บทความวิชาการ

การรอดชีวิตของจุลินทรีย์โพรไบโอติกในน้ำผลไม้

สวามินี นวลแขกุล[1*] และ Dimitris Charalampopoulos[2]

บทคัดย่อ

บทความฉบับนี้มุ่งประเด็นไปที่การรอดชีวิตของจุลินทรีย์โพรไบโอติกในอาหารที่มีค่าความเป็นกรดสูง โดยเฉพาะในน้ำผลไม้ ในปัจจุบันผู้บริโภคกังวลเกี่ยวกับผลของอาหารสุขภาพ อาหารฟังก์ชั่นนอลได้รับความสนใจมากเพื่อส่งเสริมสุขภาพที่ดีนอกเหนือจากโภชนาการขั้นพื้นฐาน ผลิตภัณฑ์อาหารสุขภาพรวมส่วนประกอบที่เป็นฟังก์ชันนอลเช่น จุลินทรีย์โพรไบโอติก พรีไบโอติก เปปไทด์ สารฟีนอล กรดไขมันและอื่น ๆ จุลินทรีย์โพรไบโอติกในทางการค้าส่วนใหญ่มีอยู่ในผลิตภัณฑ์นม เช่น นมหมัก โยเกิร์ต ไอศครีมและเนยแข็ง รวมไปถึง ผลิตภัณฑ์ที่ไม่ใช่นม เช่น เครื่องดื่ม อาหารเช้าธัญพืช เนื้อหมัก อาหารแห้ง และอาหารเสริม นวัตกรรมของจุลินทรีย์โพรไบโอติกที่ได้ออกตลาดในช่วงที่ผ่านมาเป็นเครื่องดื่มที่ทำจากผลไม้และธัญพืช อย่างไรก็ตาม ผลไม้มีสภาพแวดล้อมที่แย่สำหรับจุลินทรีย์โพรไบโอติกมากกว่านมหมักเพราะมีค่าความเป็นกรดและสารประกอบฟีนอลอยู่ในระดับสูง ผู้บริโภคมีความคาดหวังว่าผลิตภัณฑ์ที่พวกเขาซื้อประกอบไปด้วยปริมาณของจุลินทรีย์โพรไบโอติกที่ระบุไว้ในฉลากตามเวลาที่กำหนด ดังนั้น การพัฒนาเพื่อเพิ่มการอยู่รอดของจุลินทรีย์โพรไบโอติกระหว่างการเก็บรักษาจึงเป็นสิ่งสำคัญของงานวิจัย และมีผลกระทบมากในอุตสาหกรรมอาหาร มันเป็นไปได้ที่จะทำให้จุลินทรีย์โพรไบโอติกมีความแข็งแรงทนต่อสภาพแวดล้อมภายนอกเช่น ในสภาพความเป็นกรดสูง โดยการมีเกราะห่อหุ้มเซลล์ในพอลิเมอร์ชนิดต่างๆ และมีการเคลือบเม็ดพอลิเมอร์นั้น

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[1]ภาควิชาคหกรรมศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ

[2]Department of Food and Nutritional Sciences, The University of Reading. UK

*ผู้นิพนธ์ประสานงาน, e-mail: sawaminee@swu.ac.th

Survival of Probiotics in Fruit Juices

Sawaminee Nualkaekul[1*] and Dimitris Charalampopoulos[2]

ABSTRACT

This paper aimed to address the issue of survival of probiotic bacteria in acidic foods, and in particular fruit juices. In recent years, driven by the consumers' concern about the effect of diet on health, functional foods have received a lot of attention, as their aim is to promote health and well-being beyond basic nutrition. These products include those containing functional ingredients, such as probiotic microorganisms, prebiotic carbohydrates, bioactive peptides, phenolics, fatty acids, and others. Commercial probiotic products include primarily dairy products (fermented milks, yoghurts, ice cream, cheeses), and to a lesser extent non-dairy products (beverages, breakfast cereals, fermented meats, dry-foods) and dietary supplements. Novel products containing probiotics have been launched recently, mainly beverages based on fruits and cereals. However, fruit juices are more adverse environments than fermented milks for probiotics as they have high acidity and high levels of phenolic compounds. Consumers on the other hand demand that the product they purchase contains the concentration of probiotic cells stated on the package at the time of consumption. Therefore, identifying the factors influencing probiotic survival in juices and developing ways to enhance probiotic survival during storage is an important area of research with considerable impact for the food industry. It is possible to make probiotic cells more robust to external conditions, such as those of highly acidic juices by encapsulate the cells within various polymer matrices and coating the beads with polymers.

