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Original Research

Effect of Free or Immobilized *Lactiplantibacillus plantarum* T571 on Feta-Type Cheese Microbiome

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Abstract

Background: Cheese microbiome plays a key role in determining the organoleptic and physico-chemical properties and may be also used as an authenticity tool for distinguishing probiotic cultures. Due to significant reduction of cell viability often witnessed during food production processes and storage, immobilization is proposed to ascertain high probiotic cell loads required to confer the potential health benefits. Hence, the aim of the present study was to investigate the effect of free or immobilized *Lactiplantibacillus plantarum* T571 on whey protein on feta cheese microbiome. **Methods**: Next-Generation Sequencing technology was used to investigate cheese microbiome. Cheese samples containing free or immobilized *Lactiplantibacillus plantarum* T571 (a wild type strain isolated from Feta cheese brine) on whey protein, along with products containing commercial starter culture, were analyzed. **Results**: The results showed a great diversity of bacteria and fungi genera among the samples. An increased presence of *Lactobacillus* OTUs in cheese with immobilized cells on whey protein was witnessed, highlighting the survival of the strain in the final product. The immobilized culture had also a significant impact on other genera, such as *Lactococcus*, *Leuconostoc* and *Debaryomyces*, which are associated with improved technological characteristics and health benefits. **Conclusions**: Enrichment of feta cheese with immobilized potential probiotics to secure cell viability consists of an industrial challenge and leads to distinct microbiome composition that may be used as a valuable food authenticity tool.

Keywords: probiotics; microbiome; immobilization; next-generation sequencing; feta cheese; whey protein; food authenticity

1. Introduction

Nowadays, a growing interest in developing novel foods enriched with beneficial microorganisms and proteins that promote human health is witnessed [1–3]. According to the latest definition of FAO/WHO, probiotics are defined as "viable microorganisms (bacteria or yeasts) which, when administered in adequate amounts, confer a health benefit on the host" [4]. Probiotic foods are products that contain viable probiotic microorganisms in a suitable matrix and in adequate concentration [5]. To induce the health benefits, probiotic products need to contain a sufficient number of live cells, able to survive the transit through the gastrointestinal (GI) tract and subsequently proliferate and colonize in the gut [6,7]. The food industry has adopted the recommended levels of 10^7 cfu probiotic cells/g of food product at the time of consumption, according to International Probiotics Association (IPA) Europe recommendations. Hence, a daily intake of at least 10^9 viable cells, which could be achieved with a daily consumption of at least 100 g of probiotic food, has been suggested as the minimum intake to provide a health effect, although it is advisable that the minimum dose should be determined for each strain separately.

Incorporating probiotics into foods and ensuring high cell loads constitutes a real bottleneck for the food industry, not only because of cell interactions with food constituents, but also because of the severe conditions often employed during food processing and storage, as well as during the GI transit until they reach the desired site in the body. Such contingencies often lead to important losses in cell viability [7]. To overcome this deficiency, cell immobilization has been proposed to assure the active and functional form of probiotic cells and cell survival enhancement [8,9]. In this vein, immobilized probiotics on milk and whey proteins have been successfully tested as adjunct cultures in cheeses [10,11], as they are efficient natural vehicles for probiotic cells in food products, due to their structural and physicochemical properties [12,13].

However, knowledge on the effect of the probiotic cultures on the food microbiome is missing. The impact of probiotics colonizing a food matrix depends not only on their ability to graft in the microbiota, but on sharing genes and metabolites, supporting challenged microbiota.

Until present, culture-based microbial methodologies were employed to explore the dynamics of a microbial com-

munity in foods, with critical limitations on detecting no-cultivable or no-abundant microorganisms during cheese ripening. Recently developed advanced methodologies, such as next-generation sequencing (NGS), are extremely useful because of the enhanced sequencing depth that can be achieved compared to previous technologies. To date, NGS methods have been applied mostly to describe the human microbiome, but they have also been used to describe a vast array of environmental and agricultural ecologies [14,15]. This technology has also been applied for the investigation of cheese bacterial diversity [16,17].

Greek Feta cheeses are white-brined products and their microbiota differ through time and depend on the environmental, processing and ripening conditions. Feta cheese microbiota are originating from starter or adjunct cultures, the milk microbial cultures of indigenous sheep and goat breeds if no pasteurized milk is used, and the secondary microflora. The starter lactic acid bacteria (SLAB) are inoculated cultures, involved in acid production during manufacture and contribute to the complexity of the ripening process. Secondary microorganisms are not responsible for acid production during manufacture, but generally play an important role during ripening, as these bacteria contribute to the lipolysis and proteolysis [18]. The secondary microbial flora consists of non-starter lactic acid bacteria (NSLAB), which grow internally and are often unique to specific cheese varieties. Specifically, the microbial ecology of cheese is based on the complex interaction among SLAB and NSLAB.

Hence, the aim of the present study was to investigate the effect of immobilized *Lactiplantibacillus plantarum* T571 (formerly known as *Lactobacillus plantarum* T571) [19] on whey protein on Greek feta cheese microbiome. *L. plantarum* T571 properties have been previously studied [20] and the immobilized culture has been tested as adjunct culture in pilot-scale feta production [21].

2. Materials and Methods

2.1 Lactiplantibacillus plantarum T571 Culture

Lactiplantibacillus plantarum T571 was isolated from Feta cheese brine, as described before [20]. The strain was retrieved from a –80 °C stock culture in 10 mL of MRS broth (LABM, Lancashire, UK) and incubated overnight at 30 °C. A subculture was made in 10 mL MRS broth and incubated at 30 °C for 24 hours. For the manufacturing of probiotic Feta cheese, the cells were extracted by centrifugation at 10,000 g for 5 minutes, washed twice with 1/4 strength Ringer's solution (LABM, Lancashire, UK), and resuspended in Ringer's solution to provide a final population of 10^7 cfu/g in the milk [21].

