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Entrapment of *L. casei* ATCC393 in the viscus matrix of *Pistacia terebinthus* resin for functional myzithra cheese manufacture

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Abstract

Pissa Paphos, a natural mastic resin (*Pistacia terebinthus*) was evaluated as an encapsulating and matrix-forming material for the immobilisation of the probiotic bacterium *Lactobacillus casei* ATCC 393. The immobilized biocatalyst was added as an adjunct for the production of functional myzithra cheese. In total, four myzithra cheeses were manufactured: a. cheese with *L. casei* cells entrapped in a *P. terebinthus* matrix (Pissa Paphos) b. cheese with free *L. casei* cells and *P. terebinthus* resin, c. myzithra cheese with free *L. casei* cells without the resin and d. traditional myzithra cheese. *P. terebinthus* resin provided antimicrobial properties by suppressing the growth of fungi/yeasts in myzithra cheese during refrigerated storage. On the contrary, the presence of the resin did not affect the cell counts of the probiotic microorganism which maintained in high populations (above 9 log CFU g^{-1}) during hole storage period. Additionally, the viscus matrix of the resin seems to confer a protective effect on entrapped *L. casei* cells since higher populations were observed over free cells. Moreover, all myzithra cheeses with incorporated resin were characterized by an exceptional mastic gum aroma and pleasant coherent texture which indicates the product's high commercialization potential.

Keywords: *Pistacia terebinthus* resin; probiotics; encapsulation; terpenes; myzithra cheese.

Chemical compounds studied in this article: α-pinen (PubChem CID: 6654), β-pinen (PubChem CID: 14896), α-terpineol (PubChem CID: 442501), 4-terpineol (PubChem CID: 11230), eukalyptol (PubChem CID: 2758), terpinolene (PubChem CID: 11463), myrtenol (PubChem CID: 10582), pinocarveol (PubChem CID: 88297),

3-carene (PubChem CID: 26049), o-cymene (PubChem CID: 10703), verbenol (PubChem CID: 61126).

1. Introduction

Resins are plant products that exude naturally (surface resins) or can be obtained by incision or infection (internal resins). They are insoluble in water but soluble in organic solvents (Dell & McComb, 1979). The most popular resins origin from Pistacia plants (*Pistacia lentiscus, Pistacia terebinthus*) which native to the Mediterranean region from Morocco and Portugal to Greece, Turkey and Syria (Rauf et al., 2017). Most of the plant parts including fruits, fruit fatty oil and resin were used as food and traditional medicine in the region since ancient times (Lardos, 2006).

Pissa Paphos, the mastic gum obtained from *Pistacia terebinthus L. (Anacardiaceae* family) tree, grows mainly on dry rock slopes and hill sides or in pine forests of Cyprus especially in Paphos and Limassol district. The tree's aromatic resin, called by the locals "*Paphos pissa"* and/ or "*pissa Pafitiki"* has a significant contribution to the local economy (Lardos, 2006). Over the years, different parts of *P. terebinthus* tree have been reported to provide several ethnopharmacological utilizations such as an antiseptic, diuretic, anti-inflammatory, antipyretic, antibacterial and antiviral agent, for wound treatments , eczema, burns and stomach-aches (Rauf et al., 2017; Topçu et al., 2007).

Lately, consumers awareness has focused on safe and high-quality food products, leading the food companies and related industries to implement novel methods for the production of functional foods (Bogue, Collins, & Troy, 2017). One major category of functional foods is probiotic dairy products. Probiotics are lactic acid bacteria that when presented as live microbial supplements can confer a beneficially affect to the host by improving its intestinal microbial balance (Fuller & Gibson, 1998). It has been established that a minimum level of probiotic lactic acid bacteria (10^6 ~ 10^7 CFU g^{-1}) contained viable in dairy products is necessary for

improving human health (Shori, 2015). Thus, scientists have recently targeted on the development of novel methods that will enhance probiotic viability in dairy products like cell immobilisation, microencapsulation, addition of prebiotics, drying (freeze-drying, spray-drying) etc. (Bosnea, Moschakis, Nigam, & Biliaderis, 2017). Techniques like immobilisation and microencapsulation of probiotics, even though represent a great challenge, have been established in order to improve the survival of probiotic bacteria giving promising results since they increased survival rates and stability of probiotics during fermentation processing and storage (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011; Terpou, Bekatorou, Kanellaki, Koutinas, & Nigam, 2017). Also, cell encapsulation in dairy fermentation is a rapidly expanding research area because of its attractive technical and economic advantages compared to the conventional free cell systems (Bosnea, Moschakis, et al., 2017; Morales & Ruiz, 2016).