Keywords: survival, probiotic, fruit juices, high acidity, encapsulation

[1]Department of Home Economics, Faculty of Science, Srinakharinwirot University

[2]Department of Food and Nutritional Sciences, The University of Reading, UK

*Corresponding author, email: sawaminee@swu.ac.th

Introduction

The application of probiotic bacteria in foods for promoting health benefits is based on the concept that the maintenance of a healthy gut microflora provides protection against gastrointestinal disorders including infections and inflammatory syndromes of the bowel [1]. *Lactobacillus* and *Bifidobacterium* are the two genera that have been most commonly used as probiotics and have been widely investigated for their beneficial effects [2]. Probiotics are currently used as co-starter or adjunct cultures for the production of fermented milks [3] and other dairy products, such as yoghurt [4], ice cream [5], cheese [6], and to a lesser extent fermented meats, cereals, vegetables, and fruits [7]. They are delivered in two main formats, either as a dried nutraceutical product or incorporated in various foods, such as fermented dairy products. The viable cell concentration of the probiotic bacteria in a food product or nutraceutical formulation should be as high as possible because a significant number of bacterial cells die during storage and passage through the stomach and the small intestine [7-10]. Taking into account the fact that the minimum effective dose is suggested to be 10^8-10^9 cells per day [7, 11], in order to exert their health benefits, the minimum concentration of live probiotic should be at least 10^6-10^7 CFU per gram of product at the time of consumption.

The development of non-dairy probiotic products is a challenge to the food industry in its effort to utilise the abundant natural resources by producing high quality functional products. In this respect, fruit juices can be used as alternative vehicles as they are suitable for lactose intolerant consumers and contain high amounts of sugars, vitamins and minerals, which are advantageous for probiotic survival during storage [12, 13]. In addition, fruit juices are consumed frequently and loyally by consumers [14].

Factors influencing probiotic survival

The main parameters affecting the viability of probiotics in food products included the species/strain used [15], effect of probiotic production process [16], the composition of the food product [9], the storage temperature and time [17], the oxygen levels (especially in the case of *Bifidobacterium* species) [9], and the type of container [18]. The following sections are discussed in more detail.

- Genera/Species/Strains

The type of probiotic affects considerably its robustness and technological properties [3, 18]. In general, *Bifidobacterium* species seem to be more sensitive than *Lactobacillus* species [9, 19]; it must be noted though that most studies looking at different species within a genus have been carried out with lactobacilli. The most robust *Lactobacillus* species seem to be

L. casei and *L. rhamnosus;* these have been shown to survive better than *L. acidophilus* in cheese products during storage at low temperatures [7]. In another study, similarly, *L. rhamnosus* showed good survival during storage in fruit juice at 4°C for 80 days, whereas two *L. acidophilus* strains showed the lowest stability among all strains tested [18]. Moreover, Sadaghdar et al. (2012) [20] reported that among various *Lactobacillus* strains, a *L. casei* strain exhibited the highest survival during refrigerated storage in flavoured fermented milk products (5°C, 21 days), while a *L. acidophilus* strain showed the lowest survival.

- Effect of probiotic production process

Probiotics are produced through a fermentation process, during which the cells are grown in bioreactors and are subsequently harvested, re-suspended in a suitable cryo-protectant medium and freeze dried. Although no considerable amount of research has been conducted in this area, it has been suggested that both the upstream and the downstream processing are likely to affect the robustness of the probiotic during its incorporation into the food product and its subsequent storage. Key fermentation parameters likely to play a role include the fermentation pH, the medium composition, the growth time and the gas atmosphere, as they can potentially affect the cell physiology in a contradiction way which contributes to the stability of the processed cells [16]. For example, Palmfeldt and Hahn-Hagerdal (2000) [21] reported that growing L. reuteri at pH 5 rather than at pH 6 enhanced the viability of the cells during freeze drying; the highest survival rate (approximately 80%) was observed when the cells were grown at pH 5 and harvested after 2.5 h in the stationary phase. The same authors also reported that the cells' response to starvation can also protect the cells from other stresses. Moreover, it has been shown that stationary phase cells are generally more tolerant to stressful conditions compared with exponential-phase cells [16, 22]. Although it has not been studied, it might be the case that cells that are considered to be more technological robust can survive better in food products during storage.

Probiotic cells can be added into a food product either as fresh, dried or frozen concentrated cultures [17]. Freeze-drying has been the typical method used for producing dry bacterial powders, as freeze dried cells are easier to handle than frozen cells [17]. There is very little information on the effect of the drying process on the subsequent survival of probiotics in food products [23]. In the only study found, Saarela et al. (2006) [24] reported that fresh *L. rhamnosus* cells added into apple juice mixed with an oat mix (oat flour with 20% β-glucan) survived better than freeze-dried cells. The authors suggested that the freeze dried cells were probably injured during the freeze drying process, and were thus more sensitive to the acidic conditions of the product. Further research is however needed in this field to establish this relationship, which will hopefully lead to the development of better production practices.

- Composition of food products

The viability of probiotic bacteria also depends on the composition of the food product, e.g. the pH, the content in organic acids, sugars, dietary fibre, protein and phenolics, and the water activity [9, 18, 25, 26]. These effects can probably explain the differences in cell survival between various food products. For example, the survival of *L. rhamnosus* was higher in chocolate-coated breakfast cereals compared with apple juice [24], whereas the survival of *B. animalis* subsp. *lactis* E-2010 (Bb-12) in milk was higher than that in fruit juices [17].

