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Effect of Immobilized *Pediococcus acidilactici* ORE5 Cells on Pistachio Nuts on the Functional Regulation of the Novel Katiki Domokou-Type Cheese Microbiome

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Featured Application: A new Katiki Domokou-type cheese was developed containing a novel *Pediococcus acidilactici* strain isolated from kefir grains, immobilized on pistachio nuts, positively affecting the cheese microbiome.

Abstract: Nowadays, functional foods supplemented with health-promoting microorganisms have attracted consumer attention due to their health benefits. However, maintaining high cell loads, which consist of an essential requirement for conferring the health effect, is a real bottleneck for the food industry due to viability declines during food processing and storage. Hence, freeze-drying and cell immobilization have been suggested to enhance cell viability. The aim of our study was to assess the effect of freeze-dried immobilized *P. acidilactici* ORE5 on pistachio nuts on the functional regulation of the Katiki Domokou-type cheese microbiome. Supplementation of Katiki Domokou-type cheese with free or immobilized *P. acidilactici* ORE5 culture resulted in cell loads > 8.5 logcfu/g up to 7 days of storage. Both free and immobilized *P. acidilactici* ORE5 cells suppressed the growth of *L. monocytogenes* after deliberate inoculation, acting as a protecting shield. HS-SPME GC/MS analysis showed that the incorporation of *P. acidilactici* ORE5 culture in cheese resulted in an improved volatile compounds profile, as verified by the preliminary sensory evaluation. According to Next-Generation Sequencing analysis, a wide range of bacterial diversity was revealed among samples. The most abundant genus was *Lactococcus* in all samples, while the results showed an increased presence of *Pediococcus* spp. in cheese fortified with *P. acidilactici* ORE5 culture, highlighting the ability of the strain to survive in the final product. Furthermore, the incorporation of *P. acidilactici* ORE5 culture in cheese had a significant impact on cheese microbiome composition, as the presence of spoilage bacteria, such as *Chryseobacterium*, *Acinetobacter* and *Pseudomonas*, was significantly less compared to the control cheese, indicating quality improvement and prolongation of the product's shelf-life.

Keywords: *Pediococcus acidilactici* ORE5; Katiki Domokou cheese; probiotic fortification; biopreservation

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1. Introduction

There is currently an upsurge of interest in functional foods as consumers become more conscious of their health and seek dietary choices that offer additional benefits beyond basic nutrition. Functional foods consist of a variety of components, including vitamins, essential fatty acids, antioxidants, prebiotics and probiotics. Foods enriched with probiotic

microorganisms are considered the main part of the functional foods market. Defined by the FAO/WHO as "microorganisms (bacteria or yeasts) which, when administered in adequate concentrations, provide health benefits to the host", probiotics are gaining increasing scientific and industrial interest [1]. In recent years, there have been numerous reports on the biological effects exhibited by probiotics, including their role in digestion, production of immune-modulating bioactive compounds, detoxification of toxins, and influence on the activity of the gut-brain axis [2].

To confer health benefits, probiotic foods should contain high cell loads able to survive during gastrointestinal tract (GI) passage and colonize the gut epithelium. According to the International Probiotic Association (IPA), the recommended cell levels in the foods are 10^7 cfu/g at the time of consumption in order to achieve a daily dose of 10^9 cells, considering the consumption of 100 g product. However, maintaining such high cell loads during food processing and storage consists of a real bottleneck for the food industry. Hence, cell immobilization is recommended as an alternative solution to overcome such obstacles, as it has been reported that it may enhance cell survival rates and ensure cell stability [3,4]. In addition, considering that the food industry favors dried products due to their prolonged shelf life, ease of storage and transportation, and cost-effectiveness resulting from the elimination of refrigeration needs, freeze-drying is commonly employed for delicate ingredients that require special care [5].

The selection of a suitable food carrier is of great importance, as it has a significant impact on the adhesion of cells and their ability to colonize the gut effectively [6], along with the sensory properties. Prebiotics, as non-digestible compounds that stimulate the growth or activity of beneficial bacteria in the gut, such as dietary fibers, seems a promising option. Pistachio nuts (*Pistacia vera* L.) contain dietary fibers, and their administration to diabetic rats resulted in functional modulation of gut microbiota [7] and beneficial alteration in fatty acids profile [8].

Dairy products and especially cheese, have been commonly served as food vehicles for probiotics to the human gut due to their chemical and physical attributes, such as a low pH value and high titratable acidity, contributing to enhanced transit tolerance of bacteria and facilitating the effective delivery of probiotics to the GI tract [9]. Katiki Domokou, also known as Tsalafouti, is a traditional soft cheese manufactured in the Domokos area in Greece from goat's and ewe's milk and has been recognized as a Protected Designation of Origin (PDO) since 1994. The process begins by pasteurizing and cooling the milk at temperatures 27–28 °C. Coagulation is then achieved with or without the addition of rennet, followed by a resting period at temperatures ranging from 20–22 °C. The resulting curd is subsequently filtered and placed into cloth sacks to allow drainage. Notably, during cheese manufacturing, no commercial starter cultures are usually employed. Instead, the manufacturing process relies mainly on the natural acidification of the milk, accomplished by inoculation with fresh product. Thus, the indigenous microflora is enhanced, contributing to the milk's acidification during the ripening phase. This practice helps to establish a desired microbial population that promotes the development of specific flavors and textures in the cheese as it matures. The final product retains a high moisture content (approximately 75%), low levels of salt (around 1%) and maintains a pH 4.3–4.5. Storage is conducted at temperatures of 4–5 °C for up to 4 days [10,11].

The microbiota of dairy products, such as cheese, consist of starter or adjunct cultures, as well as non-starter lactic acid bacteria [12]. The incorporation of functional cultures in dairy products has a profound impact on their microbiome, particularly the composition, functionality, and sensory characteristics. More specifically, lactic acid bacteria ferment lactose and produce lactic acid, creating an acidic environment that inhibits the growth of undesirable microorganisms, while promoting their growth since they are well-adapted to thrive in this environment [12]. Notably, when proper safety measures are neglected during the production process (improper pasteurization, post-pasteurization contamination, etc.), cheese constitutes an optimal environment for the proliferation of pathogenic bacteria, including *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* [13]. Moreover, soft

and semi-soft cheese products are susceptible to the growth of molds and bacteria that produce harmful toxins [14]. Microbial hazards have a great economic and legal impact on the food industry, intensifying the need for preservatives. Considering consumer demand for natural alternatives, several studies have suggested the use of lactic acid bacteria as biopreservatives [15]. Additionally, they contribute to the breakdown of proteins and lipids, releasing flavor-enhancing compounds and enzymes that influence the sensory attributes of the cheese. Mechanistically, lactic acid bacteria adhere to the surface of cheese curds, forming biofilms that protect against contamination and provide an environment for microbial interactions. They also produce exopolysaccharides, contributing to the cheese's texture and rheological properties [16]. Hence, the incorporation of probiotics in cheese production not only enhances nutritional value and promotes health benefits, but also shapes microbial dynamics, ultimately influencing the quality, flavor, and texture of the cheese.

Several lactic acid bacteria, such as *Lactococcus*, *Lactobacillus*, and *Streptococcus* species, are commonly employed as starter cultures in cheese production [12]. However, for the selection of a suitable strain for the production of functional food, several criteria should be considered regarding its potential beneficial properties. In this vein, *P. acidilactici* ORE5, a wild-type strain isolated from kefir, was initially evaluated for potential probiotic characteristics, and subsequently, it was used for the production of a novel functional Katiki Domokou-type cheese enriched with immobilized *P. acidilactici* ORE5 on pistachio nuts, assessing cell survival, along with the physicochemical and microbiome changes during storage. Moreover, the potential growth suppression of *L. monocytogenes* was investigated after deliberate contamination, a food-borne pathogen usually associated with cheese safety.

2. Materials and Methods

2.1. Microbial Cultures

Pediococcus acidilactici ORE5 was isolated from kefir grains, and species identification was based on 16S rRNA sequencing, according to Li et al. [17]. The results were compared with the sequences deposited in the GenBank database using BLAST analysis on the National Center for Biotechnology Information (NCBI).

Lactiplantibacillus plantarum ATCC 14917 was obtained from ATCC (LGC Standards, Middlesex, UK). Both *P. acidilactici* ORE5 and *L. plantarum* ATCC 14917 were grown on de Man, Rogosa and Sharpe (MRS) broth (Condalab, Madrid, Spain) at 37 °C for 24 h. The medium was sterilized at 121 °C for 15 min prior to use.

Listeria monocytogenes NCTC 10527 serotype 4b (kindly provided by the Laboratory of Clinical Microbiology, Sismanoglio General Hospital, Athens, Greece) was grown in Brain Heart Infusion (BHI) broth (Condalab) at 37 °C for 24 h. The medium was sterilized at 121 °C for 15 min before use.

2.2. In Vitro Screening for Probiotic Properties

2.2.1. Resistance to Low pH, Pepsin, Pancreatin and Tolerance to Bile Salts

The assessment of resistance of bacterial cells to pH 2.0, 3.0 or 4.0, as well as the tolerance to bile salts, was carried out, according to Argyri et al. [18]. Regarding the resistance of the bacterial cells to pepsin and pancreatin, a method described by Plessas et al. [19] was performed.

2.2.2. Antibiotic Susceptibility

The determination of the antibiotic susceptibility was assessed using the M.I.C. Evaluator® strips (2018) and expressed as Minimum Inhibitory Concentration (MIC), following the method described by Plessas et al. [19].

2.3. Production of Freeze-Dried Immobilized P. acidilactici ORE5 on Pistachio Nuts

Cell immobilization on pistachio nuts was carried out as described previously [3]. The immobilized cells were transferred to −80 °C overnight and freeze-dried on a BenchTop

Pro (Virtis, SP Scientific, Warminster, PA, USA) for 24 h at ~30–35 Pa with the condenser temperature at −100 °C. For comparison reasons, freeze-dried free (non-immobilized) *Pediococcus acidilactici* ORE5 cells were also produced.

2.4. Functional Katiki Domokou-Type Cheese Production

Katiki Domokou cheese ("Oreines plagies", Domokos, Greece) (pasteurized goat and sheep milk, 13% fat, 1% salt) purchased from a local market was enriched with freeze-dried immobilized *P. acidilactici* ORE5 cells on pistachio nuts (sample KIP), resulting in 8 logcfu/g of cheese. For comparison reasons, Katiki Domokou cheese containing freeze-dried free cells (sample KFP) was also prepared by incorporating in cheese, resulting in the same cell levels as the KIP sample. Additionally, a regular commercial product with no *Pediococcus acidilactici* ORE5 cells (sample KC) was used as a control (Figure S1). All products were stored at refrigerated temperature (4 °C) for 14 days to monitor self-life. Samples were taken after 3 days and subjected to physicochemical, microbiological and molecular analyses.

2.5. Resistance to Spoilage Assessment

All products were deliberately inoculated with *L. monocytogenes* (inoculum 10^5 cfu/g) and stored at a refrigerated temperature (4 °C) for 14 days to determine cell levels.

