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Developing Stable Freeze-Dried Functional Ingredients Containing Wild-Type Presumptive Probiotic Strains for Food Systems

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Featured Application: The present study contributes to the design and development of ready-to-use dry food ingredients containing high numbers of functional microbes to be directly incorporated into food systems.

Abstract: Designing stable dried functional food ingredients and foods containing live probiotic cultures maintaining high viable cell loads at the time of consumption is a challenge for the industry. The aim of the present study was the development of stable freeze-dried functional food ingredients with enhanced shelf-life during long storage. Zea flakes, pistachios, and raisins were used as immobilization supports for the wild-type presumptive probiotic strains *Pediococcus acidilactici* SK and *Lactiplantibacillus plantarum* F4, while *L. plantarum* B282 was used as a reference strain. Cell survival was monitored during storage at room and refrigerated temperatures for up to 6 months. Levels of freeze-dried cultures were maintained up to 7.2 logcfu/g after 6 months storage at room temperature and up to 8.5 logcfu/g at refrigerator temperature, in contrast to free cell levels that ranged <7 logcfu/mL, suggesting the positive effects of immobilization and freeze-drying on cell viability. Of note, levels of freeze-dried immobilized *P. acidilactici* SK cells on zea flakes and pistachios remained stable after 6 months of storage at 4 °C, ranging 8.1–8.5 logcfu/g (survival rates 98.2 and 99.7%, respectively). The technology developed presents important advantages for the maintenance of cell viability during storage, assuring stability of ready-to-use functional food ingredients that could be directly incorporated in food systems.

Keywords: probiotics; prebiotics; functional foods; cell immobilization; freeze-drying; storage

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1. Introduction

Functional foods consist of nutraceuticals, pharma foods, medical foods, probiotics, prebiotic dietary fibers, and vitamin foods [1].

Foods enriched with probiotic microorganisms, such as lactic acid bacteria (LAB) and/or bifidobacteria, make up a large part of the commercial market. According to the most recent definition by the FAO/WHO, "probiotics are microorganisms (bacteria or yeasts) which, when administered in adequate concentrations, provide health benefits to the host" [2].

One of the main ways that probiotics exert their beneficial effects is through alterations in the composition of intestinal microbiome [3]. The effect of functional (probiotic) microorganisms and prebiotic dietary fibers on the composition of the intestinal microbiota has been well documented over the last years [4]. Prebiotic dietary fibers constitute of compounds that display health benefits towards digestive health.

Cereals contain fiber, nutrients, and are rich in vitamins and minerals. Daily cereal intake has been associated with a decreased risk of chronic diseases, such as obesity, type-1 diabetes (T1DM) and type-2 diabetes mellitus [5], and cardiovascular disease [6]. Fiber acts as a substrate for microbial fermentation in the gut, thereby facilitating the maintenance and/or growth of beneficial microbial populations. Consumption of dried fruits [7] and nuts has been linked to beneficial health and glycemic control benefits [8]. Raisins (*Vitis vinifera* L.) are rich in vitamins, minerals, and antioxidants, such as polyphenols (e.g., resveratrol). Metabolites of phenolic compounds have the ability to promote the growth of specific microbial species [9,10]. Pistachios (*Pistacia vera* L.) contain high loads of monounsaturated fatty acids (MUFA), fiber, and phytochemicals, which have beneficial effects on health [11]. More specifically, they contain high percentages of oleic acid, along with phenolic compounds, such as resveratrol and pro-anthocyanins [12]. The beneficial effects of pistachio consumption on gut microbiome and lipid profiles have been recently demonstrated in a streptozotocin- induced T1DM animal model [13,14]. Cereals, pistachios, and raisins could therefore be proposed as foods with prebiotic properties, which may potentially regulate the levels of microbial populations contributing to maintenance of human health [13,15]. Similarly, three-day consumption of a granola meal improved glycemic regulation in a recent study [16].

Immobilization technology enables enhanced survival of probiotic cells [17] and can be employed in the production of novel functional foods, enriched with immobilized functional cultures in natural food carriers. Immobilization is defined as limiting cells to a solid matrix, separated from the main liquid phase. Many studies have demonstrated that the use of immobilized cultures in the production of probiotic foods led to increased cell viability [17–19], while the delivery vehicle is possible to affect the adhesion and therefore the colonization properties of functional cells [20,21]. The use of natural food carriers, containing prebiotic dietary fibers for the immobilization of probiotic cells, may lead to the production of synbiotic functional components [22]. Prebiotics and probiotics contribute to intestinal microbiome homeostasis by enhancing the presence of beneficial bacterial species and exerting benefits for the host immune system in diseases, such as T1DM [23].

In general, dried products are preferred by the food industry as they have an extended shelf life, are easy to store and transport, and reduce storage costs since refrigeration is not required. Freeze-drying is usually used for sensitive ingredients, as it is employed under vacuum and at low temperatures [24]. However, freeze-drying can cause many negative effects on cells, such as rupture of cell walls, due to water vapor transport to the surface and sublimation of water molecules, protein destruction, and cell shrinkage. It is estimated that the formed crystals in the cooling step cause more adverse effects on cells with a large surface area, e.g., freeze-drying of enterococci has better results than freeze-drying of rod-shaped bacteria of the genus *Lactobacillus* [25]. Nevertheless, freeze-dried cultures have been used for food and beverage production, such as sausages [26,27], dairy [28], and wine products [19].