In addition, synthetic chemical additives are used as preservatives (antimicrobials, antioxidants and anti-browning) to ensure the products self-life and safety or as flavor enforcements for improvement of products characteristics (Carocho, Barreiro, Morales, & Ferreira, 2014). However, many studies have confirmed that the excessive consumption of synthetic food additives is related with gastrointestinal, respiratory, dermatological and neurological adverse reactions and as a result consumers avoid the consumption of such products (Caleja et al., 2016; Carocho et al., 2014). Therefore, an alternative solution for enhancement of self-life and safety of food products is the use of natural additives. Mastic gum and its essential oils are very promising food additives since numerous resent studies have demonstrated its antimicrobial and flavoring effect (Aksoy, Duran, & Koksal, 2006; Daifas et al., 2004; Paraschos et al., 2011; Schoina et al., 2014). Moreover, the use of

mastic gum (*Pistacia lentiscus*) as immobilisation support has recently been proposed by Morkhade (2017) as a successful microencapsulating and matrix-forming material for sustained drug release..

Thus, the aim of the present study was to examine the capability of *Pistacia terebinthus* resin as a probiotic microencapsulation matrix and its use as an adjunct for functional myzithra cheese making. The main targets of the study were to investigate *Pistacia terebinthus* resin as a natural encapsulation matrix for the probiotic bacterial strain *Lactobacillus casei* ATCC 393 (Saxami et al., 2012) and the effects on probiotic cell viability in myzithra cheeses during 30 days of storage (4^{o}C) and product's shelf-life and finally the influence on the aromatic profile of the produced myzithra cheeses.

2. Materials and methods

2.1 Pistacia terebinthus resin for probiotic cell encapsulation

The probiotic Gram^{+} *Lactobacillus* bacterial strain *Lactobacillus casei* ATCC 393 (DSMZ, Braunschweig, Germany) was used for the microencapsulation process. The probiotic *L. casei* ATCC 393 was selected according to its *in vitro* and *in vivo* studies of the microbial survival in GI tract, adhesion to the intestine and modulation of the intestinal microflora in rats (Saxami et al., 2012).

L. casei cells were grown at 37°C in de Man-Rogosa-Sharpe (MRS) liquid medium (LabM, UK) for 48-72 h. Wet biomass was harvested by centrifugation (Sigma 3K12, Bioblock Scientific, France) at 5000 rpm for 10 min. The cultivated wet biomass was introduced in MRS liquid medium along with small particles of sterile freeze-dried *Pistacia terebinthus* resin (pissa Paphos'), as described previously by Antonia Terpou et al. (2017). Freeze drying was important for immobilisation performance since the structure opens and creates holes where the probiotic LAB cells can be entrapped. The system was placed in an incubator at 37°C and agitated periodically for immobilisation to be achieved (approximately 48h). When immobilisation bioprocess was performed (glucose in the liquid culture was <1 g/L), the fermented liquid was decanted *Pistacia terebinthus* resin with immobilized probiotic cells was washed twice with sterile Ringer's ¼ solution targeting the removal of any free cells (Schoina et al., 2015). All media were autoclaved at 120°C at 1–1.5 atm for 15 min prior to use.

2.2 Verification of encapsulation of probiotic cells by scanning electron microscopy and microbiological analysis

Pieces of *Pistacia terebinthus* resin (pissa Paphos) in comparison with pieces of the encapsulated biocatalyst were coated with gold in a Balzers SCD 004 Sputter coater (Bal-Tec, Schalksmühle, Germany) for 2 min. The samples were examined in a JSM-6300 scanning electron microscope (JEOL, Tokyo, Japan), operated at an accelerating voltage of 20kV. Scanning electron micrographs were obtained in order to investigate probiotic cell encapsulation in *Pistacia terebinthus* matrix.