2.2 Feta Cheese Production

Feta cheese samples were prepared in a dairy industry in Greece (Ecofarma Peloponnese Dairy Industry). In brief, pasteurized goat and ewe's milk was inoculated with either the commercial Feta starter culture containing *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* (FTC, control trial), or the aforementioned starter culture and *L. plantarum* T571 immobilized on whey protein (FTI) [10], or the aforementioned starter culture and *L. plantarum* T571 free cells (FTF) [21]. The inoculum of the cultures added to pasteurized milk was estimated at 7 log cfu/mL. The curd was sliced and poured into molds. Dry salt was also added, and the molds were turned over at regular intervals for 24 hours. The cheese was placed in 15 kg metal jars with brine (7% w/v NaCl) the next day, and ripening took place in two stages: (a) 12 days at 18 °C (1st ripening), and (b) 76 days at 4 °C (2nd ripening). Then, the cheese was sliced into 400 g pieces and stored in plastic containers with fresh brine (7% w/v NaCl) [21]. Samples were taken at the end of ripening and subjected to NGS analysis.

2.3 DNA Extraction, PCR Amplification and 16S rRNA Sequencing

Total DNA was extracted using the NucleoSpin® Food (MACHEREY-NAGEL GmbH & Co. KG, Germany), following the manufacturer's instructions. NGS was performed using MiSeq sequencing by MR DNA (http://www.mrdnalab.com, Shallowater, TX, USA). The V1-V3 region of the bacterial 16S rRNA gene was amplified from cheese genomic DNA with 27F/519R primers (AGRGTTTGATCMTGGCTCAG/GTNTTACNGCGGCKGCTG) and the highly variable Internal Transcribed Spacer (ITS) regions of fungi ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and situated between the Small Subunit-coding sequence (SSU) and the Large Subunit-coding sequence (LSU) of the ribosomal operon was amplified with the primers ITS1/ITS4 (CTTGGTCATTTAGAGGAAGTAA/TCCTCCGCTTATTGATATGC). Polymerase Chain Reaction (PCR) amplification was performed using the HotStarTaq Plus Master Mix Kit (Qiagen, Germatown, Maryland, USA), consisting of 30 cycles with the following steps: 94 °C for 3 minutes, 30 cycles of 94 °C for 30 seconds, 53 °C for 40 seconds, 72 °C for 1 minute, and the final elongation step at 72 °C for 5 minutes. PCR products were then subjected to electrophoresis in 2% agarose gel to confirm the amplification and to determine the relative intensity of bands. Then, the amplicons were purified using Ampure XP beads (Beckman Coulter, Brea, California, USA). Samples were subsequently prepared for the illumina DNA library using MiSeq sequencing, following the manufacturer's guidelines. Procession of the sequencing data was held using a proprietary analysis pipeline by MR DNA. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity) and the final OTUs were taxonomically classified using BLASTn against a curated database derived from RDPII and NCBI

(http://www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) and compiled into each taxonomic level into both "counts" and "percentage" files. The analysis identified 3332 bacterial and 421 fungal OTUs and low-abundance (<0.01), rare OTUs were removed. The analysis of raw data in OTUs level and the calculation of a- and b-diversity were performed using Rhea platform [22]. Heatmap construction was carried out using the heatmap.2 function from the gplots package [23].

2.4 Statistical Analysis

All experiments were carried out in duplicate. Significance was established at $p < 0.05$. Results were analyzed for statistical significance with analysis of variance (ANOVA). Bonferroni's multiple range test was used to determine significant differences among results [coefficients, ANOVA tables and significance ($p < 0.05$) were computed using Statistica v.12.0 (TIBCO Software Inc., Palo Alto, CA, USA)].

Principal Component Analysis (PCA) was performed using the FactoMineR (https://doi.org/10.18637/jss.v025.i01) and factoextra (https://CRAN.R-project.org/package=factoextra) R packages, while PCA plots were constructed using tools from the corrplot (https://github.com/taiyun/corrplot) R package.

3. Results

3.1 Effect of Immobilized L. plantarum T571 Culture on Bacterial Microbiome

Shannon and Simpson indices were calculated to measure bacterial diversity (Table 1). FTI replicates were more diverse compared to the replicates in FTF and FTC ($p < 0.05$). On the other hand, FTF and FTC cheese samples had similar levels of the diversity markers ($p > 0.05$ between FTF and FTC). These findings are also supported by PCA plot analysis (Fig. 1).

Table 1. Bacterial diversity indices Shannon's and Simpson's after 16S rRNA NGS sequencing in feta cheese samples, calculated using Rhea platform and a-diversity script.

16S rRNA OTUs	FTI	FTF	FTC
Shannon's Index	2.10 ± 0.10 a,b	1.65 ± 0.05	1.64 ± 0.02
Simpson's Index	0.28 ± 0.03 a,b	0.41 ± 0.01	0.47 ± 0.01

FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control. All data represent the mean ± standard deviation (SD) from two independent experiments. Statistical analysis performed by ANOVA/Bonferroni/Statistica v.12.0 Software. Significant differences: a ($p < 0.05$ *vs* FTF), b ($p < 0.05$ *vs* FTC).