2.6. Physicochemical Analysis

PH, titratable acidity and water activity (aw) were determined according to Dimitrellou et al. [20]. Moisture content was measured as described in ISO: 5534 [21].

2.7. Microbiological Analyses

2.7.1. Monitoring *Pediococcus acidilactici* ORE5 Cell Viability

In order to monitor the levels of free or immobilized *P. acidilactici* ORE5 cells, 10 g of cheese were blended with 90 mL sterile $\frac{1}{4}$ Ringer's solution, decimal diluted, plated on MRS agar and incubated at 37 °C for 72 h. Cell levels were expressed as logcfu/g of cheese.

2.7.2. Determination of Cheese Microbiota

Total aerobic counts (TAC), total psychrophilic counts, lactococci, yeasts/molds, staphylococci, *Enterobacteriacae*, coliforms and *L. monocytogenes* were determined as described by Nikolaou et al. [4].

2.8. Minor Component Analysis by HS-SPME GC/MS

Katiki Domokou-type cheese samples collected at the beginning and after 3 days of storage were analyzed for minor volatiles on an HS-SPME GC/MS system [6890N GC, 5973 NetworkedMS MSD, HP-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness), Agilent Technologies, Santa Clara, CA, USA] and semi-quantified using 4-methyl-2-pentanol. Sample analysis was carried out as previously described [22].

2.9. DNA Extraction, PCR Amplification and 16S rRNA Sequencing

Total DNA was extracted using the NucleoSpin® Food (MACHEREY-NAGEL GmbH & Co.KG, Düren, Germany), following the manufacturer's instructions. The isolated DNA was quantified using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A PCR reaction targeting the V3 region of the 16S rDNA gene was carried out for each sample, using the primers 5′-ACTGAGACACGGTCCAGACT-3′ (F) and 5′-GTATTACCGCGGCTGCTG-3′(R). For each reaction, 100 ng of DNA were mixed with 10 μL of KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), 4 μL of primers (final concentration 0.2 μM) and proper volume of Nuclease Free water to reach a final volume of 20 μL. PCR was carried out under the following conditions:—Initial denaturation at 95 °C (5 min), followed by 25 cycles of denaturation at 95 °C (30 s), annealing at 58 °C (30 s) and extension at 72 °C (15 s). PCR products were checked in a 2% *w*/*v* agarose gel and then purified using magnetic Agencourt AMPure XP beads (Beckman Coulter,

CA, USA) according to the manufacturer's instructions. An amount of 75 ng of purified PCR products was used for the preparation of the libraries with the Ion Plus Fragment Library Kit (Thermo Fischer Scientific). The libraries were then quantified and prepared for sequencing by the Ion Torrent GeneStudio™ S5 (Thermo Fischer Scientific).

Raw sequencing data were analyzed to filter out low-quality reads. Data analysis for amplicon sequencing was performed using Mothur (v.1.45.3) [23]. OTUs (Operational Taxonomical Units) were defined by clustering reads at 3% divergence (97% similarity), using the Greengenes (v.13_8_99) database. Normalization, taxonomic-binning of OTUs in phyla and genera levels and the calculation of Shannon's and Simpson's indices were performed using the Rhea platform [24].

2.10. Preliminary Sensory Evaluation

Katiki Domokou cheese samples were evaluated for their quality characteristics using a previously approved protocol in our laboratory [25]. A mixed panel of 11 random tasters familiar with cheese products was asked to evaluate the samples by providing scores on a 0–5 scale (0: unacceptable, 5: exceptional) regarding aroma (fruity, milklike, spirituous, yeasty-breadlike, piquant), texture (watery, creamy, soft, gelatinous, curdy, lumpy, ropy), taste (too acid, acid, bitter, scorched, foreign, sweet, sweet and sour, metallic, salted) and overall quality. During the evaluation, tasters consumed water and crackers between samples. Samples were coded using randomly chosen three-digit numbers.

2.11. Statistical Analysis

The experiments were conducted in triplicate, and statistical significance was determined at a significance level of $p < 0.05$. ANOVA and Duncan's multiple range test were employed to analyze the results and to identify significant differences among the outcomes. The computations for coefficients, ANOVA tables, and significance ($p < 0.05$) were carried out using Statistica version 10.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Molecular Identification

The strain was previously isolated from kefir grains, and according to identification based on 16S rRNA sequencing, the isolated strain was characterized as *Pediococcus acidilactici.*

3.2. In Vitro Screening for Probiotic Properties

The results of the in vitro assessment for potential probiotic properties of *P. acidilactici* ORE5 are shown in Table 1. In particular, the resistance to low pH was evaluated, and at pH 4, *P. acidilactici* ORE5 cells exhibited high survival rates. Yet, a significant reduction ($p < 0.05$) in cell viability was noted at pH 2. However, *P. acidilactici* ORE5 maintained high cell loads (6.4 log cfu/mL). Concerning tolerance to pancreatin, *P. acidilactici* ORE5 retained its viability at high levels, while resistance to bile salts after 4 h of exposure was recorded.

Table 1. Determination of viability of the isolated strain after exposure to the acid environment, bile salts, pepsin and pancreatin. In all tests, the probiotic *L. plantarum* ATCC 14917 served as a reference strain.

		Final Counts (log cfu/mL)	
	Time (h)	***P. acidilactici* ORE 5**	***L. plantarum* ATCC 14971**
Resistance to low pH	0	8.4 ± 0.21^{i}	$8.3 \pm 0.13^{h,i}$
pH = 2	2	6.4 ± 0.11^{a}	7.2 ± 0.07^{d}
pH = 3	2	6.9 ± 0.13^{c}	7.7 ± 0.15^{e}
pH = 4	2	$8.1 \pm 0.07^{g,h,i}$	$8.0 \pm 0.12^{g,h}$
Pepsin	0	7.6 ± 0.09^{e}	7.4 ± 0.17^{d}
	3	6.5 ± 0.19^{b}	$6.8 \pm 0.21^{b,c}$

Table 1. *Cont.*

		Final Counts (log cfu/mL)	
	Time (h)	***P. acidilactici* ORE 5**	***L. plantarum* ATCC 14971**
Pancreatin	0	8.0 ± 0.37^{f}	$8.1 \pm 0.14^{g,h,i}$
	4	7.4 ± 0.13^{d}	7.4 ± 0.21^{d}
Bile salts	0	8.4 ± 0.22^{j}	8.5 ± 0.13^{j}
	4	8.0 ± 0.19^{f}	8.1 ± 0.08^{g}

Significant differences ($p < 0.05$) are shown with different letters in superscript.

3.3. Safety Profile—Antibiotic Susceptibility

The Minimum Inhibitory Concentration (MIC) was estimated based on the diffusion in Mueller-Hinton agar plates, and their mean values ± standard deviation are presented in Table 2. In brief, *P. acidilactici* ORE5 displayed resistance to clindamycin, erythromycin and tetracycline.

Table 2. MIC (μg/mL) of antibiotics for *P. acidilactici* ORE5 as determined by gradient diffusion using M.I.C. Evaluator® strips.

Agent	***P. acidilactici* ORE 5**	**Cut-Off [a]**
	(MIC μg/mL)	
Amoxycillin	2.41 ± 0.95	n.r. [b]
Amoxycillin + Clavulanic acid	0.25 ± 0.19	n.r. [b]
Ampicillin	1.28 ± 0.58	4
Clindamycin	1.91 ± 0.27	1
Erythromycin	1.77 ± 0.19	1
Gentamycin	7.23 ± 2.08	16
Metronidazole	159.2 ± 27.8	n.r. [b]
Tetracycline	10.97 ± 1.48	8
Tigecycline	0.49 ± 0.08	n.r. [b]
Vancomycin	>256	n.r. [b]

[a] Breakpoints are referred to *Pediococcus* spp. EFSA breakpoints for other types of LABs are slightly different. Strains with MIC higher than the breakpoints are considered to be resistant according to EFSA [26]. [b] Not required.

3.4. Physicochemical Characteristics of Functional Katiki Domokou-Type Cheese

The physicochemical parameters ranged in usual levels, as published previously (pH 4.3–4.5, moisture 75%) [10,27], and the results are presented in Table 3. In cheese samples fortified with free or immobilized *P. acidilactici* ORE5 cells, the pH ranged in lower levels ($p < 0.05$) (4.08–4.15), while lactic acid concentrations were higher ($p < 0.05$) (0.11–0.15 g lactic acid/100 g cheese) compared to the control (pH 4.28–4.33, 0.85–0.95 g lactic acid/100 g cheese). After 7 days of storage, a significant ($p < 0.05$) increase in pH values was observed, whereas acidity was decreased ($p < 0.05$) in all samples. The addition of free or immobilized cells had no effect on water activity levels, ranging 0.88–0.92. The moisture content ranged at usual levels (72.7–75.5%) in KC and KFP samples. However, in the KIP sample, lower values were observed initially (63.9–65.71%), but increased to that similar to the KC and KFP sample levels after 2 days of storage. After 7 days of storage, a slight increase in moisture content was observed.

Table 3. Effect of free and immobilized *P. acidilactici* ORE5 on pistachio nuts on the physicochemical characteristics and sensory attributes of Katiki Domokou-type cheese.

Cheese [1]	Days of Storage	Water Activity (aw)	pH	Acidity (g Lactic Acid/100 g Cheese)	% Moisture Content	Overall Sensory Evaluation [2]
KC	0	0.889 ± 0.01	4.28 ± 0.05	0.085 ± 0.02	72.71 ± 0.01	3.41 ± 0.70
	1	0.892 ± 0.02	4.27 ± 0.01	0.088 ± 0.04	73.32 ± 0.01	
	2	0.889 ± 0.01	4.33 ± 0.02	0.091 ± 0.05	74.20 ± 0.00	
	3	0.899 ± 0.05	4.29 ± 0.01	0.095 ± 0.03	74.71 ± 0.01	
	7	0.906 ± 0.01	4.31 ± 0.02	0.085 ± 0.01	75.51 ± 0.05	
	10	0.908 ± 0.02	4.45 ± 0.08	0.079 ± 0.02	77.82 ± 0.01	
	14	0.888 ± 0.03	4.72 ± 0.01	0.071 ± 0.01	78.90 ± 0.00	
KFP	0	0.900 ± 0.01	4.08 ± 0.01	0.11± 0.02	73.94 ± 0.00	3.11 ± 0.74
	1	0.918 ± 0.02	4.11 ± 0.03	0.12± 0.07	74.11 ± 0.00	
	2	0.922 ± 0.03	4.14 ± 0.01	0.20± 0.05	74.21 ± 0.01	
	3	0.925 ± 0.05	4.12 ± 0.01	0.29± 0.03	74.42 ± 0.05	
	7	0.904 ± 0.07	4.35 ± 0.01	0.22 ± 0.02	74.81 ± 0.01	
	10	0.894 ± 0.06	4.38 ± 0.02	0.17 ± 0.01	75.50 ± 0.02	
	14	0.889 ± 0.01	4.45 ± 0.01	0.09 ± 0.01	78.35 ± 0.04	
KIP	0	0.910 ± 0.01	4.15 ± 0.01	0.15 ± 0.05	63.91 ± 0.01	4.00 ± 0.54
	1	0.912 ± 0.02	4.18 ± 0.02	0.18 ± 0.05	65.72 ± 0.02	
	2	0.915 ± 0.02	4.23 ± 0.01	0.24 ± 0.01	71.51 ± 0.01	
	3	0.919 ± 0.03	4.22 ± 0.01	0.31 ± 0.02	72.11 ± 0.00	
	7	0.915 ± 0.05	4.66 ± 0.02	0.25 ± 0.03	72.91 ± 0.01	
	10	0.929 ± 0.01	4.68 ± 0.05	0.18 ± 0.01	73.51 ± 0.02	
	14	0.908 ± 0.05	4.70 ± 0.05	0.10 ± 0.01	74.13 ± 0.05	

[1] KC—control cheese, KFP—cheese with free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts. [2] The ratings ranged from 0 to 5 on a scale (0 = unacceptable; 5 = exceptional).