The aim of the present study was the development of innovative functional food ingredients, i.e., immobilized probiotic cultures on natural food constituents that can offer potential health benefits. In this vein, natural food components, containing prebiotic dietary fibers associated with functional modulation of gut microbiota [13,14], were tested as immobilization supports for selected wild-type probiotic strains isolated from traditional fermented foods or human stool, directing at designing readily processable functional food ingredients and targeting maintenance of digestive health. Immobilized bacterial strains are expected to ensure high cell viability compared to free, non-immobilized, cultures.

2. Materials and Methods

2.1. Microbial Strains

Pediococcus acidilactici SK, isolated from human stool and *Lactiplantibacillus plantarum* F4 isolated from dried figs were provided from QLCon (Patras, Greece), whereas *L. plantarum*

B282 strain, isolated from fermented table olives [29], was kindly provided from the Institute of Technology of Agricultural Products, HAO-DIMITRA (Lycovrissi, Attiki, Greece).

The strains were subcultured from a −80 °C stock culture in 10 mL of MRS broth (VWR International, Radnor, PA, USA) and incubated overnight at 37 °C. A subculture was prepared in 10 mL MRS broth and incubated at 37 °C for 24 h, prior to use.

2.2. Immobilization of Presumptive Probiotic Cells in Natural Food Carriers

Cell immobilization on natural food ingredients (zea flakes, pistachios, and raisins) was performed as described previously [19,30,31]. Briefly, the grown cell culture (300 mL) was centrifuged (7500× *g* rpm, 15 min, 4 °C) and washed with 100 mL of sterile 1/4 Ringer's isotonic solution (VWR International GmbH). Them, the cell biomass was then resuspended in sterile 1/4 Ringer's isotonic solution to the initial culture volume, and then the immobilization carrier (zea flakes, pistachios, or raisins) was introduced and the mixture was allowed undisturbed. Specifically, the experimental procedure followed was:

- 70 g of zea (*Triticum dicoccum*) flakes (product of organic farming, Antonopoulos farm, Dilofos D. Narthakiou, Greece) were immersed in 200 mL of cell suspension in a sterile conical flask and incubated at 37 °C for 10 min,
- 60 g of pistachios (*Pistacia vera* L.) (kindly provided from the Pistachio Agricultural Cooperative of Molos-Thermopyles, Greece) were immersed in 100 mL of cell suspension in a sterile conical flask and incubated at 37 °C for 24 h,
- 90 g of Sultana variety raisins (*Vitis vinifera* L.) (product of organic farming, Ampelodimiourgies, Metochi Papagianni-Skoula, Heraklion, Crete, Greece) were immersed in 100 mL of cell suspension in a sterile conical flask and incubated at 37 °C for 2.5 h.

In all cases, after the immobilization process, the immobilized cells were strained and washed with sterile 1/4 Ringer's solution, in order to remove free (non-immobilized) cells.

2.3. Freeze-Drying of Immobilized Presumptive Probiotic Cells

The cell pellet resulting from centrifugation of grown cell culture and the immobilized probiotic cultures were transferred to −80 °C for 18 h and freeze-drying was followed on a BenchTop Pro (Virtis, SP Scientific, Warminster, PA, USA) freeze-dryer under vacuum (30–35 Pa) at condenser temperature −101 °C, for 24 h. No cryoprotectants were used during freeze-drying.

2.4. Effect of Immobilization on Cell Viability during Storage

The effect of storage at room temperature (18–20 °C, RT) or at 4 °C on the viability of wet or freeze-dried free or immobilized *L. plantarum* B282, *P. acidilactici* SK, and *L. plantarum* F4 cells on zea flakes, pistachios, or raisins for a period up to 6 months was studied. Cultures were stored in plastic sterile containers and cell survival, as well as possible microbial contamination were monitored.

2.5. Determination of Immobilized Cell Levels

Levels of freeze-dried immobilized and free cells were determined both before and after rehydration (dry and wet weight, respectively). For rehydration, freeze-dried immobilized cells were immersed in sterile distilled water for 1 h at RT, followed by filtration [31]. Likewise, cell free biomass was resuspended in sterile deionized water equal to the initial volume (prior freeze-drying) at RT for 15 min. Cell enumeration was carried out as described below.

To determine the levels of immobilized cells, 5 g of samples were blended with 45 mL sterile 1/4 Ringer's solution. Accordingly, to determine free cell levels, 1 g free cell biomass or 1 mL of cell suspension (after rehydration) were transferred to 9 mL of sterile 1/4 Ringer's solution. Decimal serial dilutions in 1/4 Ringer's solution were performed, followed by plate counting on MRS agar plates after incubation at 37 °C for 72 h. Cell loads were expressed as logcfu/g immobilization carrier or logcfu/mL culture.