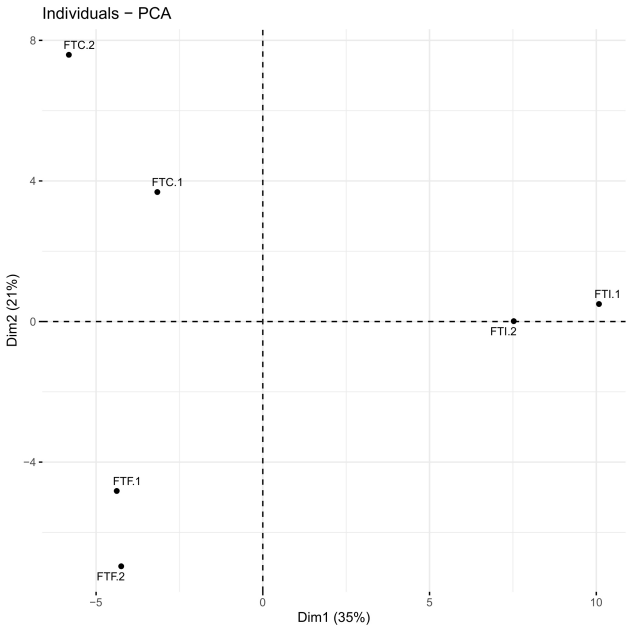

Fig. 1. Principal Coordinate Analysis (PCA) constructed using FactoMineR, factoextra and corrplot R packages. Figure is showing clustering of the feta cheese samples based on bacterial diversity (duplicates are included) in genus level. FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control.

At phyla level, *Firmicutes* was the most abundant (90–95% presence in all samples) phylum in all cases ($p < 0.05$), followed by *Bacteroidetes* and *Proteobacteria* and in lower levels by *Actinobacteria*, *Gemmatimonadetes*, *Tenericutes* and *Verrucomicrobia* (Table 2). Proteobacteria were more abundant in FTF compared to FTI and FTC samples ($p < 0.05$). No other significant differences ($p > 0.05$) among the different feta cheese samples were observed in this level.

At genera level, *Streptococcus* was the most abundant genus in all samples, with a relative abundance ranging 51–69%, and it was decreased in FTI group compared to FTF and FTC samples ($p < 0.05$) (Fig. 2). Other abundant genera were *Lactobacillus* (12–18%), *Lactococcus* (8–13%) and *Leuconostoc* (0.4–11%). *Lactobacillus* genus ranged in similar percentages ($p > 0.05$) in FTI and FTF groups, while its presence was decreased in FTC group ($p < 0.05$ *vs* FTI and FTF). *Lactococcus* spp. OTUs were higher in FTI samples ($p > 0.05$ *vs* FTF), as well as in FTC compared to FTF group ($p < 0.05$), but in similar levels between FTI and FTC ($p > 0.05$). *Leuconostoc* genus OTUs ranged in low levels (<0.4%) in both FTF and FTC groups ($p > 0.05$ between FTF and FTC), whereas in FTI group they were significantly increased ($p < 0.05$ between FTI *vs* FTC, and FTI *vs* FTF). Other genera (*Acetonema*, *Acinetobacter*, *Actinobacillus*, *Actinocorallia*, *Aeromonas*, *Agrococcus*, *Amycolatopsis*, *Bacteroides*, *Brevundimonas*, *Brochothrix*, *Carnobacterium*, *Chitinophaga*, *Christensenella*,

Table 2. Relative abundances (%) of different bacterial phyla, after 16S rRNA NGS sequencing in feta type cheese samples.

Bacterial phyla	FTI	FTF	FTC
Actinobacteria	0.18 ± 0.01	0.18 ± 0.03	0.22 ± 0.03
Bacteroidetes	2.26 ± 0.57	2.00 ± 0.31	2.60 ±0.13
Firmicutes	94.55 ± 1.27	90.18 ± 0.79	93.72 ± 0.48
Gemmatimonadetes	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.01
Proteobacteria	2.78 ± 0.65	7.40 ± 1.22 a,b	3.11 ± 0.33
Tenericutes	0.21 ± 0.06	0.20 ± 0.09	0.31 ± 0.01
Verrucomicrobia	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01

FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control. All data represent the mean ± standard deviation (SD) from at two independent experiments. Statistical analysis was performed by ANOVA/Bonferroni/Statistica v.12.0 Software. Significant differences: a ($p < 0.05$ *vs* FTI), b ($p < 0.05$ *vs* FTC).

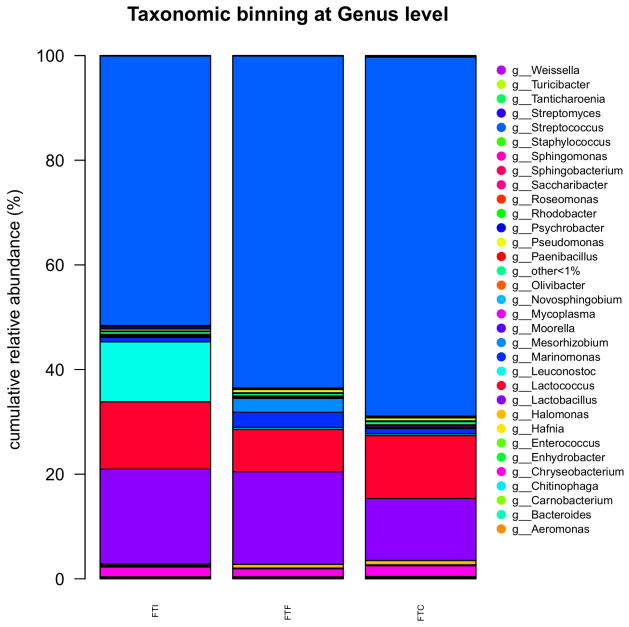

Fig. 2. Relative abundances (%) of different bacterial genera, after 16S rRNA NGS sequencing in feta cheese samples. All data represent the mean values from two independent experiments. FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control.