3.5. Microbiological Analysis of Functional Katiki Domokou-Type Cheese

The results of the microbiological analysis are presented in Table 4. Lactobacilli were detected in all samples at levels ≥6.95 logcfu/g. As expected, cell loads of lactobacilli were significantly ($p < 0.05$) higher in cheese KFP and KIP samples than in the KC product at every time point. Up to 3 days of storage, a gradual increase of cell loads was observed in samples with free or immobilized cells, resulting in 8.99 and 9.12 logcfu/g in the KFP and KIP samples, respectively. However, storage after the expiration date (4 days after package opening) affected lactobacilli levels, as a significant decline ($p < 0.05$) in cell viability was observed after 7 days of storage, resulting in 8.57 and 8.96 logcfu/g in KFP and KIP, respectively. Of note, the survival rate of lactobacilli was higher ($p < 0.05$) in KIP compared to the KFP sample. No significant differences were observed in lactococci cell loads (4.80–4.82 logcfu/g) among all samples. In accordance with lactobacilli, lactococci were increased gradually up to 3 days of storage, and their numbers were significantly reduced after the expiration date. Yeasts were detected in similar loads (6.35–6.39 logcfu/g) in all samples at the beginning of storage, but their numbers were significantly ($p < 0.05$) lower in the KFP and KIP samples compared to KC during storage. Importantly, staphylococci were detected at numbers ≤ 3.92 logcfu/g in all cheese samples. Storage affected staphylococci loads, as an increase was observed after the 3rd day, which was significantly ($p < 0.05$) higher in the KC sample compared to KFP and KIP. Significantly, coliforms, *Enterobacteriacae*, *Salmonella* spp. and *L. monocytogenes* were below the detectable limit (≤1 logcfu/g) in all samples.

3.6. Resistance of Functional Katiki Domokou-Type Cheese to Microbial Contamination

Possible resistance to microbial contamination of Katiki Domokou cheese samples fortified with free or immobilized *P. acidilactici* ORE5 culture after deliberate spiking with *L. monocytogenes* was investigated, and the results are shown in Table 5. Initial cell loads of *L. monocytogenes* were 5.08–5.11 logcfu/g in all cases, and no significant differences were observed among samples up to 2 days of storage. After 3 days and up to 7 days of storage, a gradual increase of *L. monocytogenes* levels was observed, but higher levels ($p < 0.05$) were noted in the KC than the KFP and KIP samples. During storage, a significant decline ($p < 0.05$) in both lactobacilli and lactococci counts was observed in all samples compared to the ones with no deliberate spiking with *L. monocytogenes*. More specifically, up to 3 days of storage, lactobacilli levels were 8.99 and 9.12 logcfu/g in uninoculated KFP and KIP samples, respectively, while their numbers decreased to 8.45 and 8.54 logcfu/g in KFP and KIP samples deliberately spiking with *L. monocytogenes*.

3.7. Minor Volatiles in Functional Katiki Domokou-Type Cheese

Katiki Domokou-type cheese samples fortified with free or immobilized *P. acidilactici* ORE5 cells on pistachio nuts were evaluated regarding their volatile compounds at the beginning and after 3 days of refrigerated storage by the HS-SPME GC/MS technique, and the results are presented in Table 6. For comparison reasons, Katiki Domokou cheese samples with no *P. acidilactici* ORE5 cells were also analyzed. Overall, 41 compounds were detected: 14 compounds in the KC sample, 27 compounds in the KFP sample and 37 compounds in the KIP sample. The main classes of volatile compounds identified were esters, acids, alcohols, and carbonyl compounds. From a quantitative perspective, the highest ($p < 0.05$) content of total volatiles was observed in the KFP sample (52.3 μg/g), while total volatiles were ranged from 31.6 μg/g in the KIP sample and 4.8 μg/g in the KC sample. More specifically, the incorporation of free *P. acidilactici* ORE5 cells in cheese resulted in significantly ($p < 0.05$) higher amounts of total esters, acids and hydrocarbons, while the cheese sample fortified with immobilized *P. acidilactici* ORE5 cells contained higher concentrations of total alcohols ($p < 0.05$). Regarding total carbonyl compounds, no significant differences were observed between KFP and KIP samples.

Table 4. Effect of free or immobilized *P. acidilactici* ORE5 on pistachio nuts on total aerobic count, psychrophilic bacteria, lactococci, lactobacilli, yeasts and staphylococci in the Katiki Domokou-type cheese.

Cheese [1]	Days of Storage	Total Aerobic Count (TAC) (logcfu/g)	Psychrophilic Bacteria (logcfu/g)	*Lactococci* (logcfu/g)	*Lactobacilli* (logcfu/g)	Yeasts (logcfu/g)	*Staphylococci* (logcfu/g)
KC	0	6.50 ± 0.11	4.58 ± 0.01	4.80 ± 0.05	6.95 ± 0.01	6.39 ± 0.03	3.92 ± 0.03
	1	6.44 ± 0.01	5.21 ± 0.03	5.00 ± 0.02	7.27 ± 0.01	6.70 ± 0.07	4.19 ± 0.06
	2	6.62 ± 0.02	5.38 ± 0.01	5,06 ± 0.03	7.42 ± 0.03	6.89 ± 0.03	4.33 ± 0.07
	3	6.74 ± 0.02	5.60 ± 0.08	5.43 ± 0.10	7.52 ± 0.02	7.33 ± 0.02	4.65 ± 0.01
	7	6.88 ± 0.01	5.85 ± 0.14	4.46 ± 0.09	7.08 ± 0.02	7.60 ± 0.08	5.35 ± 0.04
	10	6.74 ± 0.05	5.62 ± 0.01	4.25 ± 0.01	6.99 ± 0.01	7.99 ± 0.02	5.15 ± 0.01
	14	6.53 ± 0.01	5.52 ± 0.03	4.13 ± 0.07	6.78 ± 0.06	8.27 ± 0.01	5.07 ± 0.01
KFP	0	8.30 ± 0.13	6.92 ± 0.04	4.81 ± 0.01	8.81 ± 0.01	6.38 ± 0.01	3.80 ± 0.01
	1	8.63 ± 0.07	7.42 ± 0.02	5.10 ± 0.01	8.88 ± 0.07	6.50 ± 0.04	3.94 ± 0.05
	2	8.85 ± 0.03	7.38 ± 0.01	5.33 ± 0.02	8.91 ± 0.09	6.73 ± 0.02	4.09 ± 0.05
	3	8.96 ± 0.02	7.60 ± 0.08	5.76 ± 0.06	8.99 ± 0.09	7.03 ± 0.01	4.30 ± 0.08
	7	8.78 ± 0.05	7.85 ± 0.14	4.65 ± 0.01	8.57 ± 0.02	7.21 ± 0.05	4.70 ± 0.06
	10	8.63 ± 0.01	7.02 ± 0.01	4.42 ± 0.01	8.22 ± 0.01	7.52 ± 0.02	4.65 ± 0.02
	14	8.55 ± 0.01	6.52 ± 0.03	4.37 ± 0.04	8.09 ± 0.03	7.82 ± 0.11	4.57 ± 0.03
KIP	0	8.88 ± 0.03	6.92 ± 0.04	4.82 ± 0.01	8.89 ± 0.02	6.35 ± 0.01	3.63 ± 0.02
	1	8.95 ± 0.01	7.42 ± 0.02	5.13 ± 0.01	8.99 ± 0.05	6.43 ± 0.07	3.89 ± 0.00
	2	9.01 ± 0.04	7.38 ± 0.01	5.44 ± 0.01	9.09 ± 0.01	6.52 ± 0.03	4.02 ± 0.01
	3	9.07 ± 0.03	7.60 ± 0.08	5.97 ± 0.01	9.12 ± 0.02	6.97 ± 0.02	4.22 ± 0.05
	7	9.12 ± 0.02	7.85 ± 0.14	4.92 ± 0.08	8.96 ± 0.04	7.12 ± 0.01	4.54 ± 0.00
	10	8.99 ± 0.01	7.12 ± 0.02	4.72 ± 0.01	8.88 ± 0.01	7.45 ± 0.03	4.42 ± 0.01
	14	8.95 ± 0.02	6.52 ± 0.03	4.53 ± 0.03	8.85 ± 0.03	7.67 ± 0.02	4.38 ± 0.03

[1] KC—control cheese, KFP—cheese with free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts.

Table 5. Effect of free or immobilized *P. acidilactici* ORE5 on pistachio nuts on total aerobic count, psychrophilic bacteria, lactococci, lactobacilli, yeasts and staphylococci in the Katiki Domokou-type cheese after deliberate spiking with *L. monocytogenes*.

