CHAPTER 2
LITERATURE REVIEW

2.1 Probiotics

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1991). It is known that the human gastrointestinal tract and the intestinal micro flora are involved in a series of disease states. Especially the large intestine is a major colonization site for bacteria, viral and parasitic pathogens. The result of an overgrowth of these pathogens may be acute diarrhea disease. Probiotics can be used to balance or prevent such disturbances, but different probiotics stains are needed for specific applications (Salminen *et al.*, 1999).

The mechanism approach to probiotics first established because of many gastrointestinal dysfunctions are based on disturbances or imbalances of intestinal micro flora. Thus, probiotics were defined as viable microbial cultures that influence the health of the host by balancing the intestinal microflora and thus preventing and correcting the microbial dysfunctions. This still applies to many specific viable cultures and non-viable cultures. Examples of the proposed mechanisms of probiotics in humans are summarized in Figure 1 (Salminen *et al.*, 1999).

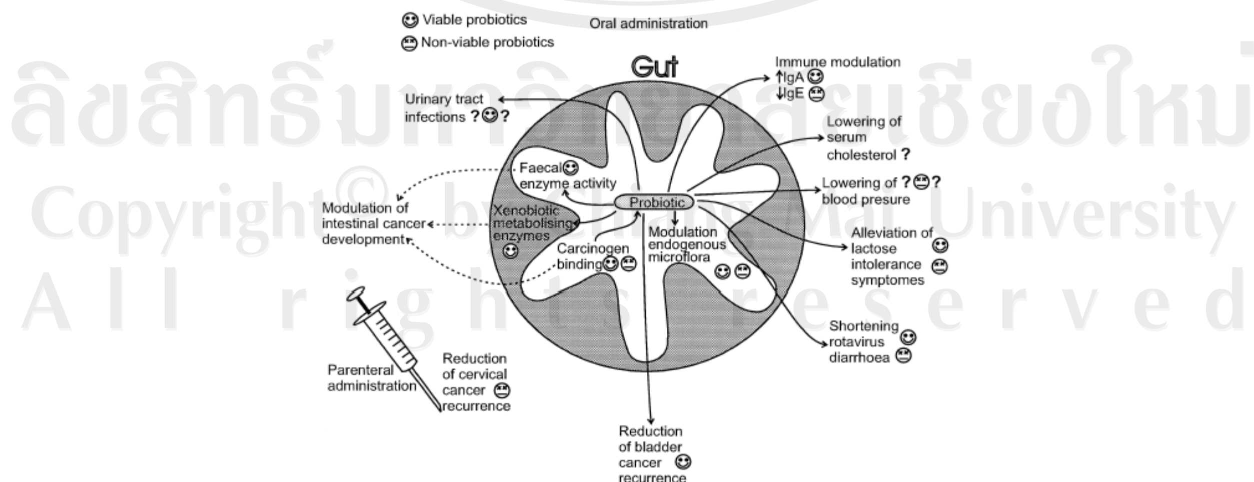

Fig. 1 Proposed mechanism of viable and non-viable probiotic health effects (Salminen *et al.*, 1999)

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2.1.1 Probiotic bacteria in fermented milk

Probiotics used in fermented milk production have mostly 3 genus; *Lactobacillus*, *Enterococcus* and *Bifidibacterium*. Probiotics strains in Genus *Lactobacillius* are *L. acidophilus*, *L. crispaths*, *L. amylovorus*, *L. gallinarum*, *L. gasseri*, *L. johnsonii*, *L. casei*, *L. paracasei*, *L. rhamnosus*, *L. reutri*, and *L. fermentum*. Both *Ec. faecium* and *Ec. faecalis* are probiotic bacteria in Genus *Enterococcus*. Example of lactic acid bacteria used in fermented milks and lactic drinks is shown in Table 1 (Nakazawa and Hosono, 1992).

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Table 1 Lactic acid bacteria used in fermented milks and lactic acid drinks (Nakazawa and Hosono, 1992)

Microorganism	Function	Type of product
Lactobacilli		
Lactobacillus bulgaricus	Flavor	Yoghurt, Bulgarian milk, Kefir, Kumis, Lactic drink
L. jugutri	Flavor	Yoghurt, Lactic drink
L. acidophilus	Flavor + Health	Yoghurt, Acidophilus milk, Lactic drink
L. casei	Flavor + Health	Drinking yoghurt, Lactic drink
L. delbrueckii	Flavor	Lactic drink
Bifidobacteria		
Bifidobacterium bifidum	Healthy	Yoghurt, Lactic drink
B. infantis		
B. breve		
B. longum		
Lactic Streptococci		
S. thermophilus	Flavor	Yoghurt
S. lactis		Yoghurt, Cultured butter milk, Cultured cream
S. cremoris		Cultured butter milk, Cultured cream
Lh. citrorum		Cultured butter milk, Cultured cream

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2.1.2 Manufacturing process for fermented probiotic products

In fermented probiotic products it is important that the probiotic culture used contributed to good sensory properties. For milk-based products the probiotic strains are often mixed with *Streptococcus thermophillus* and *Lactobacillus delbrueckii* to achieve the desired flavor and texture. The main flavor components from species often used in probiotic formulations are shown in the Table 2 (Saarela *et al.*, 2000).

Table 2 Main flavor characteristics of some strains commonly used in probiotic mixtures (Saarela *et al.*, 2000)

Culture	Main flavor components
Lactobacillus acidophilus [a]	Lactate (DL)
Bifidobacterium spp. [a]	Lactate (L+), acetate
Streptococcus thermophilus [b]	Lactate (L+), acetaldehyde, diacetyl
Lactobacillus delbrueckii ssp. *bulgaricus* [b]	Lactate (D -), acetaldyhyde, diacetyl

[a] Probiotic strains [b] Non- probiotic strains

In many cases consumers find products fermented with *L. delbrueckii* are too acidic and too heavy acetaldehyde flavor (yoghurt flavor). Therefore probiotic cultures have been developed to bring out the preferred flavors in the products in which they are used. Examples of such cultures are called ABT cultures (ABT standing for *L. acidophilus*, *Bifidobacterium* and *S. thermophilus*) (Saarela *et al.*, 2000).

The interaction between probiotic and starter bacteria might have an impact on the quality of the product. It has been shown that it is possible to produce fermented dairy products with excellent sensory properties and good survival of the bacteria by using starter and probiotic organisms together. If possible, the probiotic should be able to grow during the fermentation. This will increase the total number of probiotic resulting in a lower process cost and increased adaptation of the probiotics to the fermented food. As most probiotics grow well at 37°C a thermophillic starter might be preferable to a mesophilic one. The growth rate of the starter should be moderate allowing some growth of the probiotic bacteria. It is also important to add the probiotic before or at the same time as the starter. Addition of the probiotic after fermentation does not allow any growth but instead might result to a reduced viability as shown when *L. acidophilus* was mixed with yoghurt. The starter might improve the growth conditions of probiotic by producing substances favorable to the growth of probiotic or by reducing the oxygen pressure (Saarela *et al.,* 2000).

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2.1.3 Growth and viability of probiotic bacteria in milk

The number of viable microbial cells that should be present in a probiotic product has been the subject of much discussion, but is usually considered to be

between 10^6 and 10^8 cfu/ml. Even at expiration dates, the product must contain these minimal numbers of living microbial cells. The important factors for growth and viability of probiotic microbes in milk products are summarized as followed (Tannock, 2002).