Chryseobacterium, *Clostridium*, *Dokdonella*, *Dyadobacter*, *Enhydrobacter*, *Enterococcus*, *Gemmatimonas*, *Gordonia*, *Hafnia*, *Halomonas*, *Hirschia*, *Macrococcus*, *Marinomonas*, *Mesorhizobium*, *Microbacterium*, *Moorella*, *Mycoplasma*, *Nocardiopsis*, *Novosphingobium*, *Olivibacter*, *Paenibacillus*, *Parapedobacter*, *Parastreptomyces*, *Pedobacter*, *Phenylobacterium*, *Propionibacterium*, *Pseudomonas*, *Psychrobacter*, *Rhodobacter*, *Rhodococcus*, *Roseomonas*, *Saccharibacter*, *Sedimentibacter*, *Serratia*, *Sphingobacterium*, *Sphingomonas*, *Staphylococcus*, *Streptomyces*, *Tanticharoenia*, *Turicibacter*, *Verrucomicrobium*, *Weissella*, *Xanthomonas* and *Yersinia*) identified ranged in low percentages (<4%). *Carnobacterium* ($p < 0.05$ *vs* FTF), as well as *Psychrobacter* and *Hafnia* genera ($p < 0.05$ *vs* FTF and FTC) were more abundant in FTI samples, while *Weissella* genus OTUs were higher ($p < 0.05$) in the control sample (FTC) compared to FTI and FTF cheeses. *Aeromonas* was increased in FTF compared to FTI and FTC ($p < 0.05$) and *Rhodobacter* was decreased in FTC group ($p < 0.05$ *vs* FTI and FTF). The heatmap depicting the representation of the bacterial OTUs at genus level throughout the various samples is demonstrated at Fig. 3.

Table 3. Fungal diversity indices Shannon's and Simpson's after ITS NGS sequencing in feta type cheese samples, calculated using Rhea platform and a-diversity script.

ITS OTUs	FTI	FTF	FTC
Shannon's Index	2.12 ± 0.08	1.71 ± 0.06	1.44 ± 0.31
Simpson's Index	0.17 ± 0.02	0.32 ± 0.01	0.48 ± 0.12

FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control. All data represent the mean ± standard deviation (SD) from at two independent experiments. Statistical analysis was performed by ANOVA/Bonferroni/Statistica v.12.0 Software. No significant differences were observed.

Table 4. Relative abundances (%) of different phyla of fungi, after ITS NGS sequencing in feta cheese samples.

Fungal phyla	FTI	FTF	FTC
Ascomycota	97.43 ± 2.35	90.86 ± 0.90	98.16 ± 0.90
Basidiomycota	2.54 ± 2.34	9.10 ± 0.91	1.81 ± 0.90
Chytridiomycota	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.01

FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control. All data represent the mean ± standard deviation (SD) from at two independent experiments. Statistical analysis was performed by ANOVA/Bonferroni/Statistica v.12.0 Software. No significant differences were observed.

3.2 Effect of Immobilized L. plantarum T571 Culture on Fungal Microbiome

FTI samples had a tendency for elevated diversity as shown in Table 2. However, Shannon and Simpson indices were at similar levels between all samples ($p > 0.05$) (Table 3). PCA analysis of the samples is presented in Fig. 4.

Ascomycota was the most abundant ($p < 0.05$) phylum in all samples (91–98%), followed by *Basidiomycota* (2–9%) and in lower levels by *Chytridiomycota* (<0.04%) (Table 4). No significant differences were observed between the cheese samples ($p > 0.05$) at this level.