Cheese [1]	Days of Storage	Total Aerobic Count (TAC) (logcfu/g)	Psychrophilic Bacteria (logcfu/g)	*Lactococci* (logcfu/g)	*Lactobacilli* (logcfu/g)	Yeasts (logcfu/g)	*Staphylococci* (logcfu/g)	*L. monocytogenes* (logcfu/g)
KC	0	7.52 ± 0.04	4.69 ± 0.01	4.77 ± 0.03	6.89 ± 0.00	6.42 ± 0.03	3.99 ± 0.01	5.11 ± 0.01
	1	7.64 ± 0.06	5.42 ± 0.02	4.56 ± 0.02	6.93 ± 0.02	6.65 ± 0.01	4.11 ± 0.04	5.12 ± 0.01
	2	8.23 ± 0.01	5.53 ± 0.01	4.44 ± 0.01	7.21 ± 0.05	6.95 ± 0.01	4.42 ± 0.01	5.15 ± 0.02
	3	8.52 ± 0.03	6.28 ± 0.06	4.33 ± 0.04	7.36 ± 0.02	7.48 ± 0.02	4.65 ± 0.01	6.34 ± 0.06
	7	8.96 ± 0.02	6.01 ± 0.04	4.11 ± 0.02	6.74 ± 0.02	7.68 ± 0.03	5.25 ± 0.04	6.28 ± 0.02
	10	8.81 ± 0.01	5.89 ± 0.01	3.98 ± 0.01	6.65 ± 0.01	8.07 ± 0.05	4.79 ± 0.01	6.25 ± 0.05
	14	8.77 ± 0.04	5.7 ± 0.06	3.66 ± 0.05	6.56 ± 0.02	8.25 ± 0.06	4.72 ± 0.03	6.22 ± 0.04
KFP	0	8.13 ± 0.06	6.69 ± 0.01	4.89 ± 0.05	8.32 ± 0.01	6.43 ± 0.01	3.78 ± 0.01	5.09 ± 0.06
	1	8.54 ± 0.01	7.21 ± 0.03	5.10 ± 0.01	8.38 ± 0.04	6.54 ± 0.07	3.89 ± 0.08	5.12 ± 0.01
	2	8.57 ± 0.01	7.14 ± 0.02	5.33 ± 0.02	8.42 ± 0.07	6.85 ± 0.01	4.02 ± 0.02	5.15 ± 0.02
	3	8.62 ± 0.04	7.54 ± 0.01	5.46 ± 0.06	8.45 ± 0.02	7.11 ± 0.04	4.28 ± 0.01	5.27 ± 0.02
	7	8.56 ± 0.01	7.43 ± 0.04	4.65 ± 0.01	7.97 ± 0.03	7.32 ± 0.01	4.22 ± 0.01	5.43 ± 0.01
	10	8.28 ± 0.02	6.89 ± 0.01	4.45 ± 0.01	7.88 ± 0.04	7.68 ± 0.05	4.15 ± 0.02	5.69 ± 0.05
	14	8.18 ± 0.01	6.11 ± 0.07	4.37 ± 0.04	7.78 ± 0.04	7.96 ± 0.01	4.02 ± 0.01	5.49 ± 0.02
KIP	0	8.71 ± 0.02	6.77 ± 0.01	4.89 ± 0.01	8.42 ± 0.02	6.37 ± 0.01	3.70 ± 0.01	5.08 ± 0.04
	1	8.65 ± 0.05	7.24 ± 0.03	5.09 ± 0.01	8.49 ± 0.01	6.43 ± 0.07	3.83 ± 0.04	5.11 ± 0.04
	2	8.67 ± 0.03	7.17 ± 0.02	5.31 ± 0.03	8.51 ± 0.01	6.59 ± 0.03	3.97 ± 0.03	5.14 ± 0.01
	3	8.97 ± 0.01	7.57 ± 0.00	5.79 ± 0.05	8.54 ± 0.01	7.05 ± 0.02	4.27 ± 0.01	5.23 ± 0.01
	7	8.76 ± 0.01	7.46 ± 0.02	4.69 ± 0.05	8.28 ± 0.01	7.20 ± 0.01	4.15 ± 0.01	5.55 ± 0.01
	10	8.61 ± 0.02	6.94 ± 0.01	4.52 ± 0.01	8.11 ± 0.02	7.35 ± 0.01	4.12 ± 0.02	5.62 ± 0.04
	14	8.54 ± 0.04	6.14 ± 0.07	4.39 ± 0.04	8.06 ± 0.04	7.72 ± 0.02	4.10 ± 0.01	5.45 ± 0.02

[1] KC—control cheese, KFP—cheese with free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts.

The detected volatile compounds in cheese samples included seven esters. Among them, ethyl acetate, ethyl hexanoate and 2-phenylethyl acetate were identified in all cheese samples. However, the incorporation of free or immobilized *P. acidilactici* ORE5 cells led to significantly ($p < 0.05$) increased amounts of these compounds compared to the control sample. Of note, ethyl propanoate, ethyl butyrate, 3-methylbutyl acetate and ethyl decanoate were detected only in the KFP and KIP samples.

Among the volatile compounds, five acids were detected in cheese samples. Sorbic, octanoic, benzoic and decanoic acid were present in all samples, while hexanoic acid was found in the KFP and KIP samples after 3 days of storage. In general, the amounts of acids were higher ($p < 0.05$) in the KFP and KIP samples than in the control cheese (KC).

In total, six alcohols were detected in the samples. Amounts of 3-methyl-1-butanol and 2-phenylethanol were identified in all samples; however, higher concentrations were observed in the KFP and KIP samples ($p < 0.05$) compared to the control cheese. Of note, 2-methyl-1-propanol and 2-methyl-1-butanol were present only in KFP and KIP, while 1-hexanol and 2-methyl-phenol were detected only in the KIP sample. In all cases, the incorporation of immobilized cells in cheese led to the highest concentrations of alcohols ($p < 0.05$) among the samples.

The carbonyl compounds present in cheese samples included 6 aldehydes and 4 ketones that were more abundant in the KFP and KIP samples than in KC. Among them, only hexanal was common in all samples. In the KC sample, 2-pentanone and 2-heptanone were the only carbonyl compounds detected, apart from hexanal. 2-nonanone was observed only in the KFP sample, while heptanal was present at the beginning of storage in KFP cheese; however, it was undetectable after 3 days of storage. On the other hand, 2-methyl-propanal, 2-methyl-butanal and 3-hydroxy-butanone were present only in the KIP sample. Furthermore, 3-methyl-butanal and benzeneacetaldehyde were detected in both the KFP and KIP samples. In total, no significant differences were observed between KFP and KIP samples. Remarkably, carbonyl compounds were identified in lower concentrations ($p < 0.05$) compared to other volatile compounds detected.

Apart from the main classes of volatile compounds, 12 hydrocarbons were identified in our samples. 2-methyl-1,3-pentadiene, octane, styrene, decane, dodecane, 1,3-bis(1,1-dimethylethyl)-benzene and tetradecane were present in both KFP and KIP sample, while 2,4-dimethyl-1-heptane, 2,6-dimethyl-pyrazine and 3-ethyl-2,5-dimethyl-pyrazine were detected in the KIP sample. In general, the cheese sample fortified with free cells exhibited the highest content of hydrocarbons.

Principal Component Analysis (PCA) results are represented in Figure 1. No significant differences were noted at the beginning of the storage, and thus the samples are gathered in the left part of the plot. However, the pistachio addition (KFP samples) correlated positively to PC2. On the other hand, storage (3 days) had a significant effect on both KFP and KIP samples and correlated positively to PC1, as KFP appeared in the lower right part of the diagram and KIP in the upper right part.

Table 6. Effect of free or immobilized *P. acidilactici* ORE5 on pistachio nuts on minor volatile compounds (μg/g) of Katiki Domokou-type cheese after production (day 0) and after 3 days of storage.

Compound	Identification Method	KC		KFP		KIP	
		d0	d3	d0	d3	d0	d3
Esters							
Ethyl acetate	KI	N. D.	0.1	0.6	13.8	0.5	6.4
Ethyl propanoate	KI	N. D.	N. D.	N. D.	0.1	0.0	0.1
Ethyl butyrate	KI	N. D.	N. D.	0.1	0.2	0.2	0.1
3-methylbutyl acetate	KI	N. D.	N. D.	N. D.	0.2	N. D.	0.2
Ethyl hexanoate	KI	N. D.	0.2	0.8	1.8	0.6	0.6
2-phenylethyl acetate	KI	N. D.	0.2	1.6	8.0	1.0	3.0
Ethyl decanoate	KI	N. D.	N. D.	0.1	1.8	0.2	0.2

Table 6. *Cont.*

Compound	Identification Method	KC		KFP		KIP	
		d0	**d3**	**d0**	**d3**	**d0**	**d3**
Organic acids							
Hexanoic acid	KI	0.1	N. D.	N. D.	0.8	0.2	0.4
Sorbic acid	KI	0.5	0.5	1.0	1.5	0.4	0.2
Octanoic acid	KI	0.1	0.4	0.1	3.5	0.1	0.1
Benzoic acid	KI	0.3	0.6	0.1	1.0	0.1	0.1
Decanoic acid	KI	N. D.	0.1	0.1	1.9	0.3	0.1
Alcohols	KI						
2-methyl-1 propanol	KI	N. D.	N. D.	0.1	0.2	0.3	0.5
3-methyl-1 butanol	KI	0.2	1.0	1.4	3.1	2.4	9.4
2-methyl-1 butanol	KI	N. D.	N. D.	0.3	0.4	0.4	1.4
1-hexanol	KI	N. D.	N. D.	N. D.	N. D.	0.1	0.1
2-methyl-phenol	KI	N. D.	N. D.	N. D.	N. D.	0.1	0.1
2-phenylethanol	KI	0.2	1.3	0.9	1.3	1.1	3.1
Carbonyl compounds							
2-methyl-propanal	KI	N. D.	N. D.	N. D.	N. D.	N. D.	0.1
Butanal	KI	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
3-methyl-butanal	KI	N. D.	N. D.	N. D.	0.4	0.1	0.2
2-methyl-butanal	KI	N. D.	N. D.	N. D.	N. D.	0.1	0.1
2-pentanone	KI	N. D.	0.1	N. D.	N. D.	N. D.	N. D.
3-hydroxy-butanone	KI	N. D.	N. D.	N. D.	N. D.	0.3	0.4
Hexanal	KI	N. D.	0.1	0.2	0.1	0.1	0.1
2-heptanone	KI	N. D.	0.1	N. D.	N. D.	0.1	N. D.
Heptanal	KI	N. D.	N. D.	0.1	N. D.	N. D.	N. D.
Benzeneacetaldehyde	KI	N. D..	N. D.	N. D.	0.1	0.1	0.1
2-nonanone	KI	N. D	N. D.	N. D.	0.1	N. D.	N. D.
Miscellaneous Compounds							
2-methyl-1,3- pentadiene	KI	N. D.	N. D.	N. D.	0.1	0.1	0.2
Octane	KI	N. D.	N. D.	0.1	0.1	0.1	0.1
2,4-dimethyl-1-heptane	KI	N. D.	N. D.	N. D.	N. D.	0.3	0.4
Styrene	KI	N. D.	N. D.	N. D.	0.1	N. D.	0.1
2,6-dimethyl-pyrazine	KI	N. D.	N. D.	N. D.	N. D.	0.2	0.1
a-pinene	KI	N. D.	0.1	N. D.	N. D.	0.2	0.1
Decane	KI	N. D.	N. D.	0.1	1.5	0.7	0.9
Limonene	KI	N. D.	0.1	N. D.	N. D.	0.3	0.2
3-ethyl-2,5-dimethyl-pyrazine	KI	N. D.	N. D.	N. D.	N. D.	0.2	0.4
Dodecane	KI	N. D.	N. D.	N. D.	8.0	1.2	1.6
1,3-bis(1,1-dimethylethyl)-benzene	KI	N. D.	N. D.	0.1	1.2	0.3	0.4
Tetradecane	KI	N. D.	N. D.	N. D.	1.0	0.1	0.1
Total volatiles		1.4	4.8	6.8	52.3	12.5	31.6

KI—Tentative identification by the Kovats retention index compared to the literature. N. D.—Not detected. KC—control cheese, KFP—cheese with free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts.