Among the above mentioned factors, those that seem to affect mostly probiotic survival are the low pH and the organic acid content [7, 16]. Acids damage the bacteria cells as they enter inside the cytoplasm, where they dissociate, decreasing the intracellular pH and thus inhibit the metabolic reactions taking place. In order for the cells to maintain their intracellular pH, an increased amount of energy is required [16, 27]. In foods, this energy can be found mainly from fermentable sugars (e.g. glucose), thus enhance cell survival [28, 29]. In model systems, it was shown that at low pH conditions (pH ~ 2.0), the presence of fermentable sugars enhanced the short-term survival of several *Lactobacillus* strains; however, this effect seems to be strain specific, as it benefited more *L. rhamnosus* GG [30] than *L. rhamnosus* E800 [31]. In terms of nitrogen sources, it was shown that whey protein, which is often added in yoghurts, provided peptides and amino acids to the cells, resulting in improved survival during storage [9, 28]. Another research was shown the pH had a negative effect on *Lactobacillus plantarum* and *Bifidobacterium longum* survival, whereas citric acid, protein and dietary fibre had a positive effect, whereas ascorbic acid had no effect. The mathematical models were able to predict well cell survival in a variety of fruit juices, including orange, blackcurrant and pineapple, however they failed to predict cell survival in strawberry, cranberry and pomegranate; this was most likely due to the highly acidic character of these juices and their high content in phenolic compounds [25, 26].

The fibre is the edible part of plants that is resistant to digestion and absorption in the human small intestine, and is completely or partially fermented in the large intestine [24]. The fibre can potentially protect probiotic cells during processing and storage via a mechanism involving the physical immobilisation of the cells onto the fibre, thus improving their survival [24, 32]. Examples of fibres include oat β-glucan, the addition of which into yoghurt resulted in improved survival of *B. animalis* subsp. *lactis* during prolonged cold storage [33]. Other researchers have reported that oat flour with 20% β-glucan was able to protect *L. rhamnosus* during storage in apple juice [24]. On the other hand, prebiotic compounds, i.e. 'non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract' [34] have also been suggested to improve the survival of lactobacilli and bifidobacteria

in the large intestine [35]. Such compounds include oligosaccharides, such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), transgalacto-oligosaccharides (TOS), isomalto-oligosaccharides, xylo-oligosaccharides, and soybean-oligosaccharides [36, 37]. For example, oligofructose significantly improved the viability of *L. acidophilus* and *B. animalis* in low-fat ice cream stored at -18°C for 90 days [38]. Furthermore, Hernandez-Hernandez et al. (2012) [39] reported that GOS and lactulose supported the growth of various *Lactobacillus* strains and also improved their survival through the gastrointestinal tract.

Another important factor for the stability of probiotics during storage, in particular in dried formulations, such as dried infant formulas, is the water activity of the formulation [40-43]. More specifically, it has been suggested that cell survival is negatively affected when the food product has a water activity >0.25 [44]. For example, in a study investigating the survival of freeze-dried *L. acidophilus* during 10 weeks of storage at 20°C, it was shown that when the water activity increased from 0.11 to 0.23 and then to 0.43 the survival of probiotics progressively decreased, reaching a maximum loss of 3 log CFU/g [45]. Nualkaekul et al. (2012) [42] was to investigate the survival of freeze dried *L. plantarum* cells mixed with several freeze dried instant fruit powders (strawberry, pomegranate, blackcurrant and cranberry) during storage for 12 months as well as after reconstitution with water every month. The only factor influencing cell survival during storage in instant fruit powders was the water activity of the dried powders rather than the pH, and the citric acid, dietary fibre and total phenol concentrations. This could explain the fact that the highest cell survival was obtained in the fruit powder with the lowest water activity, i.e. blackcurrant (a_w ~ 0.14), whereas the lowest was in the fruit powder with the highest water activity, i.e. cranberry (a_w ~ 0.32).

- Storage temperature and time

Most acidic dairy products containing probiotics, such as yoghurt and fermented milks, are kept refrigerated during storage, which enhances the viability of probiotics [37]. In general, the lower the storage temperature the greater is the survival of probiotics, and for this reason refrigeration at 4°C has been generally used as the most appropriate storage temperature [17, 18]. This is most likely due to the fact that in acidic environments, the diffusion rate of acid into the cell in higher temperatures (e.g. 25°C) is faster than in lower temperatures (e.g. 4°C) [46]. Besides temperature, the storage time affects also probiotic survival; the longer the storage time the lower the survival [47, 48]. The most important criterion from a technological point of view is that the probiotic is maintained at high levels (>10^6 CFU/g or mL) for the time that a particular product would be normally stored for, e.g. for a yoghurt for 4-5 weeks and for fresh juice for up to 6 weeks, as the shelf life is mainly determined by the safety requirements and organoleptic properties.

