, microencapsulated cells with soy milk + gum arabic survived best in bile, followed by whey protein + gum arabic, soy protein + gum arabic, gelatin, and gum arabic respectively. It is to be noted that survival rates of microencapsulated *L. acidophilus* with soy milk + gum arabic reminded approximately more than 10^5 cfu/g after 3 hours in maximum bile concentration, and also after 2 hours in twice of this maximum bile concentration.

Holcomb et al. [1991] reported that the viability of *L. acidophilus* and *B. bifidum* was not affected in bile concentrations ranging from 0.15 to 0.45%. This may have been due to the lower concentrations of bile used in their study. However, bile concentration in the present study were approximately five times those used in their study, and are closer to the maximum bile reported to be present in human intestine.

One effect should be noted when comparing the present results with others. Most studies incorporate the bile salts in the growth medium. However, this study used no growth support media or buffers during exposure to bile concentrations as this was to ensure that other factors could neither enhance nor diminish the survival rates.

Davenport [1977] reported that bile concentrations in the small intestine could reach 2% during the first hour of digestion of food. Bile concentrations can then decrease to 0.5% by the second hour. In the present study *L. acidophilus* was deliberately exposed to the maximum concentration (2%) and (4%) amount of bile for 4 hours. Survival rates may have been altered if bile concentrations were lowered after the second hour.

7 Summary

Modern consumers are increasingly interested in their personal health, and expect the food that they eat to be healthy or even capable of preventing illness. Gut health in general has shown to be the key sector for functional foods in world.

The viability and stability of probiotics have both a marketing and technological challenge for industrial producers. Probiotic foods should contain specific probiotic strains and maintain a suitable level of viable cells during the product's shelf-life. The technological demands placed on probiotic strains are great and new manufacturing process and formulation technologies may often be required for bacteria primarily selected for their functional health properties. Before probiotic strains can be delivered to consumers, they must first be able to be manufactured under industrial conditions, and then survive and retain their functionality during storage as frozen, freeze-dried or dried cultures, and also in the food products into which they are finally formulated. The probiotic strains should also survive the gastrointestinal stress factors and maintain their functionality within the host. Additionally, they must be able to be incorporated into foods without producing off-flavours or textures and they should be viable but not growing. The conditions under which the products are stored are also important for the quality of products.

Future technological prospects exist in innovations finding solutions for the stability and viability problems of probiotics in new food environments. Current research on novel probiotic formulations and microencapsulation technologies exploiting biological carrier and barrier materials and systems for enteric release provides promising results.

To be beneficial, probiotic cultures require some preservation or stabilization treatments for the cells to retain their viability and fermentative activity. The objectives of this study are to evaluate: the viability and physiological changes of *L. acidophilus* after the micoencapsulation procedure and over a period of storage time; influence of heat treatments; viability and stability to some simulated conditions in the carrier foods (including the presence of salt, sugar, and organic acids); and the resistance of microencapsulated *L. acidophilus* to some simulated conditions of the human intestinal tract (including gastric juice and bile salts); which could be improved and protected by microencapsulation procedure.

- To investigate the survival of *L. acidophilus* after spray drying, *L. acidophilus* spray dried with different carrier material mixtures including: gum arabic; gum arabic+mannitol; gum arabic+pectin; gum arabic+maltodextrin; gum arabic+skim milk; gum arabic+gum guar; gum arabic+gum karaya; gum arabic+carrageenan;

gum arabic+alginate; gum arabic+ lecithine; gum arabic+glycerol; gum arabic+tomato juice; gum arabic+tween 20; gum arabic+soybean flour; gum arabic+gelatin; soy protein; soy protein+alginate; soy protein+pectin; whey protein; whey protein+alginate; whey protein+pectin; soy milk; soy milk+alginate; soy milk+pectin; gum arabic+soy protein; gum arabic+soy protein+alginate; gum arabic+soy protein+pectin; gum arabic+whey protein; gum arabic+whey protein +alginate; gum arabic+whey protein+pectin; gum arabic+soy milk; gum arabic+soy milk+alginate; gum arabic+soy milk+pectin; gelatin; gelatin+mannitol; gelatin+pectin; gelatin+maltodextrin; gelatin+skim milk; gelatin+alginate; gelatin+lecithine; gelatin+glycerol; gelatin+tween 20; gelatin+tomato juice; gelatin+soybean flour; gelatin+soy protein; gelatin+whey protein; gelatin+soy milk. It was found that the survival of *L. acidophilus* in different capsule materials high varied after spray drying. It is evident that, in general, the number of survivors decreased after spray drying for all capsule materials tested. The decreases ranged from less than 1 log cycle to 2 log cycles approximately.