2.1.3.1 Strains used and interaction between different species

Growth and survival properties vary between different bacteria strains but the combination of probiotic and traditional starter cultures is also important. Therefore the strains used in the preparation of a product must be checked carefully for compatibility. In general, lactobacilli survives better than bifidobacteria due to their greater tolerance to oxygen and low pH. The production of acetic acid by bifidobacteria can enhance the growth of *L. acidophilus* (Tannock, 2002).

L. delbrueckii ssp. *bulgaricus* may affect the survival of bifidobacteria and *L. acidophilus* due to the amount of acid products and hydrogen peroxide that are produced. Stimulatory effects on the growth of bifidobacteria in the presence of *L. delbrueckii* ssp. *bulgaricus* have been observed and were suggested to be due to proteolytic activities of the lactobacilli which increased the availability of valine, glycine and histadine. *S. thermophilus* has not been reported to inhibit probiotic microbes to the extent as was observed with *L. delbrueckii* subsp. *bulgaricus*, and may sometimes stimulate their growth or survival due to consumption of oxygen. The study of the survival of commercial strains of *L. acidophilus* and *L. rhamnosus* GG together with a mesophilic lactococcal culture showed that the strain GG was stable in this environment, whereas the number of viable cells decreased in the case of some of the *L. acidophilus* strains (Nighswonger *et al.,* 1996).

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2.1.3.2 Composition of fermentation medium

Milk composition, and the heat treatment of the milk, is of importance for the growth of starter and probiotic strains. Addition of casein or whey protein hydrolysates, yeast extract, glucose, vitamins and minerals can stimulate the growth and survival of probiotic strains and enhance the texture of the products. However, the nature and extent of additives is regulated by law in most countries. The addition of protein increase the buffering capacity of fermented milks and hence may retard the decrease in pH and prevent further pH changes during storage, thus allowing better

survival of the probiotic cells. As in the case of all starter cultures, antimicrobial substances in the milk, cleaning agents, disinfectants and bacteriophages may influence the growth of probiotic microbes.

2.1.3.3 Dissolved oxygen

Bifidobacteria are obligate anaerobes and lactobacilli are microaerophillic. Different sensitivities to oxygen have been observed among various bifidobacterial strains. De-aeration of the milk in a dairy plant before addition of probiotic microbes is important and improves the survival of both bifidobacteria and *L. acidophilus*. Packaging the product in oxygen-impermeable packages is an efficient way to achieve good survival of bifidobacteria during the shelf life of the product. This can also be of importance for lactobacilli. Incorporation of *S. thermophilus* strains that have high oxygen utilization ability lowers the level of dissolved oxygen in yoghurt and can thus improve the survival of bifidobacteria.

2.1.3.4 Size of the inoculums

Since probiotic strains often grow poorly in milk, a relatively large inoculum, 5-10% compared to 1% for traditional starters, is usually used. The probiotic strains usually do not grow well when it is added together with a supporter culture. The addition of probiotic bacteria must be at the level required in the final product. The size of the inoculums of the supporter culture may influence the survival of probiotic bacteria.

2.1.3.5 Incubation temperature

The optimum growth temperature for most probiotic microbes is 37°C. For yoghurt cultures 40 to 43°C is optimal for acid production. A production temperature at 37°C will therefore favour the probiotic organism.

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2.1.3.6 Final acidity

A reduced probiotic bacterial count both for lactobacilli and bifidobacteria can be caused by over acidification and accumulation of D-lactic acid in fermented products. The over acidification caused by the growth of *L. delbrueckii* ssp.

bulgaricus at refrigerator temperature is the main factor in the death of bifidobacterial cells in milk products.

2.1.3.7 Storage temperature

Storage temperature influences the growth and acid production of *L. delbrueckii* ssp. *bulgaricus*. Thus a low storage temperature (< 4°C) lowers the risk of over acidification and results in better survival of bididobacteria.

2.1.4 Morphology of probiotic and yoghurt starter microorganisms

Yoghurt starter bacteria are *S. thermophilus* and *L. bulgaricus* that have a different morphology. *S. thermophilus* is Gram-positive cocci that forming chains or occurring in pairs. It is anaerobic homofermentative lactic acid and produce L(+) lactate, acetaldehyde and diacetyl from lactose in milk. It is absence of growth at 15°C, whilst growth at 45°C may give rise to irregular cells and segments; most strains are able to grow at 50°C or survive heating for 30 min at 60°C (Tamine and Robinson, 1999).

The morphology of *L. bulgaricus* is a Gram-positive rod with rounded ends, of 0.5-0.8 x 2-9 μm that occurs as single or in short chain. It is obligately homofermentative and can ferment fewer sugars, produce D(+) lactate and acetaldehyde from lactose in milk and some strains produce exo-polysaccharide. Slight growth occurs at < 10°C and most strains are able to grow at 50-55°C (Tamine and Robinson, 1999).

L. acidophilus is a Gram-positive rod with rounded ends that occurs as single cells, as well as in pair or in short chains. It is non-flagella, non-mobile and non-sporeforming, and is intolerant to salt. Growth of *L. acidophilus* may occur at temperatures as high 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 *et al.,* 1998).

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2.1.5 Health benefits of *L. acidophilus*

The antibacterial action of *L. acidophilus* is occurred by producing organic acid, hydrogen peroxide and antibiotics to suppress the multiplication of pathogenic bacteria. It shows stronger antibacterial properties against Gram-positive bacteria than

Gram-negative bacteria. Pathogenic bacteria in the intestinal tract cause intestinal problem, diarrhea and digestive upsets and contribute to the maintenance of health, so, *L. acidophilus* can improve the composition of the intestinal micro flora by its by product (Mosilhey, 2003).

There are people who find it unpleasant to drink milk because of a decrease in secretion of β-galactosidase in the digestive tract when they become adults. The administration of acidophilus milk and of cow's milk containing *L. acidophilus* to such people has shown a beneficial effect. Besides anti-cholesterol factors such as cell components of lactic acid bacteria, extrabacterial metabolites are also thought to be present in fermented milks. However, in the case of fermented milk made with *L. acidophilus* it is also possible to envisage a direct cholesterol lowering action by *L. acidophilus* (Nakazawa and Hosono, 1992).

In order to exert both its therapeutic and nutritional effect in 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 (hydrochloric acid) present in the stomach. If they survive gastric digestion they become strong candidates for the interaction with the gastrointestinal micro flora. After passing through the stomach barrier, the microorganisms reach the duodenum where the secretion of bile salt take place. Thus, resistance to bile salt is an important factor to guarantee the establishment and growth of microorganisms used as dietary adjuncts within the intestinal tract (Mosilhey, 2003).

2.2 Encapsulation technique

The health benefits provided by probiotic bacteria have led to their increasing use in fermented and other dairy products. However, their viability in these products is low. Encapsulation has been suggested to protect the bacteria in the product's environment and improve their survival. There are two common encapsulation techniques, namely extrusion and emulsion, to encapsulate the probiotics for their use in the fermented and other dairy products (Krasaekoopt *et al.*, 2003).

Micro-encapsulation of various bacterial cultures including probiotics has been a common practice for extending their storage life and converting them into a powder form for ease of their use. There are several techniques such as spray drying, freeze drying and fluidized bed drying for encapsulating the cultures and converting

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them into a concentrated powdered form. In this case, the cultures are not protected from the product environment or during the passage through the stomach or intestinal tract. Encapsulation in hydrocolloid beads entraps or immobilizes the cells within the bead matrix, which in turn provides protection in an environment used in fermented and other dairy products (Krasaekoopt *et al.*, 2003).