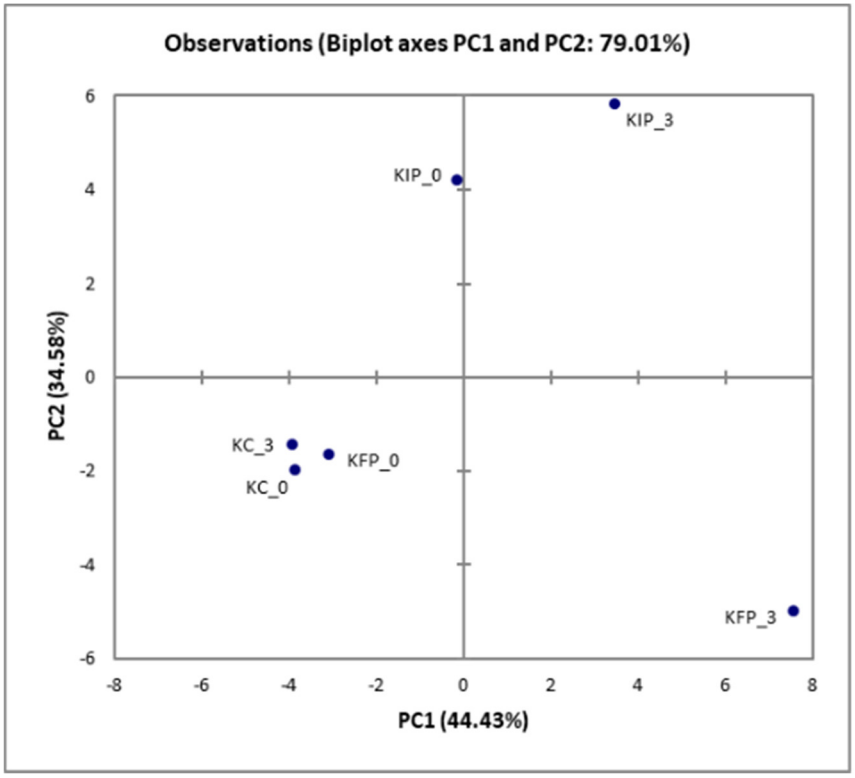

Figure 1. Principal component analysis (PCA) of minor volatiles identified in the Katiki Domokou-type cheese samples containing free or immobilized *P. acidilactici* ORE5 cells on pistachio nuts. KC—control samples, KFP—cheese samples containing free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts. The storage duration (days) is shown at the end of the sample codes.

3.8. Effect of Immobilized Pediococcus acidilactici ORE5 Culture on Bacteria Microbiome of Katiki Domokou-Type Cheese

Molecular analysis was performed in all the cheese samples after 3 days of storage, in order to evaluate the composition of the bacterial microbiome. Microbial diversity was assessed using Shannon and Simpson indices, as shown in Table 7. The replicates of the KIP sample exhibited greater diversity compared to KC and KFP samples ($p < 0.05$). Furthermore, the KC sample replicates were more diverse than the KFP sample ($p < 0.05$).

The relative abundances of bacterial phyla and genera are presented in Figures 2 and 3, respectively. At the phyla level, the most abundant phylum (>93%) was *Firmicutes* in all samples ($p < 0.05$), followed by *Proteobacteria* (0.41–3.79%) and *Bacteroidetes* (0.22–2.88%). *Actinobacteria* were present in lower levels (0.13–0.20%), while *Saccharibacteria*, formerly known as *TM7*, were found only in the KC sample (0.02%). *Firmicutes* were more abundant in the KFP and KIP samples compared to KC ($p < 0.05$), and conversely, *Proteobacteria* and *Bacteroidetes* were in lower levels in the KFP and KIP than in the KC sample ($p < 0.05$). Regarding *Actinobacteria*, no significant differences were observed between KC and KIP samples ($p > 0.05$). However, *Actinobacteria* were present in lower percentages in the KFP sample ($p < 0.05$).

At the genera level, *Lactococcus* spp. was the most abundant genus (>78.7%) in all samples ($p < 0.05$). Notably, lower percentages were observed in the KIP sample (78.71%) ($p < 0.05$) compared to KC and KFP (87.49 and 92.77%, respectively). *Pediococcus* genus was present, as expected, in cheese samples fortified with free or immobilized *P. acidilactici* ORE5 with a relative abundance of 0.31 and 10.18% in the KFP and KIP, respectively, while in KC, it was detected in low levels (0.01%). Furthermore, the *Streptococcus* genus was identified in relative abundance, ranging 0.74–3.11%, whereas in KFP and KIP, the levels were higher compared to the KC sample ($p < 0.05$). *Lactobacillus* spp. OTUS was higher

in KIP (0.27%) compared to KC and KFP ($p < 0.05$), while no significant differences were observed between KC and KFP samples (0.10% relative abundance in both samples). The genera *Chryseobacterium*, *Acinetobacter* and *Pseudomonas* were abundant in the KC sample (2.44, 1.57 and 1.29%, respectively) and were present in significantly lower percentages in KFP and KIP ($p < 0.05$). Similarly, *Staphylococcus*, *Enhydrobacter* and *Escherichia* were detected at higher levels in KC ($p < 0.05$) than in the KFP and KIP samples. Other genera (*Allobaculum*, *Alloiococcus*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Enterococcus*, *Facklamia*, *Flavobacterium*, *Gluconacetobacter*, *Helicobacter*, *Janthinobacterium*, *Jeotgalicoccus*, *Macrococcus*, *Mannheimia*, *Methylobacterium*, *Oligella*, *Pedobacter*, *Prevotella*, *Propionibacterium*, *Psychrobacter*, *Salmonella*, *Sphingobacterium* and *Stenotrophomonas*) were identified in low percentages ($\leq$0.08%). Of note, the genera *Facklamia*, *Flavobacterium*, *Salmonella* and *Sphingobacterium* were present in the KC sample, while their presence was not detected in the KFP and KIP samples. Moreover, *Propionibacterium* OTUS were higher in the KFP and KIP (0.06 and 0.08%, respectively) ($p > 0.05$) than in the KC sample (0.03%).

Table 7. Bacterial diversity indices Shannon's and Simpson's after 16S rRNA NGS sequencing in the Katiki Domokou-type cheese samples calculated using the Rhea platform and the a-diversity script.

16S rRNA OTUs	KC	KFP	KIP
Shannon's Index	$0.71 \pm 0.05^{b,c}$	$0.50 \pm 0.03^{a,c}$	$0.89 \pm 0.01^{a,b}$
Simpson's Index	$0.23 \pm 0.02^{b,c}$	0.14 ± 0.05^{a}	0.17 ± 0.02^{a}

KC—control sample, KFP—cheese sample containing free *P. acidilactici* ORE5 cells, KIP—cheese sample with immobilized *P. acidilactici* ORE5 cells on pistachio nuts. All data represent the mean $\pm$ standard deviation (SD) from two independent experiments. Significant differences: a ($p < 0.05$ vs. KC), b ($p < 0.05$ vs. KFP), c ($p < 0.05$ vs. KIP).

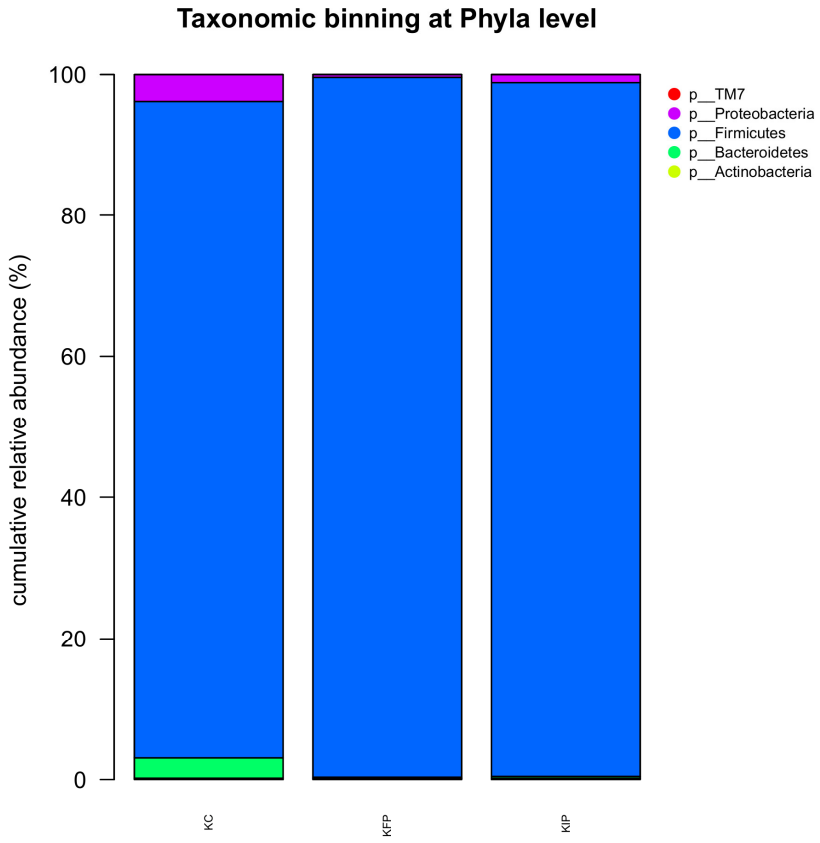

Figure 2. Relative abundances (%) of different bacteria phyla after 16S rRNA NGS sequencing in the Katiki Domokou-type cheese samples. All data represent the mean values from two independent experiments. KC—control samples, KFP—cheese samples containing free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts.

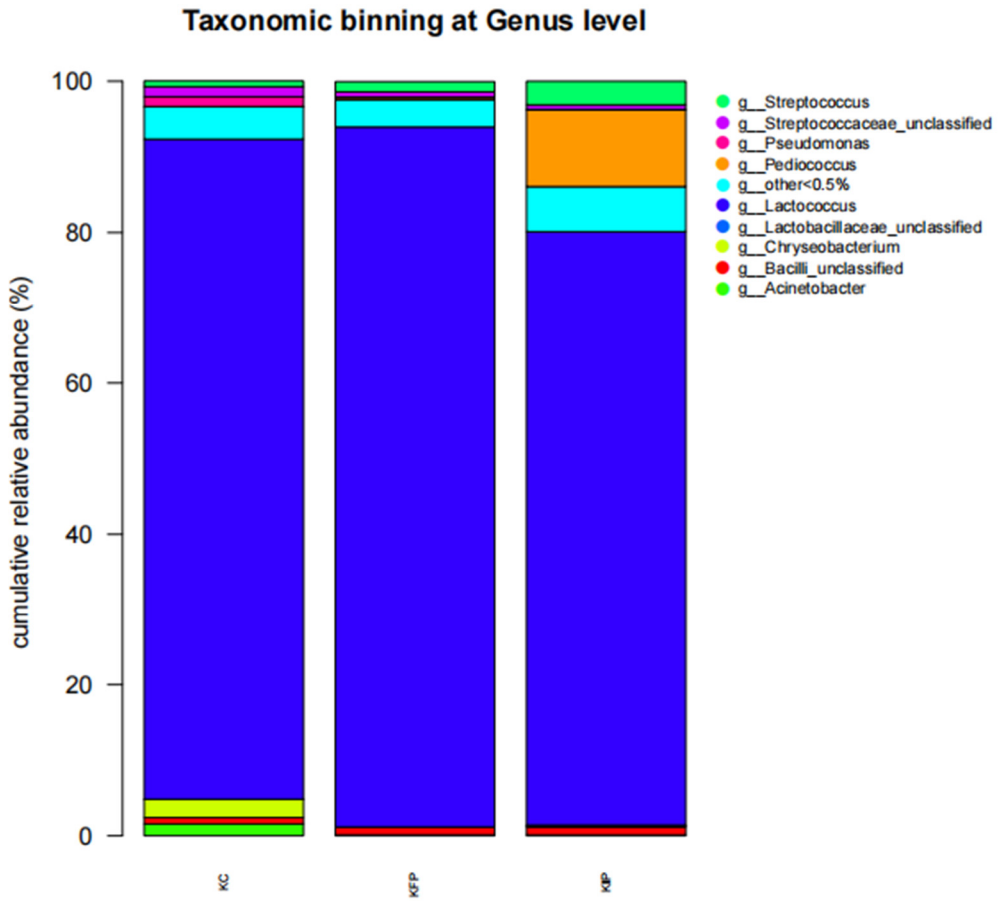

Figure 3. Relative abundances (%) of different bacterial genera, after 16S rRNA NGS sequencing in the Katiki Domokou-type cheese samples. All data represent the mean values from two independent experiments. Genera with percentages <0.5% are represented as "others". KC—control samples, KFP—cheese samples containing free *P. acidilactici* ORE5 cells, KIP—cheese with immobilized *P. acidilactici* ORE5 cells on pistachio nuts.