The counts of the entrapped *L. casei* cells into a specific amount of the resins' viscus matrix were determined. In particular, 1 g of encapsulated biocatalyst was added to 9 mL of ringer solution followed by shaking in a homogenizer for 210 s. Existing probiotic colonies of the encapsulated biocatalyst were identified by enumeration in MRS agar medium (37 °C, 48 - 72 h). To demonstrate the complete detachment of encapsulated *L. casei* cells from the resins matrix, the first homogenate solution is poured out and new ringers' solution is added. Subsequently, the mixture is shaken in a homogenizer for 210 s and the resulting liquid is tested until no grow of *L. casei* colonies is detected.

2.3 Myzithra cheese production

Myzithra is a traditional Greek whey cheese produced by heating cheese whey at 88–92 °C under continuous stirring for 40–45 min in order to obtain the protein remaining from cheese production (Litopoulou-Tzanetaki & Tzanetakis, 2011).

Sweet cheese whey (0.54% fat, 1.67% total protein, 5.35% lactose and pH 6.4) derived as an industrial by-product of Graviera hard cheese production (A.VI.GAL SA-Achaia milk industry) (Bozoudi et al., 2016) was used for myzithra cheese making as described previously by Litopoulou-Tzanetaki and Tzanetakis (2011) with small modifications. Specifically, cheese whey was gradually heated under continuous

161 stirring with a rate of 1,5 °C/ min up to 75°C and when small curd particles of whey
162 proteins were formed, the temperature was increased to 90 °C with a rate of 1 °C/ min
163 for 45 min in total. Acidification of whey to pH 5.2 was performed by the addition of
164 10% lactic acid before heating (Pappas & Voutsinas, 2009). A very thin layer of
165 coagulum was formed on the surface of the whey after 45 min of heating at 90 °C and
166 then stirring was reduced and finally stopped. The formed curd was transferred
167 gradually by a perforated ladle into a sterile perforated fabric and hung from a pole in
168 a ventilated room at room temperature (18~22 °C) for 3 h in order to drain.

169 The drained curd was divided equivalent particles and four types of myzithra
170 cheeses were prepared: (C) control myzithra cheese; (F) myzithra cheese with free
171 probiotic cells (ME) myzithra cheese with encapsulated probiotic cells and (MF)
172 myzithra cheese with *Pistacia terebinthus* resin and free probiotic cells. In the case of
173 myzithra cheeses produced by the adjunct probiotic culture, the curd was cooled after
174 heating at 37°C and homogenously 1g of free *L. casei* cells (F) or 0.5g of
175 encapsulated *L. casei* cells in *P. terebinthus* resin (ME) or 0.5g of *P. terebinthus* resin
176 with 1g of free *L. casei* cells (MF) were added respectively per 100g of cheese curd.
177 All myzithra cheeses were placed into different sterile paper containers and stored for
178 30 days at 4^{o}C.
179

180 *2.4 Microbiological analysis of myzithra cheese*

181 Representative 10g portions of myzithra cheese samples were obtained at various time
182 intervals (1^{st}, 5^{th}, 7^{th}, 14^{th}, 30^{th} storage day at 4^{o}C) and blended with 90 mL of sterile
183 trisodium citrate (2% w/v) solution and mixed in a stomacher (Bagmixer 400, Model
184 VW, Interscience). The solution was then subjected to serial dilutions of 9mL of
185 Ringer solution ¼ strength. Enumeration of viable cell counts of total aerobic

mesophilic bacteria, lactococci, lactobacilli, enterobacteria, coliforms, yeasts & fungi and staphylococci were performed in triplicate by pour plating 0.1 mL or 1mL of appropriate dilutions on the selective media for each species and according to instructions of the manufacturer as described previously by A. Terpou et al. (2017). Cell counts were expressed as log of mean colony-forming units.