2.2.1 Techniques for micro-encapsulation of bacterial cells in hydrocolloid beads

The encapsulation techniques applied to probiotic for the use in fermented milk products or biomass production can be classified into two groups, depending on the method used to form the beads: extrusion (droplet method) and emulsion or two-phase system (Figure 2). Both extrusion and emulsion techniques increase the survival of probiotic bacteria by up to 80-95% (Krasaekoopt *et al.*, 2003).

2.2.1.1 Extrusion technique

Extrusion is the oldest and most common approach to make capsules with hydrocolloids. It simply involves preparing a hydrocolloid solution, adding microorganisms to it, and extruding the cell suspension through a syringe needle in the form of droplets to free-fall into a hardening solution or setting bath. The size and shape of the beads depend on the diameter of the needle and the distance of free-fall, respectively. This method is the most popular due to its ease, simplicity, low cost and gentle formulation conditions ensuring high retention of cell viability (Krasaekoopt *et al.*, 2003). Encapsulation of lactic acid and probiotic bacteria by extrusion technique is summarized in Table 3.

The supporting material used for extrusion is alginate, which is a linear heteropolysaccharide of D-mannuronic and L-guluronic acid extracted from various species of algae. The functional properties of alginate as supporting material correlate strongly with the composition and sequence of L-guluronic acid and D-mannuronic acid. Divalent cations such as Ca^{2+} bind preferentially to the polymer of L-guluronic acid. The size of the beads is approximately 2-3 mm in diameter. Moreover, the size and sphericity of the bead depend mainly on the viscosity of the sodium alginate solution and the distance between the syringe and the calcium chloride collecting solution. As the concentration, and hence viscosity, of sodium alginate increases, the size of the beads decreases. The extruder orifice diameter is another important factor,

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which regulates droplet size. Using a 0.27 mm syringe obtained a bead size of 2-3 mm. The composition of the alginate also influences bead size; small beads result from “low guluronic” alginates (Krasaekoopt *et al.*, 2003).

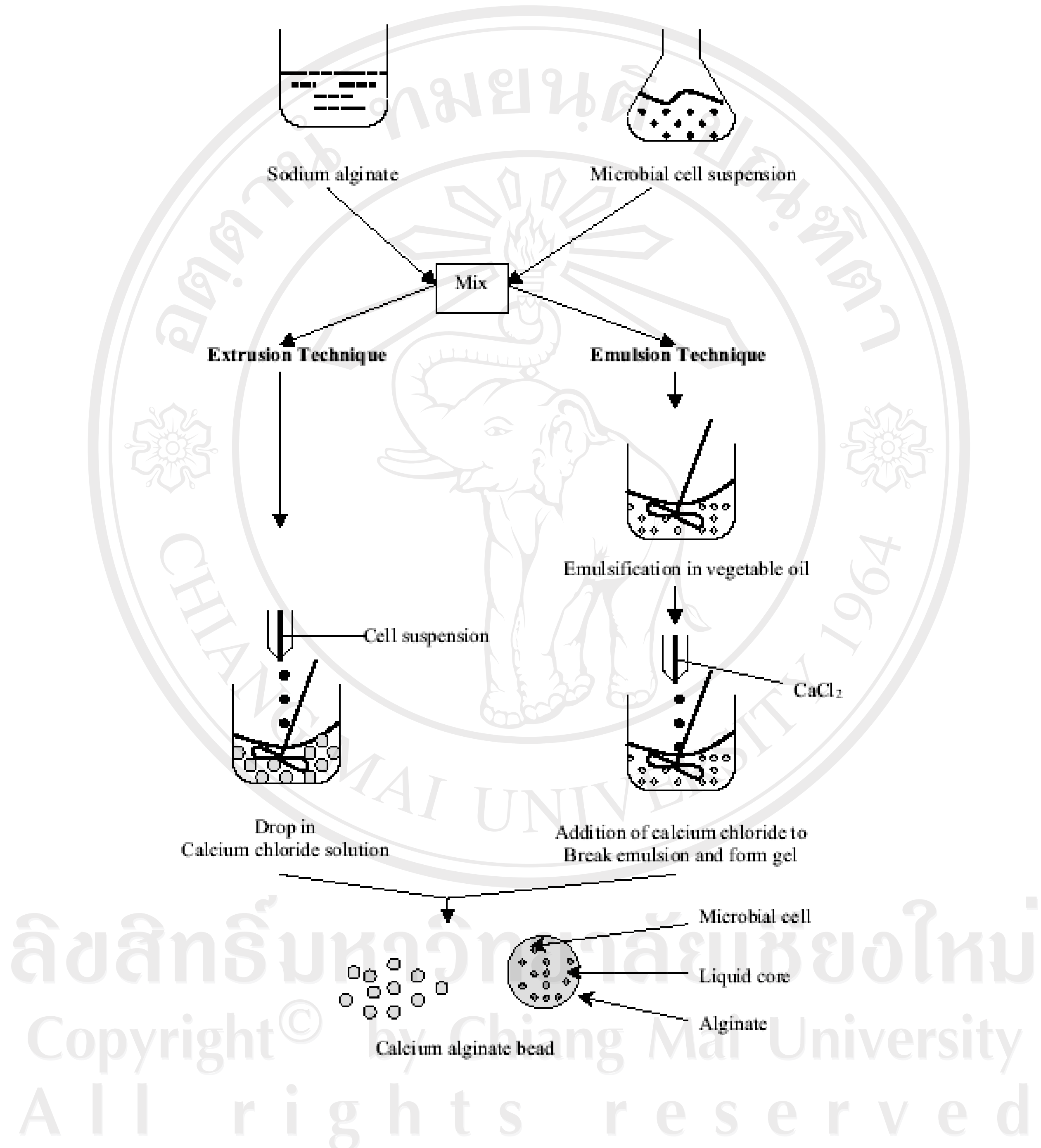

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Fig. 2 Flow diagram of encapsulation of bacteria by the extrusion and emulsion techniques (Krasaekoopt *et al.*, 2003)

Table 3 Encapsulation of lactic acid and probiotic bacteria by the extrusion technique (Krasaekoopt *et al*., 2003)

Bacteria	Supports used	Conc. of alginate (%)	Conc. of $CaCl_2$ (M)	Special treatment	Diameter of bead (mm)	Application
Lactobacillus delbrueckii ssp. *bulgaricus* *Streptococcus thermophilus*	Alginate	1.875	1.5	N [a]	2.5	Yoghurt
S. lactis ssp. *diacetylactis* *S. cremoris*	Alginate	1.875	1.5	N	2.6	Cheese
S. cremoris	Alginate	1	0.1	N	-	Phage protection
Lb. ssp. *bulgaricus* *S. thermophilus*	Alginate	1.875	1	N	2.5	Yoghurt
Lactococcus lactis ssp. *cremoris*	Alginate	2	0.05	Coated with low MW [c] chitosan	2	Biomass production
Lb. plantarum	Alginate	2	0.1	Added glycerol	2	Biomass production
Lc. lactis ssp. *lactis* bv. *diacetylactis*	Alginate	1.5	0.2	Coated with alginate	-	Cream
Lc. lactis ssp. *cremoris*	Alginate	2	0.1	N	-	Biomass production
Lb. acidophilus	Alginate	0.6	0.3	Mixed with starch	5	Biomass production

[a] N, no treatment [b] - , no record [c] MW , molecular weight.