- Oxygen levels

Many studies have shown that probiotic bacteria are affected by oxygen, although this is strain dependent [18, 49, 50]. In general, *Bifidobacterium* species are more sensitive to oxygen than lactobacilli [50, 51]. Champagne et al. (2008) [18] showed that oxygen did not affect the viability of *L. rhamnosus* R0011 in an apple-based fruit juice under anaerobic (unopened bottle) and aerobic conditions (opened bottle to air, shaking). Bifidobacteria are anaerobic, however the sensitivity to oxygen changes according to different species and even strains within species [52]. Oxygen is unable to cause any damage to the cells by itself; however, during the metabolic pathway, oxygen is partially reduced to water, leading also to the formation of reactive oxygen species, which include the superoxide anion radical (O_2^-), the hydroxyl radical (OH^+), and hydrogen peroxide (H_2O_2). These intermediates have a high oxidizing potential and thus are responsible for cellular oxygen toxicity [53]. The levels of dissolved oxygen in the fruit juice products depend on the processing operations, such as blending and homogenisation, the type of packaging and particular whether oxygen is able to diffuse through it during storage, the headspace inside the package, and the size of the product. To minimise the effect of oxygen, antioxidants are sometimes added into foods (e.g. yoghurt) to improve the survival of probiotic strains, as they can act as oxygen scavengers; an example of this is the addition of ascorbic acid or L-cysteine [7, 9].

- Type of container

The container and the packaging material are additional factors influencing cell survival. In general, the levels of oxygen within the container during storage should be as low as possible in order to avoid cell death [47]. Dave and Shah (1997) [54] studied the survival of probiotic bacteria in yoghurt filled into glass and high-density polyethylene containers for 35 days. The level of dissolved oxygen was significantly lower in the glass than in the plastic containers, and as a result, cell survival was higher in the former. Similarly, Jayamanne and Adams (2004) [55] found that *Bifidobacterium* strains in meekiri (fermented buffalo milk) survived best in glass bottles, followed by plastic containers and clay pots, when stored at 29°C for a period of over 4 days. The main reason for the low cell numbers in clay pots was that they allowed the diffusion of oxygen into the containers.

Fruit juices as probiotic carriers

There is a considerable interest in developing fruit-juice-based functional beverages with probiotics because fruit juices have taste profiles that are appealing to all age groups and are perceived as healthy and refreshing foods [56]. As a result, a moderate number of studies have

been conducted looking at the survival of lactobacilli and bifidobacteria in various fruit juices; these studies are summarised in Table 1. [1, 12, 17, 18, 24, 57,] Overall, although differences obviously exist depending on the species and even the strains used, the general conclusion from these studies is that the acidity of the juices is the most prominent factor influencing the viability of the cells. Therefore, juices that are less acidic than others, e.g. orange, pineapple and apple juice support the survival of the cells better. The rest of this section discusses in more detail the compositional characteristics of the juices that are likely to be of importance.

In general, all fruit juices contain organic acids and their pH usually varies between 2.6 and 3.9 [24]. Citric and malic acid are the main acids in fruit juices such as orange, lemon, grapefruit, blackcurrant and apple juice [58, 59]. Ascorbic acid is also present in amounts ranging from 100 to 1000 mg/L, depending on the juice [59]; fresh orange juice typically contains 500-600 mg/L ascorbic acid [60]. Fruit juices also contain minerals and polyphenols, which can potentially have antioxidant activities [61, 62].

Overall, there is no information in the literature regarding the specific role of citric and malic acids present in fruit juices on the survival of probiotic bacteria during storage. Organic acids, including citric and malic acid, are generally used as preservatives in food products due to their antimicrobial activities. The mechanism of action is that the undissociated form of the acid passes freely through the cell membrane, where it dissociates releasing protons; this leads to the acidification of the cytoplasm [63]. Therefore, organic acids are expected to have an adverse effect on the viability of probiotics. On the other hand, research has shown that ascorbic acid possibly plays a role too. In particular, it has been reported that increasing the concentration of ascorbic acid improved the survival of *L. acidophilus* in yoghurt as it acted as an oxygen scavenger; however, the outcome was not the same for bifidobacteria [9, 13], which is surprising considering that bifidobacteria are generally more susceptible to oxygen than lactobacilli. It must be mentioned though that ascorbic acid is a sensitive compound, as a variety of factors influence its stability during processing and storage, including the temperature, the concentration of salts, sugars and minerals in the juice, the pH, the oxygen levels, the presence of enzymes and light [64]. Therefore, such degradation processes that are likely taking place might have affected the published results.

In terms of the effect of sugars, the concentration of sugar in fruit juices varies between 60-200 g/L [65] and consists mainly of sucrose, glucose and fructose [66]. The high concentration of sugars might exert an osmotic pressure effect on the cells and might thus affect their viability [9, 15]. For example, Akin (2005) [67] reported that the viability of *L. acidophilus* and *B. lactis* in ice-cream decreased by ~ 0.5-1 log when the concentration of sugar increased from 15% (w/v) to 21% (w/v) during storage at -18°C for 90 days. On the other hand,

Table 1 Survival of free probiotic cells in various types of fruit juices.