For the enumeration of *L. casei* cells MRS-V agar (0.1% vancomycin) was prepared which according to Tharmaraj and Shah (2003) would disintegrate the adjunct probiotic strain and *L. bulgaricus ssp. delbrueckii* cell counts. The growth potential of *L. bulgaricus* was expected in higher number than any lactobacilli naturally occurring microflora since the whey used as raw material for myzithra cheese production was not sterilized and was obtained after Graviera cheese manufacture. Nowadays, in industrial dairies, cheese milk is at first pasteurized and then used with the addition of a starter mesophilic or thermophilic culture for Graviera cheese manufacture (Litopoulou-Tzanetaki & Tzanetakis, 2011). In this case Graviera cheese was manufactured by the addition of yogurt culture (*L. bulgaricus ssp. delbrueckii* and *S. thermophilus*) and rennet enzyme thus, the presence of *L. bulgaricus* cells can be expected. Cell counts were expressed as % of *L. casei* viability during 30 days of refrigerated storage.

2.5 Physicochemical analysis

The pH values of cheese whey during cheese production and of myzithra cheese during storage were measured using a digital pH meter by direct immersion of the electrode (EPI-BION SENTRON pH-System 1001).

Myzithra cheese samples (20 g each) were macerated with warm water (40 °C) to produce a total volume of 210 mL and then each sample was filtered and used for

211 the identification of total acidity and lactose concentration. A quantity of 25mL from
212 the above filtrate was used for titration with 0,1 N NaOH and phenolphthalein
213 indicator. Total acidity was determined to the official method by AOAC International
214 (2000) and expressed as lactic acid content. Lactose was determined by high
215 performance liquid chromatography, using a Shimadzu chromatograph with a SCR-
216 101 N stainless steel column, a LC-9A pump, a CTO-10A oven at 60 °C and a RID-
217 6A refractive index detector as described previously by A. Terpou et al. (2017).
218 Lactose concentrations were calculated using standard curves.
219

220 *2.6 Solid phase microextraction gas chromatography–mass spectrometry analysis*

221 Samples of myzithra cheese with adjunct free (F) or adjunct microencapsulated (ME)
222 *L. casei* cells were studied for terpenoid content as an aroma profile indicator using
223 SPME GC–MS analysis and compared with control myzithra cheese samples (C). For
224 the analysis, myzithra cheese samples (7.0 g each) from the 1st day of storage (4^{o}C)
225 were introduced into a 20mL headspace vial fitted with a Teflon-lined septum and
226 sealed with an aluminum crimp seal. Through the seal a syringe needle (Supelco,
227 Bellefonte, PA, USA) was inserted. The container was then thermostated for 5min at
228 60 °C with the syringe closed and when the temperature was stable the syringe was
229 introduced at the gas area of the vial for 45 min at 60 °C.

230 The absorbed volatile analytes were then analyzed by GC–MS (Shimadzu GC-
231 17A, MS QP5050, capillary column Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25
232 μm film thickness) as described previously by A. Terpou et al. (2017). The
233 identification of the absorbed terpenoid content, presented % in total area of
234 hydrocarbons, was performed by comparing the retention times with those of
235 authentic compounds, by mass spectra of the authentic compounds generated in the

236 laboratory, by mass spectra obtained from NIST107, NIST21 and SZTERP libraries
237 and by determining kovats' retention indexes compared with those reported in the
238 literature. Kovats' retention indexes (KI) were determined by injection of a standard
239 mixture containing the homologous series of normal alkanes (C7–C32) in pure hexane
240 under exactly the same experimental conditions, as described above.
241

242 *2.7 Myzithra cheese sensory evaluation*

243 Sensory evaluation of cheese is necessary in order to determine the influence of
244 cheese composition on sensory characteristics, eating quality and consumers
245 acceptability. Sensory evaluation was carried out by 10 laboratory members, priory
246 trained, using locally approved protocols. Samples were tested by two different
247 laboratories (5 members each) and all members were from different parts of the
248 country. Consumers selection criteria were between 20 ~ 45 years of age, and frequent
249 users of cheese (>once a week). The questions asked and procedure of cheese testing
250 were identical for the two laboratories. Myzithra cheese samples from the 1st storage
251 day (4^{o}C) were placed into equivalent amounts of 5 × 5 cm and served at room
252 temperature (18~22 °C). This procedure was chosen as consumers normally will
253 consume cheese directly from refrigerator. Sensory analysis was carried out in panel
254 booths conforming to international standards (International standard, 2007). The
255 samples were coded by a different 3-digital number each and were served in a
256 randomized order while the panel was asked to evaluate all myzithra cheeses (C, F,
257 ME, MF) on a 0–10 scale (the higher the number the greater the intensity) based on
258 saltiness, acidity, bitterness, sweetness, chewiness, cheese odor, mastic odor and
259 overall acceptability. Data from both laboratories were handled as one data set during
260 statistical analysis. The results are presented as a star chart of the product's attributes.