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Table 4 Encapsulation of lactic acid and probiotic bacteria by the emulsion technique (Krasaekoopt *et al*., 2003)

Bacteria	Supports used	Continuous phase	Special treatment	Diameter of bead	Application
Streptococcus thermophilus	3% κ- carrageenan and locust bean gum (2:1)	Soy oil	N [a]	0.5-2 mm	Yoghurt
Bifidobacterium pseudolongum	10% cellulose acetate phthalate	White light parafilm oil	N	-	-
Lb. delbrueckii ssp. *bulgaricus*	3 % Alginate	Vegetable oil	2% emulsifier added	-	Ice cream
Lactococcus lactis ssp. *cremoris*	4% chitosan	Mineral oil	Cross-linked with hexamethylene disocyanate	150 µm	Biomass production
Lb. delbrueckii ssp. *bulgaricus*	3% Alginate	Vegetable oil + 0.2% Tween 80	0.5% sodium lauryl sulphate added	25-35 µm	Frozen ice milk
B. bifidum B. infantis	3% Alginate	Corn oil + 0.2% Tween 80	Added glycerol	-	Ice milk

[a] N, no treatment

[b] - , no record

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2.2.1.2 Emulsion technique

In this technique, a small volume of cell-polymer suspension (discontinuous phase) is added to a large volume of a vegetable oil, canola oil or corn oil. The mixture is homogenized to form a water-in-oil emulsion. Once the water-in-oil emulsion is formed, the water soluble polymer must be insolubilized (cross-linked) to form tiny gel particles within the oil phase. The smaller the internal phase particle size of the emulsion, the smaller the final micro particles will be. The insolubilization method depends on the type of supporting material used. The beads are harvested later by filtration. The size of the beads is controlled by the speed of agitation and can vary between 25 μm and 2 mm. This technique has been used successfully to encapsulate lactic acid bacteria for batch and continuous fermentation (Krasaekoopt *et al.*, 2003). Encapsulation of lactic acid and probiotic bacteria by emulsion technique is summarized in Table 4.

2.2.1.4 Advantage and limitations of extrusion and emulsion encapsulation technique

For encapsulation of probiotics, both extrusion and emulsion techniques can be applied. Advantage and disadvantage of these techniques are shown in Table 5.

Table 5 Positive and negative features of extrusion and emulsion technique (Krasaekoopt *et al.,* 2003)

	Extrusion technique	Emulsion technique
Technological	Difficult to scale up	Easy to scale up
Cost	Low	High
Simplicity	High	Low
Survival of	80-95%	80-95%
Size of bead	2-5 mm	25 μm-2mm

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Extrusion is a relatively simple technique. It usually produces entrapped, rather than encapsulated core material, although encapsulation can be achieved through co-extrusion devices or dropping into a bath of coating material which react at the droplet surface. This method can be difficult for large-scale production because

of slow formation of beads compared with the emulsion technique (Krasaekoopt *et al.,* 2003).

On the other hand, the emulsion technique is relatively new to food industry and easy to scale up for large-scale production. It provides both encapsulated and entrapped core materials. The size of the beads formed by this method is smaller (25 µm to 2 mm) than that of beads produced by the extrusion method (2-5 mm). The size of beads from the extrusion method depends mainly on the size of the needle used, while the size of beads from the emulsion method depends on the speed of agitation and the type of emulsifier used. Due to the need for a vegetable oil, the operating cost of the emulsion technique may be higher than that of the extrusion technique (Krasaekoopt *et al.,* 2003).

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2.2.3 Applications

Sheu and Marshall (1993) reported that a product was developed to entrap culture bacteria using a two-phase (water/oil) system. It consisted of 3% sodium alginate mixed with microbial cells and suspended in an oil bath containing 0.2% Tween 80. While stirring at 200 rpm, calcium chloride (0.05 M) solution was added to break the water/oil emulsion and form calcium alginate gel. The calcium alginate beads containing microbial cells had a mean diameter of 25-35 µm (range 5-100 µm). The entrapped microbial cells were released completely from the drop shaped beads by gentle shaking in 0.1 M phosphate solution (pH 7.5) for 10 min. About more than 40% of lactobacilli survived freezing of ice milk when they were entrapped in calcium alginate than when they were not entrapped.

Iyer and Kailasapathy (2005) reported that three different complementary prebiotics (inulin, oligofructose and high amylase corn starch (Hi-maize starch)) were separately be used to co-encapsulated *L. acidophilus* CSCC 2400 or CSCC 2409 and tested for their efficacy in improving the viability of bacteria under *in vitro* acidic conditions. Additions of hi-maize TM starch to capsules containing *Lactobacillus* spp. provided maximum protection to the encapsulated bacteria after 3 h of incubation at pH 2.0 compared with the other 2 prebiotics, inulin and oligofructose. Viable counts of *Lactobacillus* spp. increased significantly ($p<0.05$) with hi-maize concentration of up to 1.0% (w/v). Further increase in hi-maize concentration did not protect the encapsulated bacteria effectively. Effects of 3 different polymers (chitosan, poly-L-

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lysine and alginate) were also tested for their efficacy in protecting the encapsulated bacteria at pH 2.0 by extrusion technique. An addition of hi-maize (0.1% w/v) to capsules containing *Lactobacillus* spp. and further coating with chitosan significantly increased ($p<0.05$) the survival of encapsulated bacteria under *in vitro* acidic and bile salt conditions and also in stored yoghurt compared with alginate encapsulated cells.

Sultana *et al.* (2000*)* reported that incorporation of hi-maize starch (a prebiotic) improved encapsulation of viable bacteria as compared to when the bacteria were encapsulated without the starch. The acidification kinetics of encapsulated bacteria showed that the rate of the produced acid was lower than that of free cultures. The encapsulated bacteria, however, did not demonstrate a significant increase in survival when subjected to *in vitro* high acid and bile salt conditions. This study was carried out in order to monitor the effects of encapsulation on the survival of encapsulated cultures of *L. acidophilus* and *Bifidobacterium* spp. in yoghurt over a period of 8 weeks. The survival of encapsulated cultures of *L. acidophilus* and *Bifidobacterium* spp. showed a decline in viable count of about 0.5 log over a period of 8 weeks while there was a decline of about 1 log in cultures which were incorporated as free cells in yoghurt. The extrusion technique did not result in an uniform bead size.

Resistant starch is starch that is not digested by pancreatic amylase in the small intestine and reaches the colon, but it can be fermented by human and animal gut microflora. In a study where rat were fed with native potato starch, an increase in the intestinal population of bifidobacteria, lactobacilli, streptococci and enterobacteria was demonstrated. The benefit of using resistant starch extends beyond traditional prebiotics, since the resistant starch can be used to ensure the viability of probiotic populations from the food to the large intestine. Resistant starch offers an ideal surface for adherence of the probiotics to the starch granule during processing, storage and transit through the upper regions of the gastrointestinal tract, providing robustness and resilience to environmental stresses. Bacterial adhesion to starch may also provide advantages in new probiotic technologies to enhance delivery of viable and metabolically active probiotics to the intestinal tract (Crittenden *et al.,* 2001).

Hansen Truelstrup *et al*. (2001) reported that *B. adolescentis* 15703, *B. breve*15700, *B. lactis* Bb-12 and *B. longum* Bb-46 with their best overall resistance, were encapsulated in alginate microspheres (a mean diameter of 20 and 70 μm) by an

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emulsion technique. *B. lactis* Bb-12 was significantly more resistant to low pH and bile than any other test strains. Preliminary trials revealed that sphere size (1-3 mm), which use gellan–xanthan and alginate-starch mixtures, is too large to allow direct incorporation in food products such as milk, yoghurt and sour cream, without adversely affecting the feel in the mouth. Reduction of the sphere size to less than 100 μm would be advantageous for texture considerations and allow direct addition of encapsulated probiotics to a multitude of food. Furthermore, survival of probiotics in alginate-starch microspheres in the size range of 0.5-1.0 mm was improved during refrigerated store in yoghurt but not affected when expose to acid and bile solutions (Sultana *et al.*, 2000).