Cells survival rate (%) was estimated according to the equation [32]

$$\text{Survival rate (\%)} = (\text{cell levels (logcfu/g) at timepoint/initial cell levels (logcfu/g)}) * 100.$$

2.6. Microbial Contamination

To check for possible microbial contamination, samples (5 g) of immobilized cells (without rehydration) or free cell biomass (1 g) were homogenized in 45 mL or 9 mL of sterile 1/4 Ringer's solution, respectively, followed by serial decimal dilutions in 1/4 Ringer's solution, and checked for:

- total aerobic counts (TAC) on Plate Count agar (Deben Diagnostics, Suffolk, UK) at 30 °C;
- coliforms on Violet Red Bile agar (LabM, Heywood, UK) at 30 °C;
- Enterobacteriacae on Violet Red Bile Glucose agar (LabM) at 37 °C;
- staphylococci in Baird Parker (LabM) enriched with egg yolk tellurite (LabM) at 37 °C;
- *Escherichia coli* in Harlequin TBGA/TBX (LabM) at 37 °C;
- yeasts/fungi in Malt Extract (LabM) at 30 °C;
- *Listeria monocytogenes* on L-Palcam agar (LabM) supplemented with X144 supplement (VWR), and
- *Salmonella* spp. in X.L.D. agar (LabM) at 37 °C.

2.7. Statistical Analysis

All experiments were carried out in duplicate. Values were expressed as average ± standard deviation (STDEV). Significance cutoff was at $p < 0.05$. Analysis of variance (ANOVA) and Tukey HSD post hoc test was used to determine significant differences among results. Coefficients, ANOVA tables, and significance ($p < 0.05$) were calculated using Statistica v.12.0 (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results and Discussion

The ingredients studied as natural immobilization supports, i.e., cereals, nuts, and dried fruits, are an integral part of the Mediterranean diet, which confers increased life expectancy and combining these beneficial effects with probiotic health related effects is a relatively new concept for the commercial market.

3.1. Effect of Immobilization and Freeze-Drying on Cell Viability

Table 1 shows the effect of freeze-drying on levels of free and immobilized *L. plantarum* B282, *P. acidilactici* SK, and *L. plantarum* F4 cells on zea flakes, pistachios, and raisins along with cell survival rates. In all cases, freeze-dried cell loads were higher than the minimum recommended concentration (>7 logcfu/g) for conferring a health effect, according to IPA Europe [33].

No cryoprotectants were used in this study, which are usually used during the freeze-drying of microbial cultures [34], in order to examine the possible positive effect of immobilization on the extension of the lifetime of the functional cultures.

Freeze-drying resulted in a significant decrease ($p < 0.05$) of the cell levels of all strains, in free form. Similar results were also reported in the literature for *L. plantarum* TISTR 2075 [35] and for *L. helveticus* LH-B02 [36].

The effect of freeze-drying on the levels of immobilized *L. plantarum* B282, *P. acidilactici* SK, and *L. plantarum* F4 cells on the natural food carriers was also studied, which ranged >7 logcfu/g, in all cases. The maximum concentration (8.5 logcfu/g) was recorded in immobilized *P. acidilactici* SK cells on pistachios. Immobilization of *L. plantarum* B282 cultures on pistachios and *L. plantarum* F4 on both zea flakes and pistachios resulted in similar ($p > 0.05$) cell loads compared to wet cells, and also higher survival rates ($p < 0.05$), compared to free cells. In contrast, survival rate of free and immobilized *P. acidilactici* SK cells on zea flakes and pistachios ranged at similar levels ($p > 0.05$).

Table 1. Effect of freeze-drying and cell immobilization on cell levels and survival rates.

Microorganisms		Immobilized Cells on Natural Food Carriers			Free Cells
		Zea flakes	Pistachio	Raisin	
L. plantarum B282	W	8.6 ± 0.2	8.4 ± 0.1	8.5 ± 0.1	9.6 ± 0.1
	FD	8.3 ± 0.1	8.5 ± 0.1	8.0 ± 0.1	10.8 ± 0.1
	FD (ww)	8.0 ± 0.1 *	8.3 ± 0.1	7.4 ± 0.1 *	8.4 ± 0.1 *
	% survival	92.6 ± 0.3 **	98.8 ± 0.2 **	86.8 ± 0.6	87.8 ± 0.8
P.acidilactici SK	W	9.0 ± 0.1	9.0 ± 0.1	8.8 ± 0.1	9.7 ± 0.1
	FD	9.0 ± 0.1	9.0 ± 0.1	8.0 ± 0.2	11.4 ± 0.1
	FD (ww)	8.3 ± 0.1 *	8.5 ± 0.1 *	7.4 ± 0.1 *	9.1 ± 0.1 *
	% survival	92.3 ± 0.4	94.3 ± 0.5	83.5 ± 1.6 **	93.7 ± 0.8
L. plantarum F4	W	8.3 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	9.3 ± 0.1
	FD	8.5 ± 0.1	8.4 ± 0.1	7.8 ± 0.2	11.4 ± 0.1
	FD (ww)	8.2 ± 0.1	8.2 ± 0.1	7.4 ± 0.1 *	8.8 ± 0.1 *
	% survival	98.2 ± 0.4 **	99.0 ± 0.5 **	89.9 ± 0.4 **	94.4 ± 0.1

W: Wet cultures after growth in MRS medium, FD: freeze-dried cultures (before rehydration), FD (ww): freeze-dried cultures after rehydration. Free cells were used as control samples. Data are expressed as mean values (average) ± standard error of the mean (STDEV). Statistically significant differences are marked *: $p < 0.05$ compared to wet cells, **: $p < 0.05$ compared to free cells.