261

262 *2.8 Experimental design and statistical analysis*

263 Myzithra cheese production and analysis was carried out in triplicate and results are
264 presented as mean values ± standard deviation. All experiments were designed and
265 analyzed statistically by ANOVA. Significant differences among results (coefficients,
266 ANOVA tables and significance) which were computed using SPSS v.8.5.

3. Results and Discussion

3.1 Rational

Whey cheeses are prepared by denaturation and precipitation of whey proteins (α-lactalbumin, β-lactoglobulin) achieved by heating whey effluent at approximately at 85 °C. In Greece traditionally, whey cheeses like myzithra cheese are consumed as table cheeses. They have high nutritional value, low fat and salt content and have good organoleptic characteristics and are therefore great vehicles for incorporation of probiotics (Papaioannou, Chouliara, Karatapanis, Kontominas, & Savvaidis, 2007).

However, freshly made whey cheeses have a pH value of greater than 6, high moisture content and a low salt concentration and are therefore considered to be extremely sensitive to microbial deterioration (Hough, Puglieso, Sanchez, & da Silva, 1999) with very short shelf-life reaching up to 7 days under aerobic conditions (Samelis, Kakouri, Rogga, Savvaidis, & Kontominas, 2003).

In contrast, *Pistacia terebinthus* resin has been proved to confer an antimicrobial effect to dairy products due to its high terpenoid characteristics (Schoina et al., 2014). Moreover, immobilisation in various natural supports has been proved to enhance the viability of probiotic cells by a protective film that is formed by each immobilisation support protecting cells against the acidic environment of dairy products (Bosnea, Kopsahelis, Kokkali, Terpou, & Kanellaki, 2017; Antonia Terpou et al., 2017; A. Terpou et al., 2017). Thus, *Pistacia terebinthus* resin was assessed as encapsulation matrix for *Lactobacillus casei* cells and incorporated in myzithra cheese.

3.2 The probiotic encapsulated biocatalyst

Electron micrographs reinsured encapsulation of the probiotic cells within the viscous

292 matrix of *Pistacia terebinthus* (Fig. 1). The average encapsulation yield obtained in
293 the present study was reported by experiments carried out on the encapsulated
294 biocatalyst presenting an average of 1.56 g of *L. casei* cells successfully encapsulated
295 in 100 g of *Pistacia terebinthus* (data not shown). More analytically, in each 5g of the
296 resin 7.8% of the initial probiotic culture is encapsulated (data not shown).
297 Subsequently, 4 log CFU of *L. casei* cells were proved to be encapsulated in each
298 gram of *Pistacia terebinthus'* viscus matrix.
299

300 *3.3 Effect of the encapsulated biocatalyst on growth of foodborne pathogens, cheese*
301 *microflora, and spoilage microorganisms*

302 Myzithra cheese samples were tested for their microbial stability as a shelf-life
303 indicator, through refrigerated storage for 30 days (Table 1). No coliforms,
304 enterobacteria or *Staphylococcus aureus* were detected in any myzithra cheese. There
305 was observed a significant amount of yeast and fungi in all myzithra cheeses from the
306 1st storage day (2.6 ~ 2.7 log CFU g^{-1}) which in the case of control cheeses (C)
307 increased significantly until the 30th storage day (4.7 log CFU g^{-1}). On the other hand,
308 yeast and fungi showed a sharp decrease in the case of cheese samples with
309 incorporated *Pistacia terebinthus* resin either added as an encapsulation support (ME)
310 or as an adjunct (MF). Myzithra cheese which is usually consumed within 7 days
311 from production and does not have yeast and fungi as a naturally occurring
312 microflora. When high numbers of the prementioned microorganisms are detected the
313 cheese is most likely accompanied by a bad odor and cannot be consumed.