Lee and Heo, (2000) showed that *B. longum* encapsulated in Ca-alginate spheres survived exposure to simulated gastric juice (SGJ) (pH 1.55) significantly better than free cell. Survival decreased with decreasing sphere size (diameter 1-2.6 mm) and increase with increasing alginate concentration (1-3%). The Cryo-SEM of *B. lactis* Bb-12 alginate bead was shown in Figure 3.

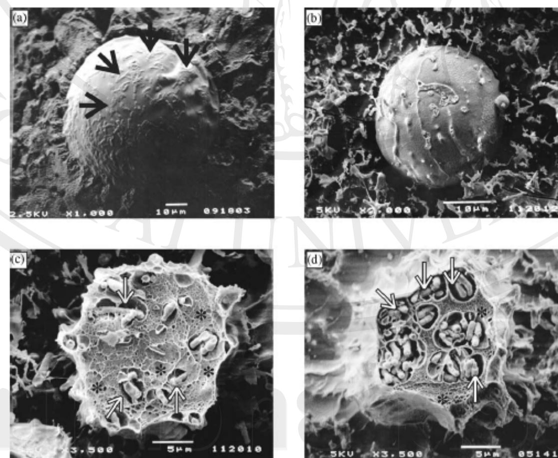

Fig. 3 Cryo-SEM of whole and fracture small alginate microsphere loaded with *Bifidobacterium lactis* Bb-12 after exposure for 1 h at 37°C in simulated gastric juice (Hansen Treulstrup *et al.*, 2002)

2.3 Yoghurt manufacture

Yoghurt is probably the most popular fermented milk. It is made in a variety of compositions (fat and dry matter content), either plain or with added substances:

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fruits, sugar or gelling agents. The nutrition values of milk and yoghurt are different as could be seen in Table 6.

Table 6 Comparison between a nutrition value of milk and yoghurt (Deeth and Tamine, 1981)

Constituent (units/100g)	Milk		Yoghurt		
	Whole	Skim	Full fat	Low fat	Fruit
Calories	67.5	36	72	64	98
Protein (g)	3.5	3.3	3.9	4.5	5
Fat (g)	4.25	0.13	3.4	1.6	1.25
Carbohydrate (g)	4.75	5.1	4.9	6.5	18.6
Calcium (mg)	119	121	145	150	176
Phosphorus (mg)	94	95	114	118	153
Sodium (mg)	50	52	47	51	-
Potassium (mg)	152	145	186	192	254

2.3.1 The yoghurt bacteria

The essential flora of yoghurt consists of the thermophilic lactic acid bacteria *S. thermophilus* and *L. bulgaricus*. For a satisfactory flavor to develop, approximately equal numbers of both species should be present. They have a stimulating effect on each other's growth (protocooperation in Figure 4). The proteolytic rods enhance growth of streptococci by forming small peptides and amino acids, the main amino acid being valine. Milk containing too little of these amino acids and the cocci proteolytic, form the acid slowly. The cocci enhance growth of the rods by forming formic acid out of pyruvic acid under anaerobic conditions and by a rapid production of CO_2. The stimulatory of formic acid remains unnoticed in intensely heated milk because in this milk formic acid has been formed by decomposition of lactose. The production of formic acid by the cocci is, however, essential in industrial practice, where more moderate heat treatments of yoghurt milk are applied, e.g., 5-10 min at 85°C. Due to mutual stimulation during combined growth of the yoghurt bacteria in milk, lactic acid is produced much faster than would be expected on the basis of the acid production by the individual pure cultures. Some antibiosis also occurs in

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yoghurt in that the cocci cannot grow after certain acidity has been reached. The rods are less susceptible to acid and continue to grow. Protocooperation and antibiosis are the great importance in the growth of the yoghurt bacteria as well as for the quality of yoghurt (Walstra *et al.,* 1999).

The cocci as well as the rods contribute significantly to the properties of yoghurt. The properties of bacterial strains used should be matched to each other because not every combination of strains is suitable. Furthermore, both species should be present in large numbers in the product, hence in the starter. The optimum ratio of diplococci to rods depends on the properties of the strains and is often approximately 1:1. This ratio between the yoghurt bacteria is best maintained if the inoculums percentage is 2.5 and the incubation time is for 2.5 h at 45°C (Walstra *et al.,* 1999).

Initially, the streptococci grows faster due to the formation of growth factors by the rods, and probably also due to the latter compounds being added via the inoculums (especially in the manufacture of set yoghurt). Afterward, the cocci are slowed down by the acid produced. Meanwhile, the rods have started to grow faster because of the growth factors (CO_2 and formic acid) formed by the cocci. As a result, the original ratio is regained. The yoghurt should then have attained the desired acidity. Continued incubation or inadequate cooling causes the rods to become predominant (Walstra *et al.,* 1999).

Fig. 4 Outline of the stimulation and the inhibition of the growth of yoghurt bacteria in milk. ———, formation of lactic acid; - - - - - - , formation of growth factor; -·-·-·-, stimulation; ··········· , inhibition (Walstra *et al.,* 1999).

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2.3.2 Manufacture of set yoghurt.

The most common type of set yoghurt are plain varieties made by simple fermentation of milk or milk products using microorganism, without additives, or varieties with stabilizers such as agar, gelatin or pectin added, sweetened varieties with added sugar, and flavored varieties with fruit juice or flavoring added (Nakasawa and Hosono, 1992).