3.9. Preliminary Sensory Evaluation

The cheese samples fortified with free or immobilized *P. acidilactici* ORE5 cells (KFP and KIP samples, respectively) were compared with cheese samples without *P. acidilactici* ORE5 cells (KC sample) and cheese sample with pistachio nuts (KP sample) regarding their sensory characteristics (Table 3). In general, an improvement in overall quality and taste, as well as an expected difference in appearance, was evident by the addition of pistachio nuts in the Katiki Domokou cheese with or without *P. acidilactici* ORE5 cells. All samples were characterized by a milk-like and yeasty-bread-like aroma, while their texture was mainly curdy and creamy. Furthermore, the KC and KFP samples were evaluated as acid and salted, whereas pistachio nuts in the KP and KIP samples also added a sweet and sour taste.

4. Discussion

Kefir grains are rich in potentially probiotic microorganisms, and it is established that they exhibit noteworthy health-promoting properties [28]. *P. acidilactici* ORE5, isolated from kefir grains, was initially screened for potential probiotic properties, and adequate survival rates at low pH were observed. This is an important finding, as a candidate probiotic strain should persist at pH 3.0 or even lower [18]. Furthermore, it exhibited resistance to pancreatin and bile salts. Pancreatin and bile salts are considered important parameters regarding food digestion. Specifically, pepsin is an enzyme that catalyzes protein digestion, while pancreatin contributes a significant role in lipid metabolism. On the other hand, bile

salts assist in the digestion and absorption of fats. Likewise, all the above are significant requirements for probiotic efficiency [29]. Regarding antibiotic susceptibility, resistance to tetracycline and clindamycin was monitored, which is also in agreement with the study of Kastner et al. [30], while resistance to erythromycin has also been reported by Rojo-Bezares et al. [31]. However, these reports are few in the literature, and they lack information about the resistance character (intrinsic or acquired).

Meanwhile, the demand for functional foods containing probiotic cells is growing as consumer awareness about the positive impact of probiotics on health is increasing. Notably, the incorporation of probiotics in dairy products is already common, as it has been suggested that milk functions as a buffering agent, protecting bacteria during the passage through the GI tract [20]. In addition, cell immobilization on food carriers may improve this protective effect, resulting in high cell viability. Pistachio nuts have been previously evaluated as natural carriers, and the positive impact of the consumption of pistachio nuts fortified with probiotic cells on the lipid profile of diabetic rats was recently reported [3,8]. In this vein, our strategy involved the incorporation of immobilized *P. acidilactici* ORE5 cells on pistachio nuts in the Katiki Domokou cheese and the evaluation of the potential effect of immobilized probiotic cells on the cheese microbiome.

Katiki Domokou is a soft cheese made from goat's and ewe's milk and, owing to its pH (4.3–4.5), may be considered a suitable food carrier for probiotic cells to maintain high cell viability during storage and after consumption. This suggestion has been corroborated by the high cell loads of *P. acidilactici* ORE5 cells achieved during refrigerated storage of cheese, as lactobacilli levels were >8.5 logcfu/g up to 7 days of storage. The incorporation of immobilized probiotic cells resulted in higher cell viability compared to the cheese sample fortified with free cells, indicating the positive impact of cell immobilization on the maintenance of high cell loads. These results are in accordance with previous studies that demonstrated the enhancement of cell survival by cell immobilization [4,32,33]. Indeed, the incorporation of immobilized *L. casei* ATCC 393 on apple pieces and casein in yogurt resulted in cell loads > 7 logcfu/g up to 28 days of storage [32], while Shehata et al. (2022) reported that soft cheese with immobilized *L. paracasei* cells on wheat bran exhibited high cell loads (>7.5 logcfu/g) after 30 days of storage [33]. Of note, a significant increase in lactobacilli numbers was observed up to 3 days of storage, which may be attributed to the ability of lactobacilli to adapt well to the milk environment [32]. Furthermore, in accordance with our results, Terpou et al. [34] reported that yeast numbers were significantly lower in cheese samples with free and immobilized *L. paracasei* cells after the ripening period compared to control cheese, indicating that *L. paracasei* strain provided an antifungal effect on cheese. According to Dimitrellou et al. [35], the incorporation of whey cheese with *L. casei* or *L. bulgaricus* culture resulted in significantly lower cell loads of staphylococci, coliforms and enterobacteria. It has been proposed that lower pH values and high acidity combined with lower moisture content may have developed an unfavorable environment for spoilage bacteria growth [35].

The growth of spoilage bacteria in foods consists of a global issue affecting the food industry with subsequent impact on the economy and health system. Dairy products are usually susceptible to spoilage due to their high moisture content, nutrient-rich composition, sensitivity to temperature fluctuations, as well as the possibility of contamination during production. A wide range of microorganisms has been indicated for spoilage in dairy products, such as *L. monocytogenes*, *S. aureus*, *Salmonella* spp. and *Penicillium* spp. [36,37]. Meanwhile, biopreservation by lactic acid bacteria is gaining a good deal of scientific interest, as they produce metabolites, such as organic acids with antimicrobial activity potential [15,37].

With the intention of eliminating food hazards, the resistance of Katiki Domokou cheese samples fortified with *P. acidilactici* ORE5 culture to deliberate contamination of *L. monocytogenes* was investigated. The growth rate of *L. monocytogenes* was significantly limited in cheese samples with free and immobilized *P. acidilactici* ORE5 culture, indicating its activity against food-borne pathogens. The study of Gonzales-Barron et al. [38]

highlighted the anti-listeria effect of intentionally added lactic acid bacteria in artisanal semi-hard cheese, while Afzali et al. [39] reported that *L. brevis* strains incorporated in yogurt drinks exhibited an antifungal activity. In addition, Belessi et al. [40] noted a correlation between yeast and *Listeria* levels, as increased yeast cell loads may favor the viability of *Listeria* in Feta cheese by affecting physicochemical characteristics of cheese, leading to more favorable pH values for spoilage growth.

Physicochemical characteristics are crucial for the determination of the quality of the cheese, as these parameters affect the sensory evaluation, texture and overall acceptability of the cheese. In this vein, pH values, acidity, water activity and moisture content were evaluated. It has been noted that the samples fortified with *P. acidilactici* ORE5 culture exhibited lower pH values and, subsequently, higher lactic acid concentrations than the control cheese as the storage continued. According to the literature, studies about functional dairy products have reported similar results attributing them to the production of metabolites of probiotic strains [41]. More specifically, post-acidification was observed in yogurts fortified with *L. casei* ATCC 393 cells after 7 days of storage, according to Dimitrellou et al. [32]. In agreement with moisture content levels detected during storage, Mileriene et al. [42] reported lower moisture values in curd cheese fortified with immobilized *L. lactis* cells on raisins, indicating that there is a correlation between pH values and moisture content. More specifically, it has been reported that low pH values (<5.0) in cheese may affect protein-to-protein interactions, resulting in a decreased ability of proteins to interact with water and, subsequently, increased syneresis and lower moisture levels [43].

The sensory evaluation of dairy products significantly determines their quality. Subsequently, the development of a unique profile of aromatic compounds in cheese consists of a challenge for the food industry. According to previous studies, esters, acids, alcohols and carbonyl compounds are considered the most important volatile compounds contributing to flavor development in cheese samples. Nevertheless, cheese flavor is rather a result of a balance between the concentrations of volatile compounds detected in cheese than a contribution of particular components [20]. For the HS-SPME GC/MS analysis, samples at the beginning and after 3 days of storage were tested. The incorporation of free or immobilized cells affected the aroma profile of cheese, in accordance with previous studies on dairy products fortified with lactic acid bacteria [32,33]. Indeed, regarding esters, Dimitrellou et al. [20] noted that cheese samples fortified with free *L. casei* cells exhibited higher amounts of ethyl hexanoate and ethyl decanoate compared to control cheese, as well as to cheese samples with immobilized cells on whey protein. Similarly, Shehata et al. [33] reported the detection of ethyl decanoate in soft whey cheese with free and immobilized *L. paracasei* cells; however, it was undetectable in the control cheese. In total, the esters identified in cheese samples are known to produce a fruity aroma in foods [34,44]. The acids found at increased concentrations in the KFP and KIP samples were sorbic, octanoic and decanoic acid, which were at higher amounts in the KFP samples—results that are in accordance with previous studies in dairy products fortified with probiotics [33,34]. Among alcohols, the most abundant were 3-methyl-1-butanol and 2-phenylethanol in all samples and are described as exerting malt notes and burnt aroma [4]. Carbonyl compounds detected in cheese samples belong to aldehydes and ketones, but aldehydes were in low concentrations due to their reduction to alcohols [35]. 3-methyl-butanal and hexanal detected only in the KFP and KIP samples were similarly identified in cheese samples fortified with free or immobilized lactic acid bacteria, in accordance with previous studies [20]. Of note, 3-methyl-butanal is also present in other cheeses like Camembert and aged Cheddar, adding a fruity aroma [45]. Furthermore, hydrocarbons were also present in cheese, mainly in the KFP and KIP samples. In general, hydrocarbons do not directly affect the aroma profile of cheese. However, they act as precursors to other volatile compounds [46]. Notably, PCA, which was carried out in order to evaluate the effect of *P. acidilactici* ORE5 on the volatile profile of Katiki Domokou cheese, revealed that the incorporation of free and immobilized cells affected the concentrations of volatile compounds, which can be attributed to their fermentation activity, while similar results have been reported in previous studies [20,46].