Immobilization of cells on zea flakes or pistachios may protect cells from the stress to which they are subjected during the freeze-drying process and enhances cell survival, in a manner that depends on the food ingredient and on the microbial strain [37]. Similar results were obtained after immobilization of kefir culture on grape skins that led to increased viability of lactobacilli compared to free cells, even when no cryoprotectant was applied during freeze-drying [32].

In contrast, cell immobilization on raisins resulted in decreased ($p < 0.05$) survival rates of *P. acidilactici* SK and *L. plantarum* F4 cells; however, no significant differences ($p > 0.05$) were observed between free and immobilized *L. plantarum* B282 cells. The reduced survival rates of immobilized cells on raisins may be due to the antimicrobial activity of specific fruit ingredients, mainly polyphenols [38].

3.2. Effect of Immobilization and Freeze-Drying on Cell Viability during Storage

The effect of storage at RT and 4 °C on cell viability is an important factor for industrial practice [37,39]. As probiotic cultures may be used as ingredients in the production of functional foods, it is important that cell levels are maintained at higher than or at least equal to the recommended concentration (≥7 logcfu/g) during storage and until the time of consumption. In this vein, the effect of culture form (free or immobilized cells), culture condition (wet or freeze-dried cells) and storage on cell levels and on survival rate was studied both at RT and at 4 °C. A proper immobilization support ought to maintain cell viability, be easy to handle, and have a good cost–benefit rate [17]. Bacterial injury and protein inactivation can also occur during storage; thus, maintaining the viability of immobilized probiotics throughout storage consists of a main challenge in the food industry [40].

3.2.1. Storage at RT

Storage of freeze-dried cultures is strongly recommended at temperatures 1–4 °C, as storage at RT or higher results in rapid cell death [41]. However, since refrigerated storage and maintaining an unbroken cold chain implies an increase in cost, storage at RT is advantageous [42].

The effect of freeze-drying, cell immobilization, and storage time on cell concentration and survival rates is presented in Table 2a–c. All factors (culture form, cell condition, and storage time) significantly affected cell viability ($p < 0.05$).

Levels of wet free cells ranged <7 logcfu/mL after 40 days of storage, for all strains. Counts of freeze-dried free cells ranged >7 logcfu/mL after 40 days of storage, but dropped <7 logcfu/mL after 90 days of storage, which is the minimum recommended concentration for conferring a beneficial effect, as already reported [33].

Table 2. (**a**–**c**) Effect of immobilization and storage at RT on cell levels and survival rates.