- The purity and identity of the cultured microencapsulated powder after spray drying was proved by API test, and plating and counting on MRS agar (for *lactobacilli* counts), plate count agar (for general viable counts of bacteria), and malt extract agar (for yeasts and molds counts). The results showed that the cultured microencapsulated powder has only *L. acidophilus* and neither other kinds of bacteria nor yeasts or molds.

- The next question addressed in this study concerned the stability of microencapsulated *L. acidophilus* during prolonged storage over a period of time under refrigeration. The same capsule materials mixtures as for spray drying were used. The results showed a great variability in the survival ability of microencapsulated *L. acidophilus* during storage at 5°C ranging from 4 to 15 weeks, which could be highly dependent on the different kinds of capsule materials. The decline in viability during refrigerated storage was more rapid for microencapsulated cells when gum arabic or gelatin alone was used as capsule materials without soy protein, whey protein, or soy milk. The mixtures of capsule materials using gum arabic+soy protein, gum arabic+ whey protein, and gum arabic+ soy milk had the best viability. The results investigated that after 10-11 weeks refrigerated storage, microencapsulated *L. acidophilus* using gum arabic+soy milk, gum arabic+ whey protein, and gum arabic+soy protein could maintain their viability which corresponds to the advised therapeutic-minimum dose. However, the present results concluded that microencapsulated cells may present another approach for reducing viability losses under refrigerated storage.

- The following viability and stability experiments were conducted on *L. acidophilus* microencapsulated with gum arabic, gealtin, soy protein+gum arabic, whey protein+gum arabic, and soy milk+gum arabic. Free cells served as a control.

- Free *L. acidophilus* exposed to 37, 40, 45, 50, 55, and 60°C/30 min. 60°C was chosen as a lethal temperature, due to the extreme reduction of the cfu at this temperature. On the other hand, the results for microencapsulated cells demonstrated more thermotolerant at 60°C as compared to the free cells. While, microencapsulated cells were less thermotolerant at higher temperatures (63 and 65°C) used. However, microencapsulated cells with whey protein+gum arabic, soy protein+gum arabic, and soy milk+gum arabic showed better viability than those microencapsulated with gum arabic and gelatin, which were approximately similar to the free cells.

- One aim of this study was to determine the viability of *L. acidophilus* during storage in different sucrose concentrations (5.0, 10.0, 15.0, 20.0, and 25.0%). Free cells showed little viability differences in sucrose concentrations of 5.0, 10.0, and 15.0% as compared to the control, while were more sensitive to both 20.0 and 25.0% sucrose concentrations. The stability of microencapsulated cells was weakly affected by the different sucrose concentration used. 20.0 and 25.0% sucrose had moderate effect on the microencapsulated cells. Microencapsulated cells with soy protein+gum arabic, whey protein+gum arabic, and soy milk+gum arabic showed the best viability in the high sucrose concentrations. They retained the recommended therapeutic-minimum numbers after 5-6 weeks with an exception that this value was reached after 4-5 weeks for the higher sucrose concentration used (25.0%).

- The survival of free and microencapsulated *L. acidophilus* in 1.0, 2.0, 3.0, 4.0, and 5.0% sodium chloride during storage at 5°C was investigated. The viability of the free cells decreased steadily during three weeks storage in 1.0, 2.0, and 3.0% NaCl concentrations, and this declined rapidly in 4.0 and 5.0% of NaCl solutions after 2 and 1 week(s) respectively. Similar results were observed for gum arabic. For gelatin, the numbers decreased slower and more steadily after 4 weeks in 1.0 and 2.0% NaCl; 3 weeks in 3.0 and 4.0% NaCl; and 2 weeks in 5.0% NaCl. Microencapsulated *L. acidophilus* with soy protein+gum arabic maintained high viability after 5 weeks in 1.0 and 2.0%; 4 weeks for 3.0%; 2 weeks in 4.0%; and 1 week in 5.0% NaCl. Microencapsulated *L. acidophilus* with whey protein+gum arabic showed high viability after 6 weeks in 1.0%; 5 weeks for 2.0%; 3 weeks for 3%; and similar results as gelatin for 4.0 and 5.0% NaCl. Microencapsulated *L. acidophilus* with soy milk+gum arabic showed the best stability to salt compared to the other capsule materials. They retained high viability after 6 weeks in 1.0 and

2.0%; 5 weeks in 3.0%; and after 2 weeks in both 4.0 and 5.0% NaCl concentrations.