Strains	Fruit juices	pH	Survival conditions	Observations from survival data	References
L. salivarius UCC118, *L. salivarius* UCC500, *B. lactis* Bb-12, *L. casei* DN-114 001, *L. rhamnosus* GG and *L. paracasei* NFBC43338	Orange, pineapple and cranberry	OJ ~ 3.65, PJ ~ 3.40, CJ ~ 2.50	Stored at 4°C for 12 weeks	The initial cells from 10^8 log CFU/mL, the viability above to 10^7 CFU mL in orange juice and above to 10^6 CFU/mL in pineapple juice after 12 weeks	[1]
L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis type Bi-04 and *B. lactis* type Bi-07	Orange and apple	~ 2.8	Stored at 4°C for 6 weeks	No viable bacteria present by week five	[12]
B. animalis subsp. *lactis* E-2010 (Bb-12)	Orange, grape and passion fruit	3.7	Stored at 4 and 20°C for up to 6 weeks	At 20°C, the viability decreased below log 4 CFU/mL from 7.5-8.4 log CFU/mL	[17]
L. rhamnosus R0011	Apple-pear-raspberry blend	3.6-3.9	Stored for up to 6 weeks at 2 and 7°C under anaerobic (unopened bottles) and aerobic conditions (opened bottle to air, shaking)	For unopened bottles, viability dropped by 75% after 5 weeks of storage at 7°C, while in opened bottles between 20% and 40%.	[18]
L. rhamnosus E-97800	Apple	3.5	Added oat flour with 20% β-glucan, stored for 12 weeks at 4°C and at 20°C	Storage at 4°C better than at 20°C; no protective effect by oat flour	[24]
L. acidophilus LB2, LB3 and LB45, *L. brevis* LB6, *L. rhamnosus* LB11 and LB24, *L. fermentum* LB32, *L. plantarum* LB42 and *L. reuteri* LB38.	Pineapple, apple, orange, pear and/or grape, passion fruit, lemon	4.2	Stored at 4°C for 80 days	High viabilities observed for most strains after 80 days of storage	[57]

Charalampopoulos et al. (2003) [30] reported that the viability of *L. plantarum* during storage for 4 hours in phosphate buffered medium (pH 2.5) increased progressively, in some cases by up to 2 logs, as the glucose and maltose concentrations increased from 1.5 to 8.33 g/L. Another study showed that the presence of glucose at concentrations even lower than the previous mentioned study, ranging from 0.18 to 3.5 g/L mM, enhanced the survival of *L. rhamnosus* in simulated gastric juice (pH 2.0) over 90 min by ~ 2 log CFU/mL. It was suggested that glucose provided ATP enough via glycolysis, enabling proton exclusion and thereby enhancing cell survival during gastric transit [31].

Other components, which are present in the fruit juices and are likely to affect probiotic survival include phenolics. Although no data are available from studies with juices, it was shown in model systems for example that *L. rhamnosus* was sensitive to polyphenols; a minimum inhibitory concentration of at least 125 μg/mL was reported [68]. Other studies have also reported an antimicrobial activity by phenolic acids (62.5-1000 mg/mL) against various *Lactobacillus* strains including *L. paraplantarum, L. plantarum, L. fermentum, L. brevis* and *L. coryniformis* [69]. Finally, in addition to the above components, fruit juices contain natural microbial growth inhibitors or additives such as KCl, aspartame, flavorings and colourings, which can potentially affect cell survival, depending on the concentration of the particular ingredient and the strain [70].

Encapsulation approaches for improving probiotic viability

Encapsulation is a process in which the cells are entrapped within an encapsulating polymer with the aim to reduce cell injury or cell loss and thus improve cell survival [37]. Encapsulation can be used to protect probiotic cells from adverse environments, such as mild heat treatment during processing, or high acidic conditions in the food product [71], and thus it reduces the likelihood of cell injuries and cell death during processing and storage [72]. Several techniques can be used for encapsulating or immobilising probiotic cells; these include spray drying, extrusion, emulsion, co-extrusion, spray coating [37, 73-75]. Each of these generates beads with different characteristics in terms of size, shape and texture properties [51]. The most commonly used methods are the extrusion and the emulsion method [37].

- Extrusion method

The extrusion process is carried out by adding the cell suspension into a sodium alginate solution or another polymer, and then the solution is extruded through a needle into a calcium chloride solution. Figure 1 presents a schematic diagram of the process. This method is economical, simple to operate, produces uniformly shaped and sized beads with gentle operations

which makes cell injuries minimal and causes relatively high viability of probiotic cells. However, it is difficult to scale up to production due to the slow formation of the beads [37, 76] also in an industrial scale rather than one very large system, it is more likely to have several systems running in parallel to each other (scale out). The concentrations of alginate and calcium chloride used to form the gel generally vary between 0.6-3% and 0.05-1.5 M, respectively [37, 43, 77]. The size and shape of the formed beads depend on the viscosity of the sodium alginate solution, the diameter of the needle, and the distance between the syringe and the calcium chloride solution [73, 78], which is usually around 2-3 mm in diameter [37]. Lee and Heo (2000) [79] studied the survival of *B. longum* encapsulated with 2, 3, and 4% sodium alginate and demonstrated that the concentration of alginate and the bead size affected cell survival during exposure to simulated gastric juices and bile salts. In another study, Sun and Griffiths (2000) [80] investigated the survival of *B. infantis* in pasteurized yoghurt after refrigerated storage for 5 weeks, as well as simulated gastric juice at pH 2.5, 2.0 and 1.5 following encapsulation in gellan-xanthan beads (0.75% gellan and 1% xanthan gum, diameter ~ 3 mm). The result demonstrated the survival of encapsulated cells remained significantly better than that of the free cells; for example in the difference between encapsulated and free cells was more than 8 logs at pH 2.5.