(a) Storage at RT—*L. plantarum* B282								
		logcfu				**% Survival**		
		d0	**d40**	**d90**	**d180**	**d40**	**d90**	**d180**
zea flakes	W	8.6 ± 0.2	<1	<1	<1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	FD(ww)	8.0 ± 0.1	6.9 ± 0.2	3.9 ± 0.1	3.1 ± 0.1	86.2 ± 2.0	48.6 ± 0.3	39.4 ± 0.6
	FD	8.3 ± 0.1	6.6 ± 0.1	4.0 ± 0.1	3.3 ± 0.1	79.4 ± 1.5	48.7 ± 0.7	39.2 ± 0.4
pistachios	W	8.4 ± 0.1	6.8 ± 0.1	5.0 ± 0.1	3.0 ± 0.1	81.8 ± 0.8	60.0 ± 0.2	36.2 ± 1.5
	FD(ww)	8.3 ± 0.1	6.0 ± 0.1	4.4 ± 0.1	3.7 ± 0.1	73.1 ± 1.0	53.3 ± 1.3	44.5 ± 0.3
	FD	8.5 ± 0.1	7.0 ± 0.1	5.1 ± 0.1	4.4 ± 0.1	82.0 ± 0.6	60.0 ± 0.3	52.3 ± 0.4
raisins	W	8.5 ± 0.1	<1	<1	<1	NC	NC	NC
	FD(ww)	7.4 ± 0.1	<1	<1	<1	NC	NC	NC
	FD	8.0 ± 0.0	<1	<1	<1	NC	NC	NC
free cells	W	9.6 ± 0.1	3.1 ± 0.1	<1	<1	31.9 ± 0.8	NC	NC
	FD(ww)	8.4 ± 0.1	7.3 ± 0.1	2.7 ± 0.1	1.0 ± 0.1	86.3 ± 0.7	32.5 ± 0.7	12.3 ± 0.7
	FD	10.8 ± 0.1	8.6 ± 0.0	4.0 ± 0.0	2.2 ± 0.2	79.7 ± 0.3	37.1 ± 0.2	20.0 ± 1.8
(b) Storage at RT—*P. acidilactici* SK								
		logcfu				**% Survival**		
		d0	**d40**	**d90**	**d180**	**d40**	**d90**	**d180**
zea flakes	W	9.0 ± 0.1	<1	<1	<1	NC	NC	NC
	FD(ww)	8.3 ± 0.1	8.0 ± 0.1	7.6 ± 0.1	7.2 ± 0.1	96.8 ± 1.1	91.2 ± 1.2	86.6 ± 1.3
	FD	9.0 ± 0.1	8.3 ± 0.1	8.2 ± 0.1	7.8 ± 0.1	91.3 ± 0.4	90.8 ± 1.0	86.7 ± 1.0
pistachios	W	9.0 ± 0.1	7.0 ± 0.1	6.3 ± 0.1	5.8 ± 0.1	76.9 ± 2.4	70.3 ± 0.5	64.5 ± 0.8
	FD(ww)	8.5 ± 0.1	7.8 ± 0.1	7.2 ± 0.1	7.0 ± 0.1	91.8 ± 1.1	85.4 ± 0.9	82.5 ± 0.2
	FD	9.0 ± 0.1	8.1 ± 0.1	7.9 ± 0.1	7.6 ± 0.1	90.6 ± 1.6	87.8 ± 0.8	84.2 ± 0.5
raisins	W	8.8 ± 0.1	<1	<1	<1	NC	NC	NC
	FD(ww)	7.4 ± 0.1	<1	<1	<1	NC	NC	NC
	FD	8.0 ± 0.2	<1	<1	<1	NC	NC	NC
free cells	W	9.7 ± 0.1	4.9 ± 0.1	<1	<1	50.1 ± 0.2	NC	NC
	FD(ww)	9.1 ± 0.1	8.5 ± 0.1	5.5 ± 0.1	3.1 ± 0.1	93.9 ± 1.1	60.1 ± 0.4	34.5 ± 0.7
	FD	11.1 ± 0.1	9.7 ± 0.1	7.5 ± 0.1	5.0 ± 0.1	84.7 ± 0.5	65.8 ± 1.0	44.0 ± 0.3
(c) Storage at RT—*L. plantarum* F4								
		logcfu				**% Survival**		
		d0	**d40**	**d90**	**d180**	**d40**	**d90**	**d180**
zea flakes	W	8.3 ± 0.1	4.8 ± 0.1	<1	<1	57.1 ± 0.4	NC	NC
	FD(ww)	8.2 ± 0.1	6.0 ± 0.1	4.0 ± 0.1	2.7 ± 0.1	73.0 ± 0.5	48.3 ± 0.8	32.6 ± 0.2
	FD	8.5 ± 0.1	6.5 ± 0.1	4.1 ± 0.1	3.0 ± 0.1	77.1 ± 1.0	48.3 ± 1.5	34.9 ± 0.8
pistachios	W	8.2 ± 0.1	6.4 ± 0.1	5.2 ± 0.1	2.3 ± 0.1	77.3 ± 1.3	63.3 ± 1.4	28.4 ± 0.7
	FD(ww)	8.2 ± 0.1	6.1 ± 0.1	4.9 ± 0.1	4.0 ± 0.1	74.1 ± 0.9	60.5 ± 1.5	49.0 ± 0.1
	FD	8.3 ± 0.1	6.7 ± 0.3	5.4 ± 0.2	4.5 ± 0.1	80.9 ± 1.6	64.3 ± 1.9	54.4 ± 1.1
raisins	W	8.2 ± 0.1	<1	<1	<1	NC	NC	NC
	FD(ww)	7.4 ± 0.1	<1	<1	<1	NC	NC	NC
	FD	7.8 ± 0.2	<1	<1	<1	NC	NC	NC
free cells	W	9.3 ± 0.1	2.5 ± 0.1	<1	<1	26.4 ± 0.2	NC	NC
	FD(ww)	8.8 ± 0.1	7.4 ± 0.1	4.0 ± 0.1	2.2 ± 0.1	83.9 ± 1.2	45.6 ± 0.3	24.8 ± 0.7
	FD	11.4 ± 0.1	8.9 ± 0.1	6.2 ± 0.1	3.5 ± 0.1	78.3 ± 0.2	54.6 ± 1.2	30.9 ± 1.3

Wet and freeze-dried free cells were used as control samples. Data are expressed as mean values (average) ± standard error of the mean (STDEV). W: Wet cultures, FD: freeze-dried cultures (before rehydration), FD (ww): freeze-dried cultures after rehydration. d0: day 0, d40: day 40, d90: day 90, d180: day 180, NC: not calculated.

Regarding wet immobilized cultures, in all cases, levels < 7 logcfu/g were recorded, except for immobilized *P. acidilactici* SK cells on pistachio, which retained cell levels ≥ 7 logcfu/g after 40 days of storage. However, the levels decreased <7 logcfu/g at 90 days of storage.

During storage at RT, levels of freeze-dried immobilized *P. acidilactici* SK cells on zea flakes and pistachios remained high throughout the storage period and increased survival rate was observed compared to wet cells ($p < 0.05$). Specifically, after 180 days of storage, cell

levels ranged 7.0 and 7.2 logcfu/g (survival rates of 82.5% and 86.6%) for immobilized cells on pistachios and zea flakes, respectively, underlying the positive effect of immobilization on maintenance of cell viability, as also reported elsewhere [43].

Additionally, at 40 days of storage, freeze-dried immobilized cells on raisins were detected at levels < 1 logcfu/g. The survival of presumptive probiotic cells on raisins for only a short period of time is probably related to the antimicrobial activity of certain fruit components, like phenolic compounds [38]. Indeed, specific polyphenols, such as catechin, procyanidins, and quercetin have been associated with antimicrobial activity [44,45] (Table 2a–c).

Storage at room temperature could facilitate distribution and vast usage of probiotics, yet scarce studies focus on the subject. Freeze-dried encapsulated *L. acidophilus* LA02ID 1688 cells using sodium alginate, magnesium hydroxide, and chocolate coatings as immobilization agents, resulted in increased cell viability throughout the final products' (cereal bars) shelf life and survival rates > 94% and >90% during storage at 25 °C, for 60–90 and 120 days, respectively [46].