- The present study evaluated the stability of free and microencapsulated *L. acidophilus* during refrigerated storage in different lactic, citric, and acetic acids solutions with different pH values (3.0, 4.0, and 5.0). In general, the total viable counts decreased in all pHs tested with the time progress during storage. The most observed decreases were at pH 3.0 followed by 4.0 and 5.0 respectively as compared to the control (pH 6.4). It should be mentioned also that the decline was more rapid for free cells than for microencapsulated cells. Little variability was observed between the different acids used at the same pHs. The protein- and soy milk-containing capsule materials could highly protect and help *L. acidophilus* to survive better the low pHs harsh conditions.

- This research also evaluated the "*in vitro*" tolerance of both free and microencapsulated *L. acidophilus* to pH and bile levels similar to those encountered in the human stomach and intestine, respectively. Cells inoculated into HCl solutions with pH values of 1.0, 2.0 and 3.0. The bile concentrations tested were 1.0, 2.0 (maximum concentration found in the human small intestine) and 4.0% (twice the maximum concentration found in the human small intestine). Cells after inoculation in HCl and bile solutions incubated aerobically at 37°C, and subsequently plated out at intervals of 0, 1.0, 2.0, 3.0 and 4.0 hours after incubation. Free cells exhibited intolerance to pH 1.0 and 2.0 and were more acid tolerant at pH 3.0. Nevertheless, microencapsulated cells in all capsule materials were found to be slightly more acid tolerant at pH 1.0, and obviously more acid tolerant at pH 2.0 and 3.0. Microencapsulated cells were likely to be buffered by the protein carrier matrix and were thus not likely to be exposed to the low pH extremes. Free cells were very sensitive to bile concentrations of 2.0 and 4.0% and decreased gradually in the presence of 1.0% bile. Among the five used capsule materials, microencapsulated cells with soy milk+gum arabic survived best in bile, followed by whey protein+gum arabic, soy protein+gum arabic, gelatin, and gum arabic respectively. It is interesting to note that the survival rates of microencapsulated *L. acidophilus* with soy milk+gum arabic remained approximately more than10^5 cfu/g after 3 hours in maximum bile concentration (2.0%), and after 2 hours in twice maximum bile concentration (4.0%).

8 References

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9 Appendix

List of Tables

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List of symbols and abbreviations

Aft.Sp.dry.	after spray drying
BAT	Bifidus-Acidophilus-Thermophilus
Bef.Sp.dry.	before spray drying
°C	degree Celsius
cfu	colony forming units
DE	degree of estrification
FDA	U.S. (United States) Food and Drug Administration
FOSHU	Food for Specified Health Use
g	gram
GRAS	Generally Recognized as Safe
h	hour

l	liter
L.	*Lactobacillus*
LAB	lactic acid bacteria
mg	milligram (10^{-3} g)
min	minute
ml	milliliter
MRS	Mann Rogosa and Sharpe
MW	molecular weight
n	number
O.D	optical density
pH	$-\log_{10}[H^+]$
rpm	rotation per minute
RSM	reconstituted skim milk
SCFA	short-chain fatty acids
sp.	species (singular)
spp.	species (plural)
ssp.	sub-species
WPC	whey protein concentrates
WPI	whey protein isolates

Glossary [Sources, Butterworths Medical Dictionary, 1978; Dorland`s Illustrated Medical Dictionary, 1994; Krämer, 1997; Stedman`s Medical Dictionary, 2000]

Allergy A state of hypersensitivity induced by exposure to a particular antigen (any substance which is capable, under appropriate conditions, of inducing a specific immune response and reacting with the products of that response) resulting in harmful immunologic reactions on subsequent exposures.

Anaerobiosis Existence in an oxygen-free atmosphere.

Ascites An abnormal accumulation of fluid in the peritoneal cavity.

Assimilation The transformation of food into living tissue; anabolism.

Bacteriocins Proteins that are produced by certain bacteria possessing bacteriocinogenic plasmids (a genetic element of bacteria, additional to the normal genome, which replicates independently of the chromosome but co-ordinately with the cell) and that exert a lethal effect in closely related bacteria; in general, bacteriocins have a narrower range of activity than antibiotics do and are more potent.

Bile A greenish-yellow to brown fluid secreted continuously by the liver and stored and concentrated in the gall bladder, whence it is expelled into the duodenum under suitable stimuli.

Bowel Intestine.

Cancer A malignant (threatening life or tending to cause death) tumor arising from epithelial cells; more loosely, any malignant growth.

Carcinogenic Causing cancer.

Cholesterol $C_{27}H_{45}OH$, the most abundant steroid in animal tissues specially in bile and gall-stones, and present in food, especially that rich in animal fats.

Colitis Inflammation of the colon.

Colon The part of the large intestine which extends from the cecum (the first part of the large intestine) to the rectum (the terminal portion of the digestive tube).

Colon Epithelium Thin layer of cells covering the internal and external surfaces of colon organ.