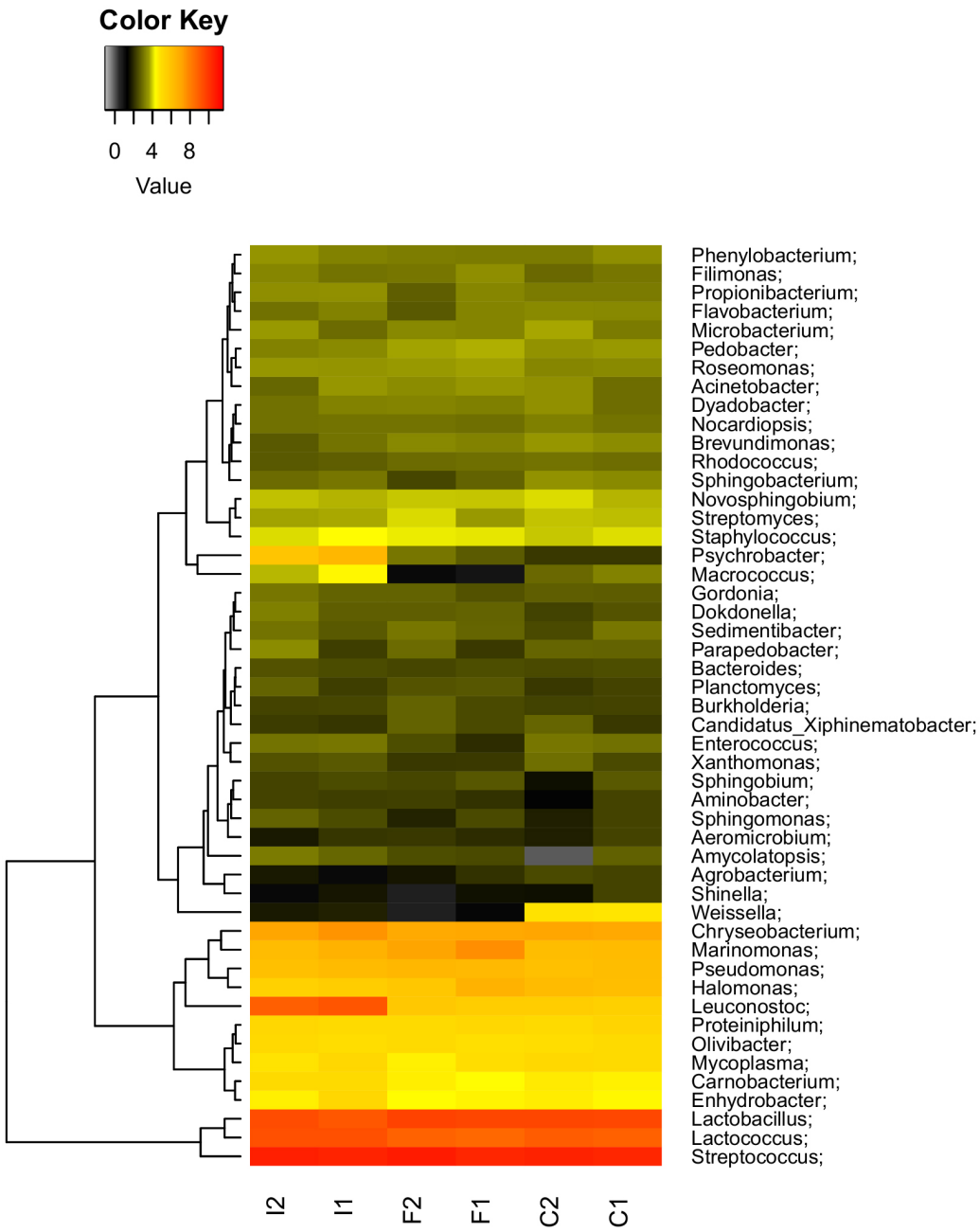

Fig. 3. Heatmap, constructed using heatmap.2 function from the gplots package, illustrating the normalized (logarithmic) counts of the bacterial OTUs (genus level) of the various samples. X axis presents the various independent samples shown individually, Y axis presents the different identified genera and their phylogenetic relationships and increasing abundances are indicated with yellow to red colors. FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control.

At genera level, *Cryptococcus* was more abundant in FTF group compared to FTI and FTC ($p < 0.05$), while in FTI and FTC it was found in similar ($p > 0.05$) levels (Fig. 5). Furthermore, *Debaryomyces* genus OTUs were higher in FTI group compared to FTF and FTC ($p < 0.05$) ($p > 0.05$ between FTF and FTC). On the other hand, *Galactomyces* was the predominant genus in FTF group with elevated ($p < 0.05$) presence than in FTC and FTI groups. Similarly, *Kluyveromyces* and *Saccharomyces* spp. were more ($p < 0.05$) abundant in the control group (FTC) compared to the two probiotic groups (FTI and FTF). *Candida* OTUs were in similar levels in all groups ($p > 0.05$). Other genera (*Acremonium*, *Arthrobotrys*, *Capronia*, *Clavispora*, *Cylindosporium*, *Davidiella*, *Dipodascus*, *Epulorhiza*, *Fusarium*, *Kodamaea*, *Lecythophora*, *Malassezia*, *Myxozyma*, *Ochroconis*, *Penicillium*, *Rhodotorula*, *Russula*, *Talaromyces*, *Tortispora*, *Trichosporon*, *unclassified-Dipodascaceae*, *unclassified-*

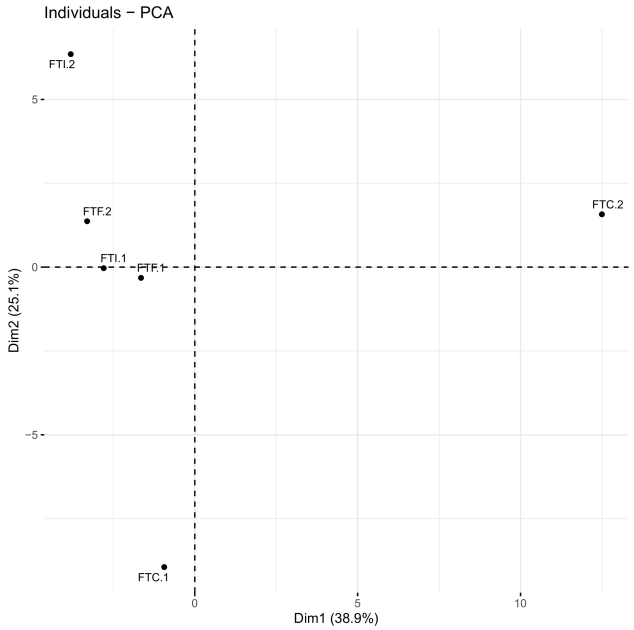

Fig. 4. Principal Coordinate Analysis (PCA) constructed using FactoMineR, factoextra and corrplot R packages. Figure is showing clustering of the feta cheese samples based on fungi diversity (duplicates are included) in genus level. FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with *L. plantarum* T571 free cells; FTC, feta-type cheese control.