Dairy products, specifically cheeses, are characterized by a certain microbiome, which differs between different types of cheese, depending on a variety of factors, such as milk source, the starter and adjunct cultures, the cheese-making process, the geographical region and the terroir [36]. Although the plate count method provides a quantitative assessment of the microbial composition of the product, it has some limitations due to its sensitivity, as well as its inability sometimes to distinguish between colonies. Hence, it is often recommended to combine the plate count method with other techniques, such as molecular methods (e.g., DNA-based techniques), to obtain a more comprehensive understanding of the microbial community [47]. In this study, the effect of *P. acidilactici* ORE5 cells incorporated into Katiki Domokou cheese on the cheese microbiome was investigated after 16S rRNA sequencing.

The Shannon index, which measures the diversity of species present in a sample, as well as the Simpson index, which assesses the dominance of species within the sample, were also calculated. Concerning the results, a greater bacterial diversity was observed as a result of the incorporation of immobilized cells in the cheese sample, which has also been noted by Mitropoulou et al. [48]. Of note, increased bacterial diversity can upgrade the nutritional value of fermented foods, given that different microorganisms, utilizing different metabolic pathways, are responsible for the formation of various metabolites, flavors and unique characteristics in the final product [49]. Regarding phyla, *Firmicutes* was the predominant phylum in all cheese samples, while *Bacteroidetes* and *Proteobacteria* were in higher levels in the control cheese compared to the samples with *P. acidilactici* ORE5 culture, in accordance with previous studies [47,48]. At the genera level, *Pediococcus* were detected, as expected, in higher percentages in samples with free or immobilized *P. acidilactici* ORE5 cells compared to the control. However, *Lactococcus* was the most abundant genus in all samples, which may be attributed to the presence of *Lactococcus* in cheese, as mesophilic lactic acid bacteria predominate during ripening and storage of cheese [11]. However, their relative abundance was significantly lower in cheese fortified with immobilized cells. Similarly, Dimitrellou et al. [32] reported a significant decline in the levels of yogurt starter cultures in samples with *L. casei* culture due to competitive activity of the probiotic strain. The fact that *Lactococcus* was the most abundant genus in all samples contrasts the microbiological analysis results that demonstrated higher counts of lactobacilli compared to lactococci. This may be attributed to the ability of some *Lactococcus* species to grow on MRS agar, and, therefore, not be easily distinguished from other cultures present on Petri dishes [50]. Consequently, it is possible some cultures belonging to the *Lactococcus* genus were incorrectly enumerated as lactobacilli.

Among other genera found in cheese, *Chryseobacterium* was detected in the control cheese, while it was found at levels < 0.05% in samples with free and immobilized cells *Chryseobacterium* are considered psychotropic spoilage bacteria and are usually detected in dairy products [51]. Similarly, *Pseudomonas*, which are considered aerobic psychotropic bacteria, were detectable in higher levels in the control cheese in contrast with KFP and KIP samples, where *Pseudomonas* levels were below 0.02%. It has been reported that *Pseudomonas* produce enzymes that may lead to spoilage due to their proteolytic activity [52]. Considering that Katiki Domokou cheese is made from goat's milk, the existence of *Acinetobacter* in the control cheese may be explained, as *Acinetobacter* is usually found in contaminated raw goat's milk [53]. Of note, in cheese samples fortified with free and immobilized cells, *Acinetobacter* was found in significantly lower levels, which may be attributed to the potential antagonistic activity of *P. acidilactici* ORE5 culture. Higher levels of *Staphylococcus* were noted in the control cheese. Although not all *Staphylococcus* are considered pathogens, *S. aureus* is responsible for food poisoning due to production of enterotoxin [54,55]. Moreover, *Enhydrobacter* were detected in 0.17% relative abundance in control cheese, while their levels were 0.02% in the KFP and KIP samples. The existence of *Enhydrobacter* is not commonly linked to cheese spoilage; however, some strains may have the potential to cause spoilage under certain conditions [32]. Relative abundance of *Sphingobacterium*, *Enterococcus*, *Facklamia* and *Salmonella* was recorded only in the control cheese,

and their presence may indicate cheese spoilage. Likewise, *Flavobacterium* was found only in the control cheese (KC), and it is considered part of natural cheese microflora, while some strains are associated with food spoilage [55]. Overall, NGS technology provides useful information about the microbiome composition of complex ecosystems like cheese, overcoming the limitations of the culture-dependent studies [48,56].

5. Conclusions

In this study, the effect of free and immobilized *P. acidilactici* ORE5 cells on pistachio nuts on Katiki Domokou cheese was evaluated. The results demonstrated that Katiki Domokou supplementation with immobilized *P. acidilactici* ORE5 positively affected the cheese microbiome, while high *P. acidilactici* cell loads were maintained, alongside growth suppression of *L. monocytogenes* after deliberate inoculation. Notably, the use of both free and immobilized *P. acidilactici* ORE5 cells improved the aroma profile of cheese, while the incorporation of immobilized *P. acidilactici* ORE5 cells on pistachio nuts increased the biodiversity of cheese, as well as enhanced the sensory characteristics of the product. Thus, the development of functional Katiki Domokou-type cheese fortified with freeze-dried immobilized *P. acidilactici* ORE5 cells on pistachio nuts holds market potential. However, more research is required in order to confirm the health-promoting effects of *P. acidilactici* ORE5 cells in experimental animal models and on humans, as well as to investigate the effectiveness of freeze-dried immobilized *P. acidilactici* ORE5 cells on pistachio nuts incorporated into Katiki Domokou cheese at an industrial scale.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13148047/s1, Figure S1: Katiki Domokou-type cheese samples. (A) Katiki Domokou-type cheese sample without *P. acidilactici* ORE5 cells—control (KC), (B) Katiki Domokou-type cheese sample fortified with free *P. acidilactici* ORE5 cells (KFP), (C) Katiki Domokou-type cheese sample fortified with immobilized *P. acidilactici* ORE5 cells on pistachio nuts (KIP), (D) Freeze-dried immobilized *P. acidilactici* ORE5 cells on pistachio nuts.

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References

1. Food and Agricultural Organization of the United Nations; World Health Organization. *Probiotics in Food: Health and Nutritional Properties and Guidelines for Evaluation*; FAO Food Nutrition Paper 85; WHO: Geneva, Switzerland; FAO: Rome, Italy, 2006. Available online: https://www.fao.org/3/a0512e/a0512e.pdf (accessed on 29 January 2023).
2. Kvakova, M.; Bertkova, I.; Stofilova, J.; Savidge, T.C. Co-Encapsulated Synbiotics and Immobilized Probiotics in Human Health and Gut Microbiota Modulation. *Foods* **2021**, *10*, 1297. [CrossRef] [PubMed]
3. Prapa, I.; Nikolaou, A.; Panas, P.; Tassou, C.; Kourkoutas, Y. Developing Stable Freeze-Dried Functional Ingredients Containing Wild-Type Presumptive Probiotic Strains for Food Systems. *Appl. Sci.* **2023**, *13*, 630. [CrossRef]
4. Nikolaou, A.; Mitropoulou, G.; Nelios, G.; Kourkoutas, Y. Novel Functional Grape Juices Fortified with Free or Immobilized *Lacticaseibacillus rhamnosus* OLXAL-1. *Microorganisms* **2023**, *11*, 646. [CrossRef] [PubMed]
5. Oyinloye, T.M.; Yoon, W.B. Effect of Freeze-Drying on Quality and Grinding Process of Food Produce: A Review. *Processes* **2020**, *8*, 354. [CrossRef]
6. Deepika, G.; Charalampopoulos, D. Surface and Adhesion Properties of Lactobacilli. *Adv. Appl. Microbiol.* **2010**, *70*, 127–152.
7. Yanni, A.E.; Mitropoulou, G.; Prapa, I.; Agrogiannis, G.; Kostomitsopoulos, N.; Bezirtzoglou, E.; Kourkoutas, Y.; Karathanos, V.T. Functional modulation of gut microbiota in diabetic rats following dietary intervention with pistachio nuts (*Pistacia vera* L.). *Metabol. Open.* **2020**, *7*, 100040. [CrossRef]
8. Prapa, I.; Yanni, A.E.; Nikolaou, A.; Kostomitsopoulos, N.; Kalogeropoulos, N.; Bezirtzoglou, E.; Karathanos, V.T.; Kourkoutas, Y. Dietary Pistachio (*Pistacia vera* L.) Beneficially Alters Fatty Acid Profiles in Streptozotocin-Induced Diabetic Rat. *Appl. Sci.* **2022**, *12*, 4606. [CrossRef]
9. Karimi, R.; Mortazavian, A.M.; da Cruz, A.G. Viability of probiotic microorganisms in cheese during production and storage: A review. *Dairy Sci. Technol.* **2011**, *91*, 283–308. [CrossRef]
10. Kagkli, D.M.; Iliopoulos, V.; Stergiou, V.; Lazaridou, A.; Nychas, G.J. Differential *Listeria monocytogenes* strain survival and growth in Katiki, a traditional Greek soft cheese, at different storage temperatures. *Appl. Environ. Microbiol.* **2009**, *75*, 3621–3626. [CrossRef]
11. Pappa, E.C.; Kondyli, E. Descriptive Characteristics and Cheesemaking Technology of Greek Cheeses Not Listed in the EU Geographical Indications Registers. *Dairy* **2023**, *4*, 3. [CrossRef]
12. Blaya, J.; Barzideh, Z.; LaPointe, G. Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment. *J. Dairy Sci.* **2018**, *101*, 3611–3629. [CrossRef] [PubMed]
13. Dimitrellou, D.; Kourkoutas, Y.; Koutinas, A.A.; Kanellaki, M. Thermally- dried immobilized kefir on casein as starter culture in dried whey cheese production. *Food Microbiol.* **2009**, *26*, 809–820. [CrossRef]
14. Araújo, V.S.; Pagliares, V.A.; Queiroz, M.L.P.; Freitas-Almeida, A.C. Occurrence *Staphylococcus* and enteropathogens in soft cheese commercialized in the city of Rio de Janeiro, Brazil. *J. Appl. Microbiol.* **2002**, *92*, 1172–1177. [CrossRef]
15. Arena, M.P.; Silvain, A.; Normanno, G.; Grieco, F.; Drider, D.; Spano, G.; Fiocco, D. Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Front. Microbiol.* **2016**, *7*, 464. [CrossRef]
16. Sherwani, K.F.; Bukhari, D.A.A. Probiotics in processed dairy products and their role in gut microbiota health. Effect of Microbiota on Health and Disease. *IntechOpen* **2022**, *2022*, 104482. [CrossRef]
17. Li, C.; Wang, S.; Chen, S.; Wang, X.; Deng, X.; Liu, G.; Chang, W.; Beckers, Y.; Cai, H. Screening and Characterization of *Pediococcus acidilactici* LC-9-1 toward Selection as a Potential Probiotic for Poultry with Antibacterial and Antioxidative Properties. *Antioxidants* **2023**, *12*, 215. [CrossRef] [PubMed]
18. Argyri, A.A.; Zoumpopoulou, G.; Karatzas, K.A.; Tsakalidou, E.; Nychas, G.J.; Panagou, E.Z.; Tassou, C.C. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol.* **2013**, *33*, 282–291. [CrossRef] [PubMed]
19. Plessas, S.; Nouska, C.; Karapetsas, A.; Kazakos, S.; Alexopoulos, A.; Mantzourani, I.; Chondrou, P.; Fournomiti, M.; Galanis, A.; Bezirtzoglou, E. Isolation, characterization and evaluation of the probiotic potential of a novel Lactobacillus strain isolated from Feta-type cheese. *Food Chem.* **2017**, *226*, 102–108. [CrossRef]
20. Dimitrellou, D.; Kandylis, P.; Sidira, M.; Koutinas, A.A.; Kourkoutas, Y. Free and immobilized *Lactobacillus casei* ATCC 393 on whey protein as starter cultures for probiotic Feta-type cheese production. *J. Dairy Sci.* **2014**, *97*, 4675–4685. [CrossRef]
21. *ISO 5534:2004*; Cheese and Processed Cheese. Determination of the Total Solids Content (Reference Method). International Organization for Standardization: Geneva, Switzerland, 2004.
22. Dan, T.; Wang, D.; Jin, R.L.; Zhang, H.P.; Zhou, T.T.; Sun, T.S. Characterization of volatile compounds in fermented milk using solid-phase microextraction methods coupled with gas chromatography-mass spectrometry. *J. Dairy Sci.* **2017**, *100*, 2488–2500. [CrossRef]
23. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J.; et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef]
24. Lagkouvardos, I.; Fischer, S.; Kumar, N.; Clavel, T. Rhea: A transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. *PeerJ* **2017**, *5*, 2836. [CrossRef]
25. Nikolaou, A.; Kourkoutas, Y. High-Temperature Semi-Dry and Sweet Low Alcohol Wine-Making Using Immobilized Kefir Culture. *Fermentation* **2021**, *7*, 45. [CrossRef]