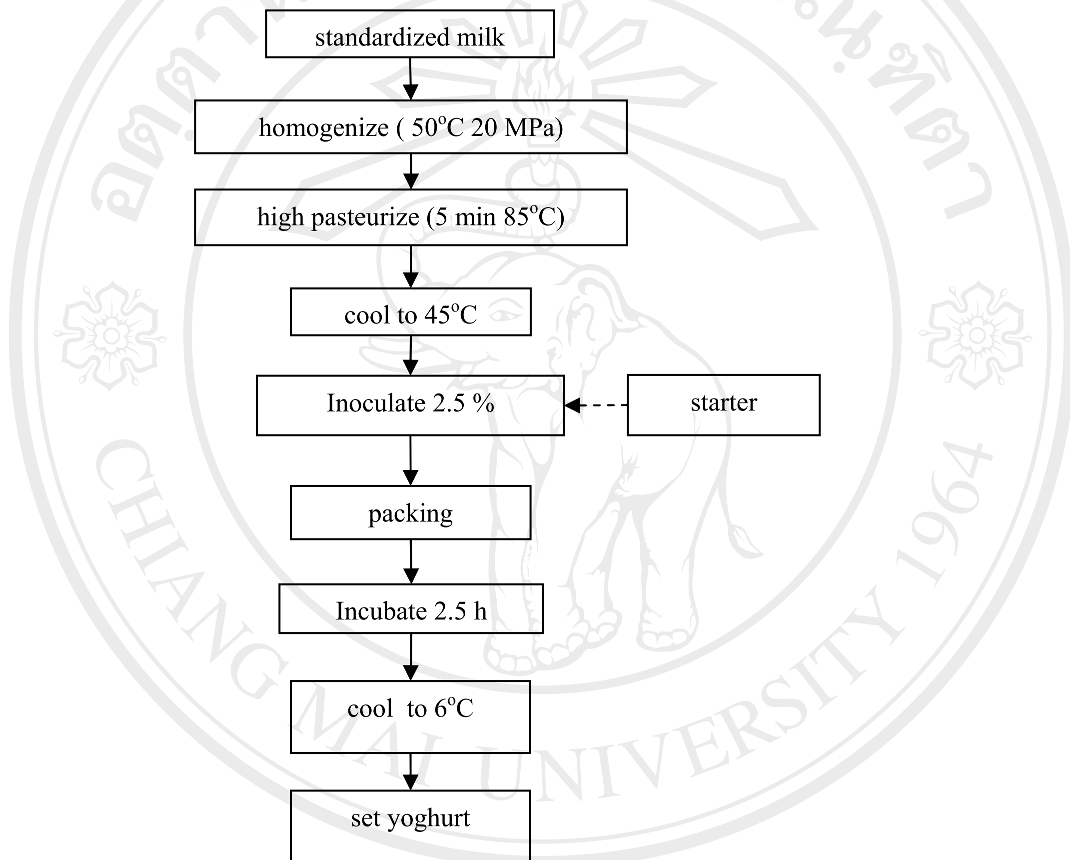

Fig. 5 An examples of the manufacture of set yoghurt (Walstra *et al.,* 1999)

2.4 Spray drying

The spray drying process transforms a pumpable fluid feed into a dried product in a single operation. The fluid is atomized using a rotating wheel or a nozzle and the spray of droplets comes immediately in contact with a flow of hot drying medium, usually air. The resulting rapid evaporation maintains a low droplet temperature so that high drying air temperatures can be applied without affecting the product. The time of drying the droplets is very short in comparison with most other drying processes. Low product

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temperature and short drying time allow spray drying of very heat-sensitive products. Some of the spray-dried products are listed in Table 7 (Master, 1991).

Table 7 Operating parameters for some spray-dried materials (Mujumdar, 1995)

Material	Moisture content		Atomizer device	Liquid-air	Air temperature	
	Inlet (%)	Outlet(%)			Inlet (°C)	Outlet (°C)
Skim milk	48-55	4	Wheel Pressure nozzle (170-200 bar)	Cocurrent	<250	95-100
Whey	50	4	Wheel	Cocurrent	150-180	70-80
Milk	50-60	2.5	Wheel Pressure nozzle (100-140 bar)	Cocurrent	170-200	90-100
Whole eggs	74-76	2-4	Wheel Pressure nozzle	Cocurrent	140-200	50-80
Coffee (instant)	75-85	3-3.5	Pressure nozzle	Cocurrent	270	110
Cream	52-60	4	Wheel	Cocurrent	500-600	>110

2.4.1 Spray drying consists of four process stages

2.4.1.1 Atomization of feed into a spray

Atomization is the most important operation in the spray drying process. The type of atomizer not only determines the energy required to form the spray but also the size and size distribution of the drops and speed, on which the final participle size depends. The chamber design is also influenced by the choice of the atomizer. The drop size establishes the heat transfer surface available and thus the drying rate. Three general types of atomizers are available. The most commonly used are the rotary wheel atomizers and the pressure nozzle single-fluid atomizers. Pneumatic two-fluid nozzles are used only rarely in very special applications. Existing spray drying systems provided various forms of the dry product-from fine powders to granules. The typical ranges of the disintegrated droplets and particle sizes of various products in a spray dryer are listed in Table 8 (Master, 1991).

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Table 8 Range of droplet and particle sizes obtained in spray dryers (µm) (Master, 1991)

Rotating wheels	1-600
Pressure nozzles	10-800
Pneumatic nozzles	5-300
Sonic nozzles	5-1000
Milk	30-250
Coffee	80-400
Pigments	10-200
Ceramics	30-200
Pharmaceutics	5-50
Chemicals	10-1000

● Selection of Atomizers (Master, 1991)

The selection of atomizer usually means the selection between wheel atomizer or pressure nozzle because the use of the pneumatic nozzle is very limited. The selection may be based on various considerations, such as availability, flexibility, energy consumption or particle size distribution of the final dry product. The Pictures of spray dry powder using different atomizers were shown in Figure 6.

Rotary atomizer	Pressure nozzle
~Easy control of particle size	Less easy control of particle size
~Large flow areas	Small flow areas
~Single atomizer for low and high capacities	Nozzle duplication for high capacities
~Handles slurries and crystalline feedstocks	Fine feed filtering required
~Particle size virtually independent of feed rate	Narrow operating feed rate range
~Capacity independent of feed rate	Capacity proportional to square root of pressure
~Large particles dried only in large-diameter drying chamber	Large particles dried only in smaller-diameter drying chamber
~Unit cost with pump comparable	Unit cost with pump comparable
~Low-pressure feed system	High-pressure feed system
~Fine-medium size particles individual, mean size up to 200 µm	Coarse free-flowing particles individual, mean size up to 350µm
~Deposit tendencies on wall at wheel level	Less tendency to deposit on wall

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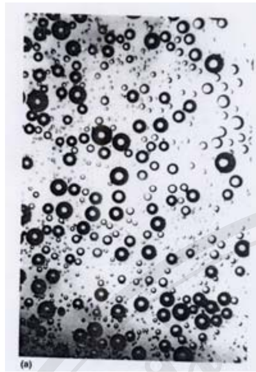

(a) Rotary atomizer

(b) Pressure nozzle

Fig. 6 Pictures of spray dry powder using a rotary atomizer (a) and pressure nozzle (b) (Master, 1991)

2.4.1.2 Spray-air contact (mixing and flow)

There are three basic types of air-droplet contact systems employed in spray drying processes (Master, 1991).

1. Co-current contact occurs when the droplets fall down the chamber with the air flowing in the same direction. It is the most common system with both wheel and nozzle atomization. Wheel atomizers are used when fine particles of heat-sensitive material are required; heat-sensitive coarse droplets are dried in nozzle tower chamber designs. The final product temperature is lower than the inlet air temperature.
2. Countercurrent contact is achieved when the drying air flows countercurrent to the falling droplets or particles. It is used for more heat- sensitive material that require coarse particles or special porosity or high bulk density. Nozzle atomization is usually used. The final product temperature is higher than that of the exit air.
3. Mixed-flow contact is employed when a coarse product is required and the size of drying chamber is limited. It has so far been the most economical system for a material that can withstand exposure to high temperature in dry form.

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How best to contact the spray cloud with drying air is dependent upon the product involved. For example, in the countercurrent arrangement, the hottest drying air contacts the dried particles as they are about to leave the chamber. If the dried product can withstand a very hot environment, and a coarse, high bulk-density product is required, this layout is highly suitable. The product particles will be of low porosity due to the reduced tendency of the droplet to expand rapidly and fracture during, evaporation. If the particle cannot withstand such high-temperature conditions, alternative contacting methods must be employed and the co-current system may be suitable. The hottest drying air contacts droplets at their maximum moisture content. The rapid evaporation prevents high droplet temperature. However, the droplets undergoing such a high evaporation rate may expand or fracture to give non-spherical porous particles and the product will often have a low bulk density (Master, 1991).

2.4.1.3 Drying of spray (moisture/volatiles evaporation)

As soon as droplets of the spray come into contact with the drying air, evaporation takes place from the saturated vapor film which is quickly established at the droplet surface. The temperature at the droplet surface approximates to the wet-bulb temperature of the drying air. Evaporation takes place in two stages. At first there is sufficient moisture within the droplets to replenish that lost at the surface. Diffusion of moisture from within the droplet maintains saturated surface conditions and as long as this lasts, evaporation takes place at a constant rate. This is termed the constant rate period or first period of drying. When the moisture content becomes too low to maintain saturated conditions, the so-called critical point is reached and a dried shell forms at the droplet surface. Evaporation is now dependent upon that of moisture diffusion through the dried surface shell. The thickness of the dried shell increases with time, causing a decrease in the rate of evaporation. This is termed the falling rate period or second period of drying (Master, 1991).