Colonic Pertaining to the colon.

Cytochrome A class of hemoprotein whose principal biological function is electron and / or hydrogen transport by virtue of a reversible valency change of the heme iron.

Digestion The process whereby ingested food is converted into material suitable for assimilation for synthesis of tissues or liberation of energy.

Diverticulitis Inflammation of a diverticulum (a pouch or sac opening from a tubular or saccular organ, such as the gut or bladder.

DNA Abbreviation for deoxyribonucleic acid.

Duodenum The first or proximal portion of the small intestine, extending from the pylorus (the distal end of the stomach which opens into the first part of the duodenum) to the jejunum [the portion of small intestine, between the duodenum and the ileum (the third portion of the small intestine)]; so called because it is about 12 fingerbreadths in length.

Endogenous Developing or originating within the organism, or arising from causes within the organism.

Enteric Relating to the intestine.

Enteritis Inflammation of the intestine, especially of the small intestine.

Epitope An antigenic determinant, in simplest form, of a complex antigenic molecule.

Gastrointestinal (GI) Relating to the stomach and intestines.

Genital Relating to reproduction or generation.

Gut The primitive digestive tube.

Hypercholesterolemic The present of an abnormally large amountsof cholesterol in the cells and plasma of the circulating blood.

Hypocholesterolemia The present of abnormally small amounts of cholesterol in the circulating blood.

Immune Protected against infectious disease by either specific or non-specific mechanisms; pertaining to the immune system and immune responses.

Immunomodulation Adjustment of the immune response to a desired level, or induction of immunologic tolerance.

Indigenous Native; natural to the place where found.

Infant A child under the age of 1 year; more specifically, a new-born baby.

Infection Invasion and multiplication of microorganisms in body tissues, which may be clinically inapparent or result in local cellular injury due to competitive metabolism, toxins, intracellular replication, or antigen-antibody response.

Inflammatory Pertaining to or characterized by inflammation (a localized protective response elicited by injury or destruction of tissues, which serves to destroy, dilute, or wall off both the injurious agent and the injured tissue).

Injure To wound, hurt, or harm.

Interferon A class of small (MW 26.000-38.000) glycoproteins that exert antiviral activity.

Irritable Capable of reacting to a stimulus (any agent, act, or influence that produces functional or trophic reaction in a receptor or in an irritable tissue); abnormally sensitive to a stimulus.

Luminal Relating to the lumen of a blood vessel or other tubular structure.

Lysozyme An enzyme hydrolyzing 1,4-β links between *N*-acetylmuramic acid and *N*-acetylglucosamine, and thus destructive to cell walls of certain bacteria; present in tears and some other body fluids, in egg white, and in some plant tissues.

Microbiota The microscopic living organisms of a region; the combined microflora and microfauna (the animal life, visible only under the microscope, which is present in or characteristic of a special location) of a region.

Mutagenic Having the power to cause mutations.

Mutant A gene or organism that has undergone genetic mutation (change in form, quality, or some other characteristics).

Pathogen Any disease-producing microorganism.

Respiratory Pertaining to respiration (the exchange of oxygen and carbon dioxide between the atmosphere and the cells of the body).

Response The reaction of a muscle, nerve, gland, or other excitable tissue to a stimulus.

Serum The fluid portion of the blood obtained after removal of the fibrin (an insoluble protein formed from the soluble protein of blood-plasma fibrinogen by the action of the enzyme, thrombin. The formation of fibrin is the fundamental process of the clotting of blood) clot and blood cells.

Surgery The branch of medicine concerned with the treatment of disease, injury, and deformity by operation or manipulation.

Syndrome The aggregate of signs and symptoms associated with any morbid (diseased or pathologic) process, and constituting together the picture of the disease.

Therapeutic Relating to therapeutics or the treatment of disease.
Toxinogenic Producing a toxin (any poisonous substance of biological origin), said of an organism.

Traveller's diarrhoea Diarrhoea (abnormal frequency and liquidity of faecal discharges) occurring among travellers, particularly in those visiting tropical or subtropical areas where sanitation is suboptimal; the most common being enterotoxigenic *Escherichia coli*.

Tumor Swelling; a new growth of tissue in which the multiplication of cells is uncontrolled and progressive.

Urogential Pertaining to the urinary (containing or secreting urine) and genital (Pertaining to reproduction or generation) apparatus.

Vaccine A suspension of attenuated or killed microorganisms, administered for the prevention, amelioration (moderation in the severity of a disease or the intensity of its symptoms), or treatment of infectious diseases.

Vehicle A substance, usually without therapeutic action, used as a medium to give bulk for the administration of medicines.

Curriculum Vitae

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