26. EFSA Panel on Additives and Products or Substances Used in Animal Feed (FEEDAP). Guidance on the Assessment of Bacterial Susceptibility to Antimicrobials of Human and Veterinary Importance. *EFSA J.* **2012**, *10*, 2740.
27. Litopoulou-Tzanetaki, E.; Tzanetakis, N. The Microfloras of Traditional Greek Cheeses. *Microbiol. Spectr.* **2014**, *2*, CM-0009-2012. [CrossRef]
28. Bengoa, A.A.; Iraporda, C.; Garrote, G.L.; Abraham, A.G. Kefir micro-organisms: Their role in grain assembly and health properties of fermented milk. *J. Appl. Microbiol.* **2019**, *126*, 686–700. [CrossRef] [PubMed]
29. Danielsen, M.; Wind, A. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int. J. Food Microbiol.* **2003**, *82*, 1–11. [CrossRef]
30. Kastner, S.; Perreten, V.; Bleuler, H.; Hugenschmidt, G.; Lacroix, C.; Meile, L. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. *Syst. Appl. Microbiol.* **2006**, *29*, 145–155.
31. Rojo-Bezares, B.; Sáenz, Y.; Poeta, P.; Zarazaga, M.; Ruiz-Larrea, F.; Torres, C. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. *Int. J. Food Microbiol.* **2006**, *111*, 234–240. [CrossRef]
32. Dimitrellou, D.; Kandylis, P.; Kourkoutas, Y. Assessment of freeze-dried immobilized *Lactobacillus casei* as probiotic adjunct culture in yogurts. *Foods* **2019**, *8*, 374. [CrossRef]
33. Shehata, M.G.; El-Aziz, N.M.A.; Darwish, A.G.; El-Sohaimy, S.A. *Lacticaseibacillus paracasei* KC39 immobilized on prebiotic wheat bran to manufacture functional soft white cheese. *Fermentation* **2022**, *8*, 496. [CrossRef]
34. Terpou, A.; Bosnea, L.; Kanellaki, M.; Plessas, S.; Bekatorou, A.; Bezirtzoglou, E.; Koutinas, A.A. Growth capacity of a novel potential probiotic *Lactobacillus paracasei* K5 strain incorporated in industrial white brined cheese as an adjunct culture. *J. Food Sci.* **2018**, *83*, 723–731. [CrossRef] [PubMed]
35. Dimitrellou, D.; Kandylis, P.; Kourkoutas, Y.; Kanellaki, M. Novel probiotic whey cheese with immobilized lactobacilli on casein. *LWT* **2017**, *86*, 627–634. [CrossRef]
36. Choi, J.; Lee, S.I.; Rackerby, B.; Goddik, L.; Frojen, R.; Ha, S.D.; Kim, J.H.; Park, S.H. Microbial Communities of a Variety of Cheeses and Comparison between Core and Rnd Region of Cheeses. *J. Dairy Sci.* **2020**, *103*, 4026–4042. [CrossRef] [PubMed]
37. Shi, C.; Maktabdar, M. Lactic acid bacteria as biopreservation against spoilage molds in dairy products—A review. *Front. Microbiol.* **2022**, *12*, 4283. [CrossRef] [PubMed]
38. Gonzales-Barron, U.; Campagnollo, F.B.; Schaffner, D.W.; Sant'Ana, A.S.; Cadavez, V.A.P. Behavior of *Listeria monocytogenes* in the presence or not of intentionally added lactic acid bacteria during ripening of artisanal Minas semi-hard cheese. *Food Microbiol.* **2020**, *91*, 103545. [CrossRef]
39. Afzali, S.; Edalatian Dovom, M.R.; Habibi Najafi, M.B.; Mazaheri Tehrani, M. Determination of the anti-yeast activity of *Lactobacillus* spp. isolated from traditional Iranian cheeses in vitro and in yogurt drink (Doogh). *Sci. Rep.* **2020**, *10*, 6291. [CrossRef]
40. Belessi, C.I.A.; Papanikolaou, S.; Drosinos, E.H.; Skandamis, P.N. Survival and acid resistance of Listeria innocua in feta cheese and yogurt, in the presence or absence of fungi. *J. Food Prot.* **2008**, *71*, 742–749. [CrossRef]
41. Hong, Q.; Wang, G.; Zhang, Q.; Tian, F.; Xiao-Ming, L.; Zhao, J.; Chen, Y.-Q.; Zhang, H.; Chen, W. Enhancement of ester formation in Camembert cheese by addition of ethanol. *Int. J. Dairy Technol.* **2017**, *70*, 220–227. [CrossRef]
42. Mileriene, J.; Serniene, L.; Kondrotiene, K.; Santarmaki, V.; Kourkoutas, Y.; Vasiliauskaite, A.; Lauciene, L.; Malakauskas, M. Indigenous *Lactococcus lactis* with Probiotic Properties: Evaluation of Wet, Thermally- and Freeze-Dried Raisins as Supports for Cell Immobilization, Viability and Aromatic Profile in Fresh Curd Cheese. *Foods* **2022**, *11*, 1311. [CrossRef]
43. Pastorino, A.J.; Hansen, C.L.; McMahon, D.J. Effect of pH on the Chemical Composition and Structure-Function Relationships of Cheddar Cheese. *J. Dairy Sci.* **2003**, *86*, 2751–2760. [CrossRef] [PubMed]
44. Hayaloglu, A.A. Comparisons of different single-strain starter cultures for their effects on ripening and grading of Beyaz cheese. *Int. J. Food Sci. Technol.* **2007**, *42*, 930–938. [CrossRef]
45. Curioni, P.M.G.; Bosset, J.O. Key odorants in various cheese types as determined by gas chromatography-olfactometry. *Int. Dairy J.* **2002**, *12*, 959–984. [CrossRef]
46. Bezerra, T.K.A.; de Oliveira Arcanjo, N.M.; de Araújo, A.R.R.; de Queiroz, A.L.M.; de Oliveira, M.E.G.; Gomes, A.M.P.; Madruga, M.S. Volatile profile in goat coalho cheese supplemented with probiotic lactic acid bacteria. *LWT-Food Sci. Technol.* **2017**, *76*, 209–215. [CrossRef]
47. Quigley, L.; O'Sullivan, O.; Beresford, T.P.; Ros, R.P.; Fitzgerald, G.F.; Cotter, P.D. High-throughput sequencing for detection of subpopulations of bacteria not previously associated with artisanal cheeses. *Appl. Environ. Microbiol.* **2012**, *78*, 5717–5723. [CrossRef]
48. Mitropoulou, G.; Prapa, I.; Nikolaou, A.; Tegopoulos, K.; Tsirka, T.; Chorianopoulos, N.; Kourkoutas, Y. Effect of Free or Immobilized *Lactiplantibacillus plantarum* T571 on Feta-Type Cheese Microbiome. *Front. Biosci.-Elite* **2022**, *14*, 31. [CrossRef]
49. Spyrelli, E.D.; Stamatiou, A.; Tassou, C.C.; Nychas, G.J.E.; Doulgeraki, A.I. Microbiological and metagenomic analysis to assess the effect of container material on the microbiota of Feta cheese during ripening. *Fermentation* **2020**, *6*, 12. [CrossRef]
50. Itoi, S.; Yuasa, K.; Washio, S.; Abe, T.; Ikuno, E.; Sugita, H. Phenotypic variation in *Lactococcus lactis* subsp. *lactis* isolates derived from intestinal tracts of marine and freshwater fish. *J. Appl. Microbiol.* **2009**, *107*, 867–874. [CrossRef] [PubMed]
51. Papadakis, P.; Konteles, S.; Batrinou, A.; Ouzounis, S.; Tsironi, T.; Halvatsiotis, P.; Tsakali, E.; Van Impe, J.F.M.; Vougiouklaki, D.; Strati, I.F.; et al. Characterization of Bacterial Microbiota of P.D.O. Feta Cheese by 16S Metagenomic Analysis. *Microorganisms* **2021**, *9*, 2377. [CrossRef]

52. Meng, Z.; Zhang, L.; Xin, L.; Lin, K.; Yi, H.; Han, X. Technological characterization of *Lactobacillus* in semihard artisanal goat cheeses from different Mediterranean areas for potential use as nonstarter lactic acid bacteria. *J. Dairy Sci.* **2018**, *101*, 2887–2896. [CrossRef]
53. Kamilari, E.; Anagnostopoulos, D.A.; Papademas, P.; Kamilaris, A.; Tsaltas, D. Characterizing Halloumi cheese's bacterial communities through metagenomic analysis. *LWT* **2020**, *126*, 109298. [CrossRef]
54. Kourkoutas, Y.; Bosnea, L.; Taboukos, S.; Baras, C.; Lambrou, D.; Kanellaki, M. Probiotic cheese production using Lactobacillus casei cells immobilized on fruit pieces. *J. Dairy Sci.* **2006**, *89*, 1439–1451. [CrossRef] [PubMed]
55. Crippa, C.; Pasquali, F.; Lucchi, A.; Gambi, L.; De Cesare, A. Investigation on the microbiological hazards in an artisanal soft cheese produced in northern Italy and its production environment in different seasonal periods. *Ital. J. Food Saf.* **2022**, *11*, 9983. [CrossRef] [PubMed]
56. Sarkar, S.; Shankar, P.S.; Kriti, G. Metabolomics of Ethnic Fermented Foods and Beverages: Understanding New Aspects through Omic Techniques. *Front. Sustain. Food Syst.* **2022**, *7*, 1040567. [CrossRef]