3.2.2. Storage at 4 °C

Likewise, the effect of storage at refrigerated temperature (4 °C) on the levels of wet and freeze-dried cultures and survival rates were also studied (Table 3a–c). All factors (culture form and condition and storage) significantly affected cell viability ($p < 0.05$) in all cases, with the exception of freeze-dried immobilized *P. acidilactici* SK cells on zea flakes and pistachios, the concentration of which was not affected by the storage period ($p > 0.05$). Specifically, the levels of immobilized *P. acidilactici* SK cells on zea flakes and pistachios ranged 8.1 and 8.5 logcfu/g (98.2 and 99.7% survival rates) after 180 days of storage, values similar ($p > 0.05$) to the initial levels, (in the 1st day of storage). In the remaining cases, storage time affected cell viability negatively ($p < 0.05$).

The greatest reduction of cell viability was observed in wet free cells after 40 days of storage (levels < 7 logcfu/mL) with survival rates ranging in significantly ($p < 0.05$) lower values compared to wet immobilized cultures. At 90 days, wet free cultures were detected ≤1.3 logcfu/mL. Freeze-dried free *L. plantarum* B282 cells ranged <7 logcfu/mL, after 90 days of storage, but levels >7 logcfu/mL for *P. acidilactici* SK and *L. plantarum* F4 after 180 days of storage were recorded, indicating a strain-specific survival, as reported elsewhere [47].

On the other hand, immobilization had a significant effect on the viability of wet cells. In particular, wet immobilized *L. plantarum* B282 and *L. plantarum* F4 cells in zea flakes ranged at levels > 7 logcfu/g up to day 90 of storage, while similar loads were recorded at immobilized cells on pistachios up to day 180. Wet immobilized *P. acidilactici* SK cells levels on raisins ranged 7.2 logcfu/g up to 40 days of storage, while for *L. plantarum* B282 and *L. plantarum* F4 strains, the limit of 7 logcfu/g was not achieved at the same timepoint. A similar study showed that cell loads of wet immobilized *L. casei* CSL3 cells in guava or kiwi ranged <6 logcfu/g after 43 days of refrigerated storage [48].

Levels of freeze-dried immobilized *L. plantarum* B282, *P. acidilactici* SK, and *L. plantarum* F4 cells on zea flakes and freeze-dried immobilized *P. acidilactici* SK and *L. plantarum* F4 cells on pistachios ranged >7 logcfu/g at the 180th day of storage and were significantly higher ($p < 0.05$) compared to freeze-dried free cells. Similarly, loads of freeze-dried immobilized *L. plantarum* B282 cells on pistachios were recorded >7 logcfu/g up to 90 days of storage, whilst at the same timepoint freeze-dried free cell levels were significantly ($p < 0.05$) lower. Regarding freeze-dried *P. acidilactici* SK cells immobilized on zea flakes, the levels ranged to similar values to freeze-dried free cells, but a higher ($p < 0.05$) survival rate was observed (92% for cells immobilized on zea flakes and 88.8% for free cells) at 180 days of storage. In contrast, levels of freeze-dried immobilized cultures on raisins did not exceed 7 logcfu/g at 40 days of storage. Raisins are well-known for their nutritional value, but they are rich in polyphenols that exert antimicrobial activity [45].

Table 3. (**a–c**) Effect of immobilization and storage at 4 °C on cell levels and survival rates.