- Emulsion method

In this method, the cell suspension and sodium alginate solution are added into a large volume of a continuous phase, which can be soybean oil, sunflower oil, canola oil or corn oil, containing an emulsifier, such as tween 80. The suspension is homogenised through stirring in order to form a water-in-oil emulsion (Figure 1). Once this is formed, the water soluble polymer becomes insoluble after addition of calcium chloride, due to cross-linking, which results to the formation of gel particles in the oil phase. Smaller particles in the water phase will lead to the formation of beads with smaller diameters [37, 71].

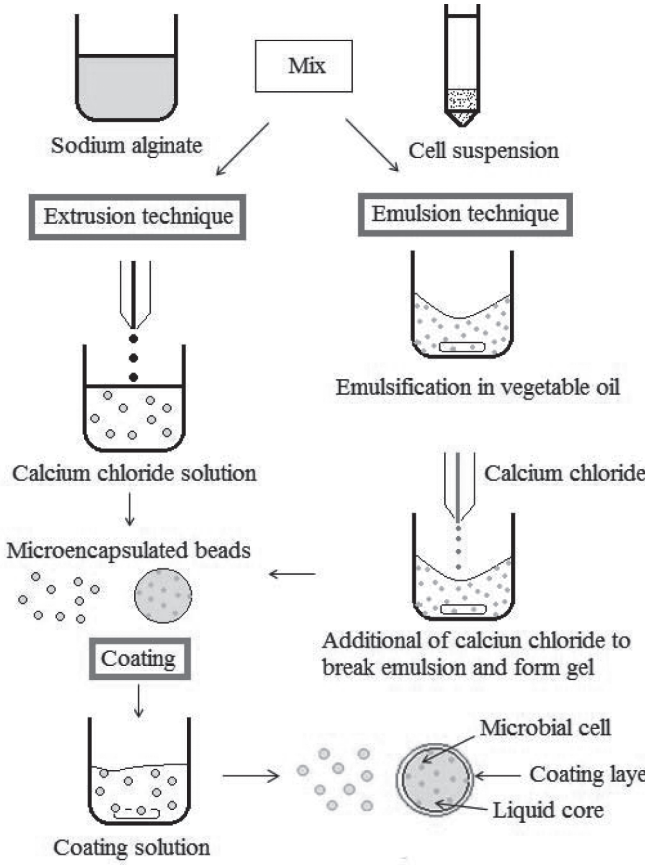

Figure 1 Flow chart of encapsulation of bacteria using the extrusion and emulsion methods and subsequent coating (adapted from Krasaekoopt et al., 2003 [37]; Cook et al., 2011 [79]).

The polymers which can be used in the emulsion method should be water soluble and should have the ability to form a gel by ionotropic gelation; such polymers include k-carrageenan, locust bean gum, cellulose acetate phthalate [81], chitosan [82], gelatin [83], and alginate [12, 84]. The size of the produced beads can vary between 25 μm and 2 mm [71]. The disadvantage of this method is that it provides a large range of size and shapes and has high cost. Moreover, due to the residual oil in the beads this method might not be suitable for the development of low-fat food products [74, 85].

Ding and Shah (2008) [12] reported that the encapsulation of *L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis* using the emulsion method improved significantly the cell survival in orange and apple juices during 6 weeks of storage at 4°C. Another study showed that microencapsulation in a calcium-induced alginate-starch emulsion increased the survival of *L. acidophilus* and *B. lactis* in yoghurts during

7 weeks of storage at 4°C by 2 and 1 log, respectively, compared to the free cells [4].

Ding and Shah (2009) [86]. investigated the stability of various probiotic strains in conditions of high acid and bile. The strains included *L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei, B. lactis,* and *B. bifidum,* and were encapsulated via the emulsion method with alginate, xanthan gum, carrageenan gum, guar gum and locust gum. In order to reduce the particle size, the emulsion was mixed thoroughly using a microfluidizer (100 bar, 22°C, 34 cycles) before adding calcium chloride. The results indicated that probiotic bacteria encapsulated in alginate, xanthan gum and carrageenan gum showed the better survival rates (>5 log CFU/mL) after 2 hours of exposure in acidic conditions (pH 2). On the other hand, the probiotic bacteria encapsulated in guar gum and locust bean gum showed the lower survival (~ 3.8 and 4.8 log CFU/mL, respectively). In all cases however, the survival of encapsulated cells was better than free cells.