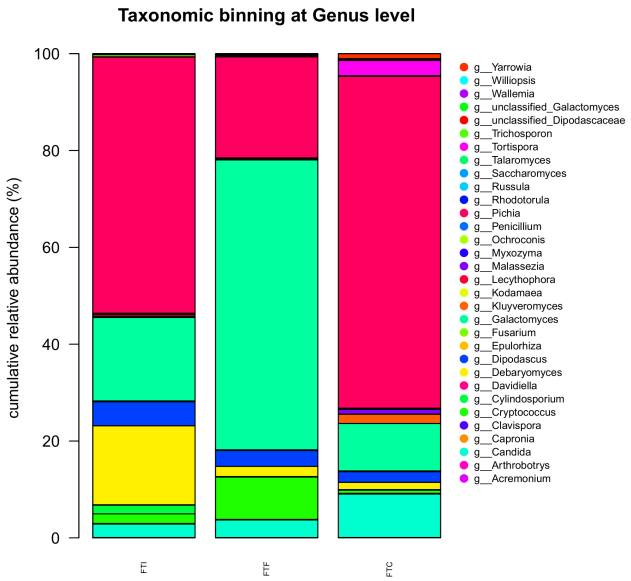

Fig. 5. Relative abundances (%) of different fungal genera, after ITS NGS sequencing in feta cheese samples. All data represent the mean values from two independent experiments. FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control.

Galactomyces, *Wallemia*, *Williopsis* and *Yarrowia*) were also detected, but in low percentages (<5%). The heatmap depicting the representation of the fungal OTUs at genus level throughout the various samples is demonstrated at Fig. 6.

4. Discussion

Food carriers are proposed to ensure the survival of probiotic cells during production, ripening, storage and passage through the GI tract, which involves exposure to low pH, due to the presence of acids in the stomach and bile salts in the small intestine [24]. Cheese owed to its high pH (4.4–4.6 for feta cheese), is regarded an excellent delivery product of viable probiotics into the human intestine, acting as a buffer against the acidic stomach environment. As a result of the dense matrix and relatively high fat content, a favorable environment for probiotic cell survival throughout GI tract transit can be achieved, providing probiotic bacteria protection.

Whey proteins have been previously tested as natural carriers for probiotic cells, and due to their structural and physicochemical properties, they can be used as a delivery vehicle [10,11]. In this vein, immobilized *L. plantarum* T571 culture immobilized on whey protein was incorporated in feta cheese and the effect of the adjunct culture on the product's microbiome was investigated. For comparison reasons, feta cheese with free *L. plantarum* T571 cells and feta cheese with no adjunct culture were also included in the study.

Each type of cheese has its own variety of microbes, making the cheese microbiome a dynamic ecosystem that evolves over time as the cheese ripens [25]. The results of our study revealed that the adjunct potential probiotic culture resulted in significant differences among the genera mapped after 16S rRNA and ITS sequencing for bacterial and fungal microbiota, respectively.

Bacterial and fungal phyla were in similar levels between all groups, with no differences attributed to probiotic supplementation, apart from an elevated abundance of *Proteobacteria* phylum in feta cheese with *L. plantarum* T571 free cells as compared to FTI and FTC samples; the relative abundance was in levels also reported elsewhere [26,27]. Adjunct immobilized probiotic resulted in a significant increase in diversity, as calculated with Shannon and Simpson indices, compared to control samples. High values for diversity indicate more diverse communities [28]. Fungal diversity was not significant different between the different samples, although at genera level, major differences were highlighted.

16S rRNA sequencing revealed distinct alterations between the probiotic (FTI and FTF) and control (FTC) cheese samples. *Streptococcus*, which was employed as one of the starter cultures, was the most abundant genus in all cases, as noted before [27,29]. However, in probiotic feta cheese with immobilized *L. plantarum* T571 cells, its presence was