(a) Storage at 4 °C—*L. plantarum* B282								
		logcfu				**% Survival**		
		d0	**d40**	**d90**	**d180**	**d40**	**d90**	**d180**
zea flakes	W	8.6 ± 0.2	8.2 ± 0.1	7.5 ± 0.1	5.9 ± 0.1	94.8 ± 1.6	86.6 ± 0.3	68.9 ± 0.2
	FD(ww)	8.0 ± 0.1	7.7 ± 0.1	7.5 ± 0.1	7.1 ± 0.1	95.9 ± 1.7	93.4 ± 0.1	88.5 ± 1.2
	FD	8.3 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	7.7 ± 0.1	99.3 ± 0.1	98.9 ± 0.1	92.5 ± 0.5
pistachios	W	8.4 ± 0.1	8.1 ± 0.1	8.0 ± 0.1	7.3 ± 0.1	97.0 ± 0.6	95.3 ± 0.4	86.9 ± 0.7
	FD(ww)	8.3 ± 0.1	7.3 ± 0.1	7.2 ± 0.1	6.5 ± 0.1	88.4 ± 0.7	87.6 ± 0.2	78.6 ± 0.4
	FD	8.5 ± 0.1	7.9 ± 0.2	7.7 ± 0.1	7.1 ± 0.1	92.8 ± 2.0	90.9 ± 0.1	83.9 ± 0.2
raisins	W	8.5 ± 0.1	5.2 ± 0.1	2.8 ± 0.1	<1	60.9 ± 1.0	33.1 ± 1.6	NC
	FD(ww)	7.4 ± 0.1	6.1 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	82.1 ± 1.1	56.0 ± 1.3	55.1 ± 1.3
	FD	8.0 ± 0.1	7.0 ± 0.1	5.0 ± 0.1	4.4 ± 0.2	87.2 ± 0.7	62.4 ± 0.4	55.5 ± 2.2
free cells	W	9.6 ± 0.1	3.9 ± 0.1	1.1 ± 0.1	<1	41.1 ± 0.6	11.0 ± 0.8	NC
	FD(ww)	8.4 ± 0.1	8.3 ± 0.1	6.4 ± 0.1	6.3 ± 0.1	98.5 ± 0.1	76.5 ± 0.7	75.4 ± 0.9
	FD	10.8 ± 0.1	10.7 ± 0.1	9.8 ± 0.1	9.6 ± 0.1	98.8 ± 0.4	90.6 ± 0.6	88.5 ± 0.3
(b) Storage at 4 °C—*P. acidilactici* SK								
		logcfu				**% Survival**		
		d0	**d40**	**d90**	**d180**	**d40**	**d90**	**d180**
zea flakes	W	9.0 ± 0.1	8.6 ± 0.1	7.9 ± 0.1	6.9 ± 0.1	96.4 ± 1.1	87.9 ± 0.4	76.9 ± 0.3
	FD(ww)	8.3 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	99.6 ± 0.2	99.1 ± 0.9	98.2 ± 1.2
	FD	9.0 ± 0.1	8.7 ± 0.1	8.6 ± 0.1	8.6 ± 0.1	95.7 ± 0.5	95.3 ± 0.1	95.1 ± 0.1
pistachios	W	9.0 ± 0.1	9.0 ± 0.1	8.7 ± 0.1	8.0 ± 0.1	99.8 ± 0.2	96.6 ± 0.1	89.1 ± 1.5
	FD(ww)	8.5 ± 0.1	8.5 ± 0.1	8.5 ± 0.1	8.5 ± 0.1	99.9 ± 0.1	99.8 ± 0.1	99.7 ± 0.3
	FD	9.0 ± 0.1	9.0 ± 0.1	9.0 ± 0.1	8.9 ± 0.1	100.1 ± 0.1	99.9 ± 0.1	99.3 ± 0.1
raisins	W	8.8 ± 0.1	7.2 ± 0.1	5.4 ± 0.1	<1	82.2 ± 1.5	61.7 ± 1.3	NC
	FD(ww)	7.4 ± 0.1	6.7 ± 0.1	6.5 ± 0.1	5.8 ± 0.1	91.4 ± 1.7	88.5 ± 2.0	78.4 ± 0.7
	FD	8.0 ± 0.2	7.2 ± 0.1	7.0 ± 0.1	6.6 ± 0.1	90.3 ± 1.0	87.5 ± 0.4	82.4 ± 0.7
free cells	W	9.7 ± 0.1	5.9 ± 0.1	1.3 ± 0.1	<1	61.1 ± 0.2	13.8 ± 0.6	NC
	FD(ww)	9.1 ± 0.1	9.0 ± 0.1	9.1 ± 0.1	8.1 ± 0.1	99.7 ± 0.1	99.3 ± 0.1	88.8 ± 0.4
	FD	11.1 ± 0.1	11.4 ± 0.1	11.2 ± 0.1	11.1 ± 0.1	98.8 ± 0.4	98.1 ± 0.2	97.0 ± 0.3
(c) Storage at 4 °C—*L. plantarum* F4								
		logcfu				**% Survival**		
		d0	**d40**	**d90**	**d180**	**d40**	**d90**	**d180**
zea flakes	W	8.3 ± 0.1	8.3 ± 0.1	7.3 ± 0.1	6.2 ± 0.1	99.1 ± 0.1	87.5 ± 0.2	73.8 ± 0.4
	FD(ww)	8.2 ± 0.1	7.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.1	95.7 ± 0.2	95.5 ± 1.0	93.9 ± 0.8
	FD	8.5 ± 0.1	8.3 ± 0.2	8.3 ± 0.1	8.3 ± 0.1	98.6 ± 1.8	98.3 ± 0.7	97.8 ± 0.5
pistachios	W	8.2 ± 0.1	8.2 ± 0.1	7.9 ± 0.1	7.5 ± 0.1	99.9 ± 0.1	96.4 ± 0.1	90.5 ± 0.4
	FD(ww)	8.2 ± 0.1	7.7 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	94.2 ± 0.1	93.2 ± 0.1	93.0 ± 0.4
	FD	8.3 ± 0.1	8.0 ± 0.1	7.8 ± 0.1	7.8 ± 0.1	95.7 ± 1.4	93.2 ± 0.4	92.5 ± 0.8
raisins	W	8.2 ± 0.1	5.8 ± 0.2	1.8 ± 0.2	<1	71.0 ± 2.6	21.4 ± 2.6	NC
	FD(ww)	7.4 ± 0.1	6.0 ± 0.1	4.0 ± 0.1	2.4 ± 0.1	81.6 ± 0.4	54.9 ± 0.8	31.9 ± 1.1
	FD	7.8 ± 0.2	6.4 ± 0.2	5.0 ± 0.1	3.1 ± 0.1	82.9 ± 2.9	64.6 ± 0.4	39.7 ± 1.6
free cells	W	9.3 ± 0.1	4.3 ± 0.1	1.0 ± 0.1	<1	46.3 ± 0.2	10.2 ± 0.7	NC
	FD(ww)	8.8 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	7.1 ± 0.1	92.7 ± 0.9	91.9 ± 1.3	80.3 ± 1.0
	FD	11.4 ± 0.1	11.2 ± 0.1	10.9 ± 0.1	9.8 ± 0.1	98.3 ± 0.7	95.9 ± 0.5	86.2 ± 0.8

Wet and freeze-dried free cells were used as control samples. Data are expressed as mean values (average) ± standard error of the mean (STDEV). W: Wet cultures, FD: freeze-dried cultures (before rehydration), FD (ww): freeze-dried cultures after rehydration. d0: day 0, d40: day 40, d90: day 90, d180: day 180, NC: not calculated.