Little comparative information is available between the extrusion and the emulsion methods. In a study by Jayalalitha et al. (2011) [87] it was shown that encapulated *Lactobacillus* and *Bifidobacterium* cells, produced through the extrusion method, survived better during storage in yoghurt for 21 days, than those produced through the emulsion method. This might be due to the fact that the extrusion method produces larger beads, which offer more protection than smaller beads [79]. The advantages and disadvantages of the extrusion and emulsion methods are summarised in Table 2.

Table 2 Advantages and disadvantages of the extrusion and emulsion methods [37, 51].

	Extrusion	**Emulsion**
Technological feasibility	Difficult to scale up	Easy to scale up
Cost	Low	High
Simplicity	High	Low
Survival of microorganism	80-95%	80-95%
Size of bead	2-3 mm	25 μm-2 mm
shaped and sized	Uniform	Non-uniform

In the case of encapsulated beads in particular, the size and texture of beads is important because it influences the sensory properties of product. In the study of Krasaekoopt and Kitsawad (2010) [88] it was reported that more than 80% of consumers accepted fruit juices containing probiotic beads, with size ranging from 100-200 μm, whereas less than 20% of consumers did not accept the probiotic beads, as the beads sometimes remained in the mouth and/or were stuck in the throat. The scores of the swallow ability of the fruit juices with probiotic beads were ~ 2 and without probiotic beads were ~ 7.

Coating of beads

Coating the beads with polymers is a method used to add extra protection to the cells; for example, to prevent the exposure of the encapsulated cells to oxygen during storage or improve their stability at low pH [43]. It has also been shown that coating of the beads increases their mechanical strength [78]. This is important as the textural properties of the beads could potentially influence cell survival and are likely to influence the organoleptic properties of the product too. The process of coating is shown in Figure 1; it usually involves placing the encapsulated beads into coating solution and the suspension mixed [89]. Another method that is widely used is spray coating; in this method a liquid coating material is sprayed over the core material and solidifies to form a layer at the surface. The advantage of spray coating is that it is easy to scale up, and can be adapted to provide multilayer coatings [51].

Possible coating materials include chitosan, poly-l-lysine, whey proteins, cellulose acetate phthalate (CAP), starch, gum and gelatin [85]; however, their selection depends on their compatibility with the encapsulating polymer. A good coating material should provide the required properties, such as strength, flexibility, impermeability and stability [75]. Most published works with coated beads have focused on chitosan [43, 90, 91]. This is because chitosan is a cationic polymer, biocompatible, biodegradable, nontoxic, is of low cost, and exhibits good film-forming abilities. The amine residues in chitosan are mostly protonated below pH 6.5, making chitosan a polycation [89]. As a result of its cationic character, chitosan is able to react with polyanions, such as alginate, giving rise to polyelectrolyte complexes [92]. Due to this interaction, alginate coated beads provide good protection of probiotic cells in acidic conditions.

Another polymer that has been used for coating alginate encapsulated beads is poly-l-lysine (PLL) [91, 93, 94]. The poly-amino acid forms a complex with the alginate, forming a semi-permeable membrane [91]. In the study of Ding and Shah (2009) [94] palm oil and poly-l-lysine were used to coat the alginate beads aiming to increase the survival of various *Lactobacillus* and *Bifidobacterium* species in acidic conditions (pH 2), after 2 hours exposure.

The results indicated that poly-l-lysine coating improved cell viability by more than 1 log CFU/mL compared to uncoated beads. The effect of coating polymers on the survival of probiotic cells is show in Table 3.

Nualkaekul et al. (2012) [77] reported in an effort to improve cell survival under acidic conditions, *L. plantarum* cells were encapsulated in alginate beads and the beads were single or double coated with chitosan. The viability of the encapsulated cells was monitored in simulated gastric solution (pH 1.5) and during storage in pomegranate juice at 4°C. The survival of the cells in simulated gastric solution was improved in the case of the chitosan coated beads by 0.5-2 logs compared to the uncoated beads. The cell concentration in pomegranate juice after 6 weeks of storage was higher than 5.5 log CFU/mL for single and double coated beads, whereas for free cells and uncoated beads the cells died within 4 weeks of storage. In simulated gastric solution, the size of the beads decreased and their hardness increased with time; however, the opposite trend was observed for pomegranate juice, indicating that there is no correlation between cell survival and the hardness of the beads. The most likely reason for the decreased hardness in the juice was that citrate sequestered the calcium ions resulting in their exchange with non-gelling monovalent ions. To this end, it was shown that the calcium concentration of the beads decreased after addition of the beads into the juice, indicating that calcium was leaching out.