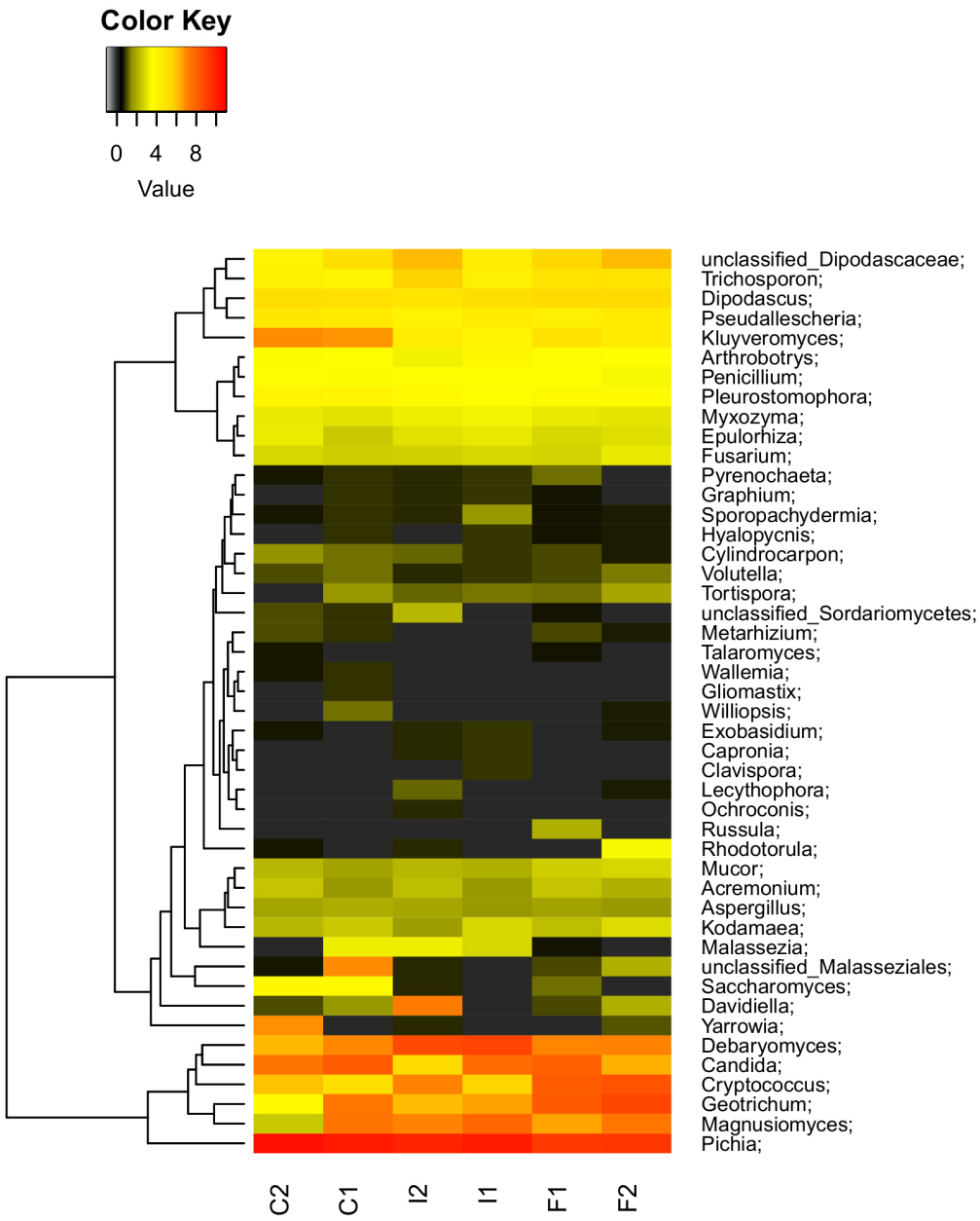

Fig. 6. Heatmap, constructed using heatmap.2 function from the gplots package, illustrating the normalized (logarithmic) counts of the fungal OTUs (genus level) of the various samples. X axis presents the various independent samples shown individually, Y axis presents the different identified genera and their phylogenetic relationships and increasing abundances are indicated with yellow to red colors. FTI, feta-type cheese with immobilized *L. plantarum* T571 cells on whey protein; FTF, feta-type cheese with free *L. plantarum* T571 cells; FTC, feta-type cheese control.

significantly lower compared to FTF and FTC samples, as a result of immobilized probiotic supplementation that enhanced the presence of other genera. Of note, all cheese products were prepared with pasteurized milk and commercial LAB starters and *L. plantarum* T571 was incorporated either as free or immobilized cells on whey protein in probiotic feta cheeses. As expected and stated elsewhere [27], the three specific species frequently used as SLAB, *S. thermophilus*, *L. delbrueckii* and *L. lactis*, were mapped with the highest abundances in their corresponding genera, although in varying percentages between the different samples.

The adjunct probiotic cultures had a significant effect on the relative LAB abundances. Supplementation of feta cheese with probiotic *L. plantarum* T571 (both immobilized and free) cells led to similar relative abundance of total *Lactobacillus* OTUs (approximately 18%). However, their presence was significantly higher compared to the control sample (FTC), underlying that probiotic addition resulted in an enhanced survival of the strain and increased the relative

abundance of the total genus. *Lactococcus* and *Lactobacillus* spp. are considered SLAB cultures and their relative proportions can vary among the final products, as described previously [27,30,31]. Feta cheese with immobilized *L. plantarum* T571 cells on whey protein (FTI sample) was also characterized by an increased abundance of *Lactococcus* compared to FTF sample, but it ranged in similar levels with the control feta cheese (FTC sample), preserving a favorable SLAB population in a normal balance. Noticeably, lactococci are often employed in cheese making and are related to aroma development [32]. In a similar experiment, microbiological analysis in feta cheeses manufactured by immobilized *L. casei* cells on whey protein-revealed an elevated presence of lactococci (approximately 8.6 logcfu/g) and lactobacilli (approximately 8.7 logcfu/g), in accordance to our results [10].

L. plantarum is used extensively in food industry as microbial starter and constitutes a widespread member of the genus *Lactobacillus*, while specific strains have been employed to produce novel functional foods and beverages with improved nutritional and technological features [33,34]. The genus has been shown to exert antimicrobial activity against pathogens and thus can be used to enhance safety and shelf-life of fermented foods [33].

Many *L. plantarum* cultures have been associated to vast a range of medical properties, including antioxidant, anticancer, anti-inflammatory, antiproliferative, antiobesity, and antidiabetic capabilities [35,36]. Recently, *L. plantarum* has been applied in medical fields for the treatment of various chronic and cardiovascular diseases, such as Alzheimer's, Parkinson's, diabetes, obesity, cancer, hypertension, urinogenital complications, liver disorders, etc. [35,37]. Interestingly, several strains have been used in clinical trials; *L. plantarum* 299v (DSM 9843) provided effective symptom relief in irritable bowel syndrome [38], and eight-week supplementation with *L. plantarum* HAC01 significantly improved HbA1c levels relative to placebo in prediabetic subjects [39].

Bacteria belonging to *Lactococcus* genus produce an antagonistic extracellular peptide called nisin, which has wide antimicrobial activity against Gram-positive spoilage and pathogenic bacteria; yet some strains have been characterized as probiotics [40].