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2.4.1.4 Separation of dried product from the air

During operation, the majority of product falls to the base of the chamber, while a small fraction passes out entrained in the air and is recovered in the separation equipment. Such equipment is usually cyclones as the dry collector is followed by wet scrubbers as the final wet collector. Alternative dry collectors are bag filters and electrostatic

precipitators. The choice of equipment is dependent upon the powder loading of the air leaving the drying chamber and acceptable efficiencies of recovery. With this system, a classification may be useful, but normally the two powder off-takes are combined and conveyed to a single discharge area. Separation of dried product from the air influences powder properties by virtue of the mechanical handling involved during the separation stage. Excessive mechanical handling can produce powders having a high percentage of fines (Master, 1991).

2.4.2 Food quality parameter

Numerous quality parameters can be defined for dried foods. Not at all are applicable for a given product or for a given application. These quality parameters may be categorized as thermal, structural, optical, sensory, textural, biological and microbiological, as well as appearance (Table 9).

Table 9 Quality changes in foods during drying (Baker, 1997)

Physical	Chemical	Biochemical
~Shrinkage structure	~Loss of chemical activity	~Degradation of cellular and biomolecules
~Loss of density	~Decomposition of some chemical constituents	~Oxidation of liquids
~Alteration of shape, size, porosity		~Denaturation of proteins
~Crystallization		~Enzymatic browning
~Change of solubility		~Maillard reaction
~Reduced rehydration		~Loss of vitamins
~Loss of aroma, organoleptic properties		

Quality degradation during drying is major concern in the selection, design and operation of a dryer for foods. The principal factors affects the quality parameters are summarized as followed (Baker, 1997);

~Chemical changes e.g. browning reactions, lipid oxidation, discoloration

~Physical changes e.g. reconstitutability, cracking, texture, aroma loss;

~Nutritional changes e.g. loss of vitamins and proteins, microbial growth

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In addition to moisture content, temperature and drying kinetics, food quality is also affected by other non-drying parameters. Thus, pH, composition of the food, feed pre-treatments and the presence of salts, solvents and oils can all have a profound effect on the quality of the dried product (Baker, 1997).

2.4.3 Spray dried yoghurt

The positive health attributes associated with the consumption of fermented milk product has stimulated considerable market interest in spray-dried dairy ingredient such as yoghurt powder. Natural flavors produced during fermented milk processing are much valued in their own right and also to complement other added flavor. Traditionally, yoghurt is produced in a hydrated form and, thus, possesses a limited shelf-life even when refrigerated. Consumption within a short time of production is advisable, particular if advantage is to be taken of putative benefits associated with the ingestion of live yoghurt cultures. The production of an instant yoghurt powder would, thus, provide benefits of shelf-life extension and convenience of preparation and storage (Tamine and Robinson, 1999).

Basically there are two methods of drying that could be employed commercially for the manufacture of dried yoghurt (spray-drying or freeze-drying) and although the latter method of drying would seem the more attractive the temperature of drying (20-35°C) is much lower than with spray drying (55-60°C) so ultimately causing the least damage to the milk constituents, and/or loss of flavor- it is far too expensive to be considered on a commercial scale. At present, powdered yoghurt is produced commercially using spray drying, but some precautionary measured should be considered. First, the concentration of yoghurt, before drying, should be carried out at 50-60°C and second, the drying conditions should be moderate to ensure a high viable cell count of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in the dried product (Tamine and Robinson, 1999).

Yoghurt can be dried in a three-stage drying plant, and on average, the yoghurt is concentrated to 35 g/100g total solid, preheated and atomized into the drying chamber with inlet and outlet temperatures at 160 and 65°C, respectively. The microbial (cfu/g) in commercial dried yoghurt of non-lactic acid bacteria, *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were as followed $< 1x10^{4}$, $1x10^{3}$ and $1x10^{4}$, respectively, whilst Pan *et al.,* 1995 reported that the count of lactobacilli in

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dried yoghurt was $7x10^5$ cfu/g. Kim and Bhowmik (1990) reported that the survival rate of the yoghurt organisms was influenced by the product inlet feed temperature to be at 30°C, the air inlet and outlet temperatures to be at 160 and 60°C, respectively, the atomizing air pressure be at 98 kPa and the hot air flow to be 0.23 m^3/min (Tamine and Robinson, 1999).

Bielecka and Majkowska (2000) reported that a synergistic set of the strains *L. delbrueckii* ssp. *bulgaricus* 151 and *S. thermophilus* MK-10 was preserved with a method of spray drying. Studies were performed on the effect of outlet air temperature in the range of 60-80°C upon the survival of yoghurt cultures, as well as the moisture content and sensory properties of yoghurt powder. Survival of yoghurt cultures was the highest at 60 and 65°C, but excessive moisture (10.2%) of yoghurt powder had a negative effect on its texture. The moisture content of the powder was lower at 80°C (4.4%), unfortunately sensory faults appeared at this temperature, and survival of bacteria cultures decreased considerably. Temperatures within the range of 70-75°C ensured satisfactory survival of yoghurt cultures (*L. delbrueckii* ssp. *bulgaricus* 13.7-15.8% and *S. thermophilus* 51.6-54.7%), maintained the proportion of yoghurt strains (*L. bulgaricus* : *S. thermophilus* = 1:3), a satisfactory moisture content (5.1-6.3%), and good sensory properties of yoghurt powder.

Kim and Bhowmik (1990) reported the survival of *Streptococcus salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was determined under various processing conditions for spray drying. Numbers of both microorganisms decreased with increased outlet or inlet air temperature and atomizing air pressure. Outlet air temperature was a major parameter affecting the number of survivors. Suitable conditions were an inlet air at 160°C, an outlet air at 60°C, an atomizing at pressure 98 kPa, a hot air flow of 0.28 m^3/min and a feed temperature at 30°C. The spray-dried yoghurt powder showed lower survival for *S. thermophilus* but a similar survival for *L. delbrueckii* ssp. *bulgaricus* as compared to freeze-dried powder.

Lain *et al.* (2002) reported that microencapsulated cells of *B. longum* B6 and *B. infantis* CCRC14633 were prepared by spray drying the cell suspension containing the test organism and 10% (w/w) of the carrier material of either gelatin, soluble starch, skim milk or gum arabic. Survival of these microencapsulated and free cells of bifidobacteria in simulated gastric juice (pH 2.0 and 3.0) and bile solution (0.5% and 2.0%) was then examined. *B. infantis* CCRC14633 was more susceptible than *B.*

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longum B6 to the stimulated gastric environment and bile solution tested. Microencapsulated bifidobacteria exhibited a lower population reduction than free cells exposure to simulated gastric environment and bile solution. Moreover, it was also observed that the protective effect exerted by encapsulation with spray drying varied with the carriers used and the strains of bifidobateria.

In spray dry process, a sample should be gel or emulsion or slurry that has total soluble solid 20-50%. This sample is shattered to droplets in the chamber and is passed by hot air. Heat transfer occurs rapidly because of small droplets. This method making a good characteristic of product depends on the shattered of sample and heat transfer rate between the droplets and the hot air. Yoghurt usually has total soluble solid 14-16% that not sufficient making spray dry process so maltodextrin is added to increase the total soluble solid of yoghurt. Maltodextrin is widely used in spray dry industry because it is more soluble, more dispersal, less absorption moisture, preserve a product flavor and more flowability. For example, maltodextrin (having dextrose equivalent (DE) 10) is used in cheese powder, fat powder and fruit juice powder.