314 The addition of the probiotic strain, either free or encapsulated, affected
315 significantly ($P< 0.05$) the total lactobacilli counts of myzithra cheese after the 1st
316 storage day (F, ME, MF), compared to control cheese (C). Moreover, in all myzithra

317 samples was detected a significant amount of Lactococci (2.9 ~ 2.0 log CFU g^{-1}) that
318 did not significantly differ among cheese samples during 30 days of storage. Their
319 presence in cheeses may occur due to non-starter lactic acid bacteria and cross-
320 contamination during cheese production (Kalogridou-Vassiliadou, Tzanetakis, &
321 Litopoulou-Tzanetaki, 1994) .
322

323 *3.4 Growth capacity of the adjunct probiotic strain during refrigerated storage*

324 Cheese presents a good vehicle for the delivery of probiotics in the intestine, while the
325 maintenance of viable probiotic cell counts at high level by the end of expiration date
326 is most crucial in such products in order to confer most health benefits. In this vein,
327 experiments were carried out in order to evaluate the effect of *Pistacia terebinthus*
328 resin and myzithra cheese storage conditions on viability of *L. casei* cells. Figure 2
329 shows the % viability of *L. casei* added either as a free culture, as an encapsulated
330 culture in *Pistacia terebinthus* resin or as a free culture along with resin particles,
331 during storage at 4^{o}C for 30 days. All myzithra cheeses in which *L. casei* was
332 incorporated (F, ME, MF), was observed a high count of viable cell (10^{9} CFU g^{-1},
333 data not shown) during storage. This result indicated the probiotic character of the
334 products.

335 More specifically, the survival rates of encapsulated *L. casei* was 8.2% by the
336 end of storage period while in contrast *L. casei* free cells along with resins
337 incorporated particles were reduced down to 4%. The lowers viability rates were
338 observed in the case of free *L. casei* cells which were reduced down to 8.2%. These
339 results occurred most likely due to the absence of *P. terebinthus* resin and its
340 antimicrobial effects allowing foodborne microorganisms like yeast and fungi (Table
341 1) to grow at the expense of probiotic cells. Since myzithra cheeses with encapsulated

biocatalyst showed the higher survival rates during storage we can assume that encapsulation acts protectively to probiotic cells against storage conditions and cheese environment.

3.5 Physicochemical characteristics of myzithra cheeses

During storage, lactose concentration, pH values and total acidity were determined for all myzithra cheeses and the results are presented in Table 2. In most whey cheeses prepared by the incorporation of lactic acid bacteria there has been observed a higher content of total acidity and a parallel with pH decrease, compared to whey cheeses prepared with the traditional recipe (Madureira et al., 2008; Madureira et al., 2015). As expected, a continuous increase of total acidity was observed during 30 storage days and ranged in acceptable levels for all myzithra cheese products (Anifantakis, 1991; Kalogridou-Vassiliadou et al., 1994). The total acidity of the whey cheeses was affected by the adjunct probiotic culture. Specifically, myzithra cheeses with *L. casei* culture (free or encapsulated) showed an acidity significantly higher ($P < 0.05$) compared to control myzithra cheeses prepared with no additional culture. In particular, total acidity increased from 0.3 to 0.5 g of lactic acid per 100 g of cheese in myzithra samples free *L. casei* cells (F) and an increase from 0.3 to 0.6 g of lactic acid / 100 g of cheese in the case of myzithra samples with encapsulated *L. casei* cells (ME). No significant differences were observed in myzithra cheese samples with free *L. casei* cells (F) and cheese samples with *Pistacia terebinthus* resin and free *L. casei* cells (MF). In parallel with total acidity increase, there was observed a pH reduction most likely as a result of continuous growth of microorganisms during refrigerated storage as it can be reinsured by microbiological analysis (Table 1). Regarding the pH of traditional whey cheese produced without an adjunct culture the presented values

367 are significantly higher (Pappa, Samelis, Kondyli, & Pappas, 2016) than those of
368 whey cheeses like myzithra produced with the incorporation *L. casei*.