The above results showed that Immobilization on pistachios and zea flakes contributed to maintenance of high cell viability for up to 6 months (Table 3a–c), higher than the recommended levels (>7 logcfu/g) when stored at 4 °C. Immobilization on natural food carriers (such as zea flakes and pistachios) can be used to preserve the viability of probiotic products during storage at 4 °C, in high doses. This technology should allow probiotics to be incorporated into food systems and allow manufacturers to place assurances on the viability and quantity of probiotics in the final products.

Our results were in agreement with other studies. Loads of freeze-dried immobilized *L. casei* CSL3 on soybean were similar to initial levels, when submitted to cold storage for 30 days [49]. Oat flakes were proposed as a suitable support for the immobilization of *L. casei* CSL3 cells, as they maintained high cell viability during storage at 4 °C for 60 days [50].

Survival of probiotic cells in high levels up to the end of the product expiration date is a crucial factor for the food industry [51]. Incorporation of freeze-dried free or immobilized *L. casei* 393 cells on oat flakes, raisins, and apple pieces on yoghurt and subsequently evaluation of its survival during storage resulted in elevated viability of immobilized cells compared to free after 60 days of storage, expanding the products usual shelf-life up to 30 days.

The positive contribution of cell immobilization in cell survival was also documented in immobilized *L. casei* 01 cells on dark or white chocolate, resulting in maintenance of cell viability after storage at 4 °C for 60 days and in higher cell survival after exposure to simulated gastric environment compared to free cells [52].

Co-encapsulation of *L. paracasei* LBC81 and ELBAL cells with a calcium alginate matrix coated with gelatin improved cell survival during 35 days of storage at low temperature, while free cells' viability decreased to 28 and 35%, respectively [43]. Moreover, the levels of encapsulated *L. paraplantarum* FT-259 in casein/pectin microparticles decreased by 0.41 and 1.26 log after 60 and 90 days of storage at 8 °C, respectively, with microbial counts remaining over 6.24 logcfu/g during the entire study period and retained high viability percentages throughout storage (over 83.2%) [40]. Of note, Hadidi et al. [53] reported that encapsulation of *L. acidophilus* probiotic cells on alginate/fish gelatin possesses protective effects, as it increased their viability by 2–3 logcfu/g during baking and 7 days of storage at 4 °C.

Studies evaluating the effect of storage on the viability of probiotic strains support that low storage temperatures prolong cell survival, compared to high storage temperatures [54,55], in agreement with the results of this study. Regarding the decrease in cell viability, it is estimated that during storage, cells increase the saturated fatty acids of their membrane, due to lipid oxidation. Lipid oxidation, which is favored at high storage temperatures, is accompanied by free radical formation, and subsequently, DNA and cell membrane damage [56].

For industrial purposes, both stability and viability of the microbial probiotic cultures consist of the most important parameters influencing manufacturing and marketing [57]. Production of probiotic supplements has to be combined with viable cell counts as shelf life of the product is crucial [58].

Results of the present study clearly demonstrate that the presumptive probiotic wild-type strains were able to survive during storage in immobilized form, highlighting that pistachios and zea flakes could be good candidates as vehicles of probiotics. The strains showed better survival ability on zea and pistachio rather than on raisins, confirming that food formulation affects the viability of probiotics during storage [59].

Furthermore, the viability of probiotic cultures during storage seems to be strain- rather than species-specific [47]. Heat, oxygen, and acid tolerance in a strain-specific manner, can be taken into account to choose strains that could achieve a high survival during storage. In general, the use of stress-resistant strains can be useful for improving stability in industrial processes [60].

4. Conclusions

Production of freeze-dried immobilized cells on zea flakes, pistachio, and raisins, followed by freeze-drying led to cell loads over the minimum levels recommended by IPA Europe (>7 logcfu/g).

As ensuring stability and high viability of probiotic bacteria is crucial for developing functional food ingredients, cell survival was monitored during storage at ambient and refrigerated temperature for up to 6 months. Freeze-drying resulted in higher cell loads

compared to wet cultures during storage. In addition, immobilization on zea flakes and pistachios enhanced cell survival and presumptive probiotic strains were encountered at levels up to 8.2 logcfu/g after storage for 3 months at room temperature, while levels up to 8.5 logcfu/g were observed after 6 months of storage at 4 °C. However, levels of immobilized cells on raisins did not exceed the recommended concentrations (<6.6 logcfu/g, after 6 months at refrigerator temperature).

Interestingly, the highest cell levels were observed in immobilized *P. acidilactici* SK, indicating a strain-specific survival potential.

Designing innovative functional foods is crucial to fulfill the consumers' increasing demands driven by the modern lifestyles. Cell immobilization technology is proposed as a potential tool to achieve increased survival levels of probiotic cultures, a critical factor for the production of functional food ingredients. However, further studies are needed to evaluate the effectiveness of immobilized cultures in real food systems.

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