Peri and Pompi (1976) reported that a spray dry process for neutralized yoghurt should use a temperature of below 80°C, and a moisture content of the final product was approximately 10%. The shelf life of product at 5°C is more than 8 months.

Metwally and Abd-El-Garad (1989) reported that the survival of different microorganisms using 2 types of atomizer. The research was conducted at an inlet temperature of 190-200°C and an outlet temperature 80-82°C. When using a centrifugal atomizer, the survival of *Streptococcus lactis* was 0.93%, *S. thermophilus* 7.75% and *L. bulgaricus* 0.92%. Whereas using a two-fluid atomizer would give a survival rate for *S. lactis* 2.2%, *S. thermophilus* 11.8% and *L. bulgaricus* 1.14%. The pressure of two-fluid atomizer 2.5 kg/cm^{2} and at this pressure, the microorganism was damage. The microorganism would be damaged if a pressure more than 1.6 kg/cm^{2} were used. Using different temperature outlet with the two-fluid atomizer, it was found that at 70-72°C, the survival of *S. lactis* was 22.0%, *S. thermophilus* 46.9% and *L. bulgaricus* 19.5%. At 82-84°C outlet temperature, the survival of *S. lactis* was 1.8%, *S. thermophilus* 12.2% and *L. bulgaricus* 4.8%. Whereas at 88-90°C outlet temperature, the survival rate of *S. lactis* was 1.57% and *S. thermophilus* was 8.8 %.

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Cai and Corke (2000) reported that maltodextrins (hydrolazed starches) were effective encapsulating agents when use in the spray drying of sensitive flavors and carotenoid. Maltodextrins were also a good compromise between cost and effectiveness. The compound was bland in flavor, had low viscosity at high solids ratio and were available in different average molecular weights (DE 4, 10, 15, 20, 25, 30 and 40). A structural analysis revealed that there were many more surface indentations and cracks in wall systems containing lower DE maltodextrins than in those containing higher DE maltodextrins. Most of the spray-dried capsules containing 25 DE maltodextrin looked like smooth sphere with hardly any surface cracks in the wall systems. Moreover, maltodextrin of 10 DE, with the lowest hygroscopicity, could be mixed with 25 DE maltodextrin (25:10 = 3:1) in order to improve overall hygroscopicity.

2.5 Vacuum Drying

To intense moisture removal and lower the drying temperature in order to protect heat-sensitive food components, vacuum drying technologies can be used. All vacuum drying systems have four essential elements a vacuum chamber, a heat supply, a device for producing and maintaining the vacuum and components to collect water vapor evaporated from the food. Vacuum drying is similar to freeze drying except that the product is not frozen and the vacuum is not too high. The evaporation of liquid water is fast due to the vacuum. The rapid movement of the liquid within the product can cause foaming or puffing of the structure. Heat transfer occurs by radiation and conduction (Ramaswamy and Marcotte, 2006).

The lower pressure allows drying temperature to be reduced and higher quality to be obtained than with classical air conventional process at atmospheric pressure. The vacuum expands air and water vapor present in the food products and crates a frothy or puffed structure (Arevalo-Pinedo and Murr, 2007).

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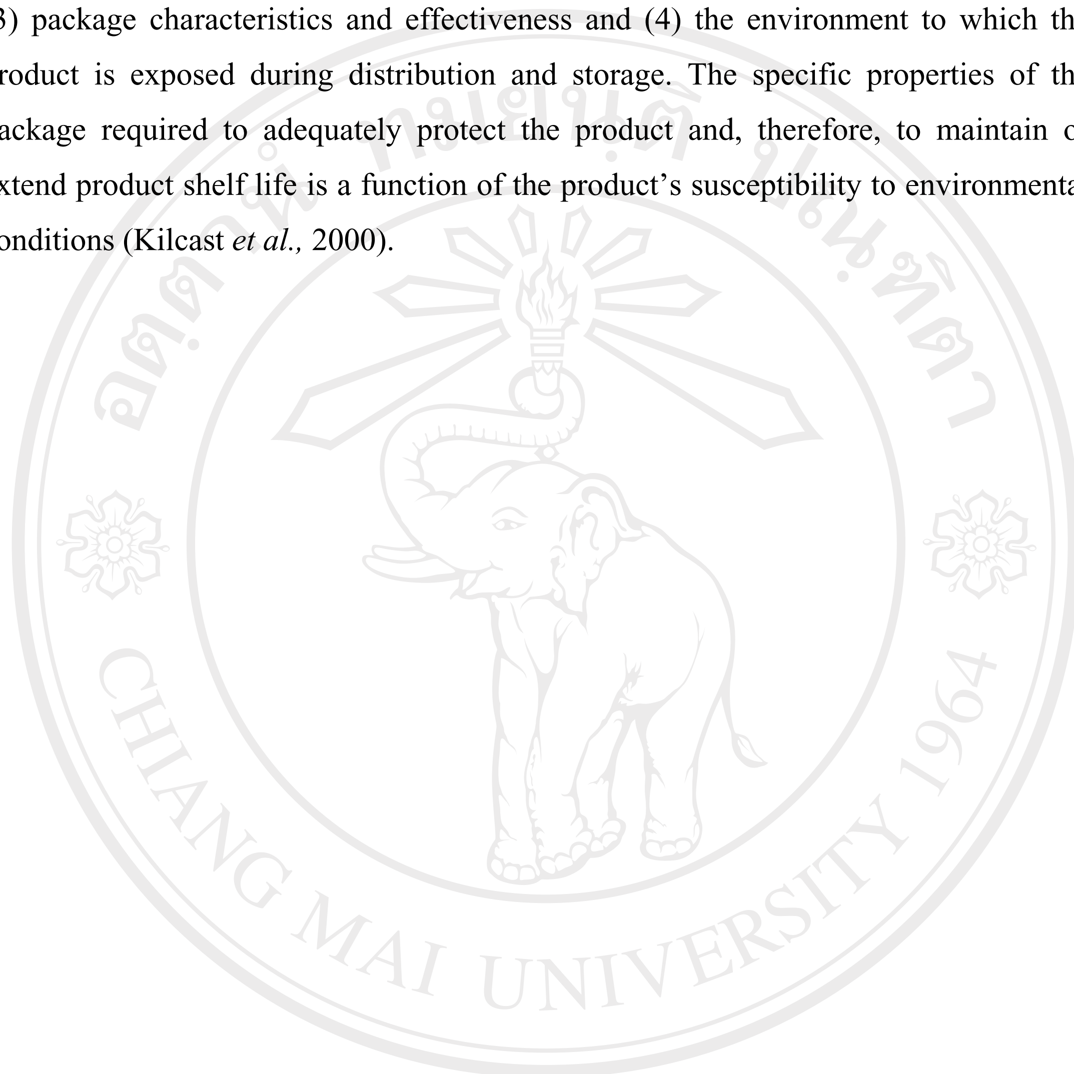
2.6 Packaging material

Product quality or shelf life is determined by the following parameters: (1) the product's physical, chemical and biological characteristics; (2) processing conditions; (3) package characteristics and effectiveness and (4) the environment to which the product is exposed during distribution and storage. The specific properties of the package required to adequately protect the product and, therefore, to maintain or extend product shelf life is a function of the product's susceptibility to environmental conditions (Kilcast *et al.,* 2000).



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