Immobilized *L. plantarum* T571 on whey protein led to increased *Leuconostoc* genus, considered as NSLAB, that was more abundant in FTI samples (compared to FTF and FTC groups), indicating that it was a result of the immobilized probiotic supplementation. *Leuconostoc* is associated with milk products, and in brine cheese production, it has been responsible for flavor development [41]. It exhibits antibacterial effect against bacteria that cause cheese spoilage. Additionally, *Leuconostoc* has been proven to inhibit pathogen growth and can be used as a safe probiotic for further research [42,43]. Indeed, several strains belonging to *Leuconostoc* genus are regarded as potential probiotics owing to various health beneficial effects [44].

Carnobacterium, a lactic acid bacterium that is associated with soft cheese was also elevated in FTI group [45], compared to FTF. *Carnobacterium* produce antimicrobial peptides, known as bacteriocins, and has been thoroughly investigated as a protective culture for preventing growth of *Listeria monocytogenes* in fish and meat products [46]. *Hafnia*, a genus associated with cheese ripening [47] and *Psychrobacter* that contributes positively to many technological cheese characteristics, especially improving the aroma properties [48], were slightly increased in FTI group compared to the other two groups. *Hafnia* produces caseinolytic protease B (ClpB) protein, which was previously identified as a conformational mimetic of α-melanocyte-stimulating hormone (α-MSH), a key anorexigenic peptide involved in the regulation of appetite [49]. Likewise, the presence of *Psychrobacter*, in another study, led to the higher production of volatile aroma compounds [50].

Mapping the fungal microbiome in different feta cheeses, striking differences were revealed, as well. Probiotic feta cheese with immobilized cells resulted in increased abundance of *Debaryomyces*, associated with the production of cheese flavor compounds [51]. *Debaryomyces* are potential biopreservative agents for use in eliminating the growth of the *Aspergillus* that produce food-contaminating mycotoxins like ochratoxin, which is associated with cancer [52]. The predominant genus in FTI group was *Pichia,* whereas *Galactomyces* was the predominant genus in probiotic feta cheese with free *L. plantarum* T571 cells (FTF sample). *Pichia* has been previously reported in cheese products [53,54], while *Galactomyces* is one of the major genera detected in cheese [51]. *Cryptococcus*, related to improved organoleptic characteristics in cheese and usually found on mature cheeses [55], was increased in probiotic cheese with free *L. plantarum* T571 cells (FTF sample). These differences support that probiotic addition led to microbial profiles and genera associated of high quality and with sensory characteristics similar to the typical characteristics of feta cheese, as reported previously [21].

Incorporation of probiotics in cheeses is widely reported in literature [10,56–59]. Whey protein can act as a natural immobilization carrier [10,11,33] and is expected to create high added value in the food sector, establishing an economically feasible, marketable, and environmentally friendly process in a series of food industrial applications by a concurrent exploitation of whey, a by-product of the dairy industry, which is difficult to dispose or treat [10]. White brined soft feta cheese, is a traditional Greek product that can be enriched with beneficial cultures, considering the increasing industrial demands and consumers' needs for the development of functional foods. Hence, the proposed technology that employs insertion of immobilized probiotic cultures on natural food carriers, able to produce feta cheese with distinct microbial composition may have a ma-

jor impact on sustainable development. Immobilization of *L. plantarum* T571 on whey protein is expected to improve cell survival during digestion and release the probiotic cells in the large intestine, given that whey protein could function as a buffering agent *in vivo*, and protect ingested bacterial strains at the upper GI tract [9].

Although NGS technology consists a key tool for the investigation of complex microbial systems (i.e., cheese microbial flora) and can be used for the culture-independent study of food microbiota, compared to time-consuming microbiological techniques, there are vast limitations that have to be considered carefully. Microbe-driven changes during food processing involve intricate relationships and especially bacterial and fungal interactions within the life of a food constituent [17]. In our case, samples after milking and before milk pasteurization, as well as in each of the two stages of feta ripening period [30] and during product storage would probably give more insight on microbiota evolvement over time. A major limitation of 16S rRNA (and ITS) NGS method is that its sensitivity is enough to detect potentially significant alterations at genus-level at best [16], thereby variations at species level or changes in gene expression are ignored.

5. Conclusions

Immobilized *L. plantarum* T571 cells on whey protein were used as functional ingredient to enrich feta cheese and the effect of probiotic supplementation on food microbiome was investigated. Enrichment with immobilized probiotic cells led to diversity both in bacteria and fungi profiles, as revealed by NGS sequencing. The presence of *Lactobacillus*, *Leuconostoc* and *Debaryomyces* in high proportions in feta cheese with immobilized cells compared to the control samples, may lead to advanced technological characteristics and contribute to possible health effects. Incorporation of viable probiotic strains into traditional cheese products consists an industrial challenge presently under investigation and resulted in distinct microbiome composition that could be a key factor in determining food authenticity. However, more research is still required in the field.

Abbreviations

GI, gastrointestinal; IPA, International Probiotics Association; NGS, next-generation sequencing; SLAB, starter lactic acid bacteria; NSLAB, non-starter lactic acid bacteria; ITS, Internal Trancribed Spacer; SSU, Small SubUnit-coding sequence; LSU, Large SubUnit-coding sequence; OTU, Operational taxonomic Units; PCA, Principal Component Analysis.

Author Contributions

YK and CT designed the research study. GM, IP, AN, NC and KT performed the research. MEG, PK and TT provided help and advice on data analysis. GM, IP and KT analyzed the data. GM, IP and YK wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest. YK is serving as one of the Editorial Board members and Guest editors of this journal. MEG is serving as one of the Guest editors of this journal. We declare that YK and MEG had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to YS.

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