Other research informed that alginate and low-methoxyl pectin were used as the core materials for the encapsulation of *L. plantarum* and *B. longum* cells, whereas three different materials were used for coating, i.e. chitosan, gelatin and glucomannan, both single and double coating; the survival of the cells was studied in pomegranate and cranberry juice. The aim was to evaluate whether there is a particular combination of encapsulating and coating material that is more suited these fruit juices, in terms of cell survival. The results indicated that free cells survived better in pomegranate juice than in cranberry juice, although in both cases no viable cells were present after 6 weeks of storage. However, for both types of juices, encapsulation (especially coupled with coating) offered considerable protection to the cells; overall cell concentrations of higher then 10^6 CFU/mL after 6 weeks of storage were achieved for certain coated beads. In terms of the encapsulation material, it seemed that pectin beads offered slightly better protection than alginate beads. In terms of coating, no significant differences were observed between the uncoated beads and the beads coated with glucomannan. On the other hand, significant improvements were observed when coating the beads (either alginate or pectin) with chitosan and gelatin, cell concentrations higher than 10^7 CFU/mL after 6 weeks of storage were achieved. In almost all cases, the size and hardness of the beads decreased during storage, which was most likely related to the acid gel character of the polymers. Overall, the results

Table 3 Effect of coating polymers on the survival of probiotic cells.

Strains	Encapsulation polymer	Coating polymer	Test conditions	Cell survival results	References
B. adolescentis 15703T	13% (w/v) gelatin	1% (w/v) alginate	Simulated gastric solution (pH 2.0, 2 h), and intestinal solution (pH 7.4, 4 h)	Coated beads improved cell survival up to 1 log compared with uncoated beads	[19]
L. plantarum 299v, *L. plantarum* 800 and *L. plantarum* CIP A159	2% (w/v) alginate	2% (w/v) of whey protein	Simulated gastric solution (pH 1.8) and Simulated intestinal solution (pH 6.5)	Coated beads improved cell survival ~ 4-5 log compared with uncoated beads	[85]
B. breve NCIMB 8807	2% (w/v) alginate	0.4% (w/v) chitosan	Simulated gastric solution (pH 2) 1 and 2 h	Coated beads improved ~ 2 log (wet beads) and ~ 1 log (dry beads) in 1 h compared with uncoated beads	[89]
L. acidophilus 547, *B. bifidum* ATCC 1994, and *L. casei* 01	2% (w/v) sodium alginate	0.4% (w/v) chitosan, 0.17% (w/v) sodium alginate, and 0.05% (w/v) poly-l-lysine	Simulated gastric juice (pH 1.55) and 0.6% bile salt solution	Coated beads improved cell survival up to 1-3 logs compared with uncoated beads	[91]
L. casei YIT 9018	4% (w/v) sodium alginate	1% (w/v) chitosan	Simulated gastric juice and 0.6% bile salt	Coated beads improved cell survival ~ 2-3 log compared with uncoated beads	[96]
L. acidophilus PTCC1643 *and* *L. rhamnosus* PTCC1637	1% (w/v) sodium alginate	0.5% (w/v) sodium alginate	Simulated gastric juice (pH 1.5, 2 h)	Coated beads improved cell survival up to 1-2 logs compared with uncoated beads	[97]
L. rhamnosus CRL 1505	Pectin (PE) and pectin-whey protein (PE-WP).	8% (w/v) of whey protein	Simulated gastric solution (pH 1.2 and pH 2)	The $MC_{PE}/_{WP}$ beads were more stable than the $MC_{PE\text{-}WP/WP}$ beads	[98]

indicated that the selection of the coating material will affect cell survival during storage but also the size and textural characteristics of the beads [95].

Knowledge gaps in the area of encapsulation

So far little is known regarding the potential application of multi-layer coating of encapsulated probiotic cells aiming to improve storage in non-dairy products (e.g. fruit juices). In particular, there is a lot of potential in using this approach to enhance probiotic survival in highly acidic fruit juices. Moreover, there is little understanding on the relationship between cell survival and the size and hardness of the beads, and on how these physiochemical properties change with storage. This would be important knowledge for designing optimal encapsulation and coating systems, in terms of the protection offered to cells, as well as from an organoleptic point of view, as the size and hardness of beads are likely affect their sensory properties. Moreover, more understanding is needed on selecting suitable combinations of encapsulation polymers and coating polymers and more data on the effects of different combinations on cell survival and on the bead's properties. Finally, more work is needed on understanding the mechanism of cell protection by uncoated and coated beads against the diffusion of acids or other antimicrobial compounds (e.g. phenolic compounds), which are likely to be present in the fruit juices.

Conclusions

The main factors affecting the viability of probiotics in food products include the species/strain used, effect of probiotic production process, the composition of the food product, the storage temperature and time, the oxygen levels (especially in the case of *Bifidobacterium* species), and the type of container. The composition of the fruit juice, i.e. the levels of citric acid, protein and dietary fibre are likely influence the protection of the cells. Encapsulation has been used to improve probiotic survival in various acidic food products, including dairy and non-dairy products that offered considerable protection to the cells. Coating the encapsulated beads with polycations can improve the chemical and mechanical stability of the beads, and consequently improve the effectiveness of encapsulation. However, before such a product reaches the market organoleptic assessment of the product needs to be carried out to ensure consumer acceptability. The sensory quality of the product is a challenge for probiotic-containing fruit juices.

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