369 Lactose accumulation was observed in all cheese samples and ranged in usual
370 levels of commercial myzithra cheeses (Kaminarides, 2015), while no significant
371 differences were observed within the samples. Nevertheless, lactose content was
372 lower in myzithra cheeses produced with either free or encapsulated probiotic cells
373 compared to traditionally made myzithra cheese. This trend was expected due to the
374 presence of live probiotic cells during storage (Kourkoutas et al., 2006; A. Terpou et
375 al., 2017).

376

377 *3.6 Effect of Pistacia terebinthus resin on volatile by-products of myzithra cheese*

378 The majority of volatiles, especially ones' that define a characteristic aroma to cheese
379 products, were identified in myzithra cheeses with encapsulated biocatalyst (ME) by
380 SPME/ GC-MS analysis and are presented as % of the total area of hydrocarbons'
381 content (Table 3). A large number of terpenoid compounds, predominantly α-pinene
382 (Table 3), appear in the produced myzithra cheeses with encapsulated *L. casei* cells
383 (ME), due solely to added *Pistacia terebinthus* resin (pissa Paphos). Additionally, in
384 Figure 3A is highlighted the plethora of terpenoid compounds of myzithra cheese with
385 encapsulated biocatalyst (ME) presented on the chromatograph of headspace analysis.
386 Concerning total terpenoid content (Figure 3B), total mono-terpenes were found to be
387 90.9% and total oxygenated mono-terpenes 9.1%. Monoterpenes have been reported
388 as potential antimicrobial agents, as antiviral agents, antifungal agents and as potential
389 antioxidants while they can also be used as ingredients of soaps, perfumes, and food
390 additives (Armaka, Papanikolaou, Sivropoulou, & Arsenakis, 1999; Prates et al.,
391 1998; Ruberto & Baratta, 2000; Zhang et al., 2016). Figure 3B shows the distribution

of terpenoid components of myzithra cheese with encapsulate biocatalyst (0.5/ 100 g of product), in which the higher content of monoterpenes was detected as is α-pinene (84.5%). These results were mostly expected as essential oils of three *Pistacia* species compile mainly of α-pinene, β-pinene, limonene and α-terpineol (Duru et al., 2003). Likewise, the significant influence of a-pinene in myzithra cheeses with encapsulated biocatalyst indicates the reins' antimicrobial effects against spoilage microorganisms (Kivrak et al., 2009) resulting to a possibly extended shelf-life of functional myzithra cheeses. Moreover, apart from α-pinen the detected α-terpineol (1.3%), eucalyptol (0.3%) and linalool (0.2%) can provide antibacterial and antioxidant effects to produced cheeses (Zengin & Baysal, 2014). In addition, according Park et al. (2012) linalool and α-terpineol provides an antimicrobial effect against periodontopathic and cariogenic bacteria. Thus, the antibacterial, antifungal and antioxidant activities of such monoterpenes result to the conclusion that *P. terebinthus* resin can be used as a natural preservative of food products with good organoleptic characteristics.

Another important influence of the incorporated resin with encapsulated probiotic cells refers to improved aromatic profile of myzithra cheeses. The numerus monoterpenes originating from the incorporated resin are well known for their contribution to the aromatic profile of products and are very often used as additives in food production (Prates et al., 1998). Most of detected terpenes are characterized by exceptional aromatic characteristics and can contribute to flavor of produced cheese due to their low threshold value. For example, α-pinene is known for its pine odor, 3-carene (0.2%) its sweet lemon odor and linalool (0.2%) for its sweet floral odor (Curioni & Bosset, 2002; Vichi et al., 2007). Apart from their floral and fruity aromas, monoterpenes are also considered important compounds because of their ability to reduce the effects of unpleasant odors caused by phenolic compounds or

417 short chain fatty acids (Curioni & Bosset, 2002).

418 The results indicated that the plethora of terpenoid compounds were detected
419 in myzithra cheese due to the incorporation of *P. terebinthus* resin. In addition, by
420 GCMS/ SPME analysis has highlighted that the use of *P. terebinthus* resin as
421 encapsulation support leads to exceptional aromatic characteristics of produced
422 myzithra cheeses, which is also in agreement with the sensory evaluation results.

423

424 *3.7 Myzithra cheese sensory evaluation*

425 Sensory evaluation of myzithra cheese samples is presented in Figure 4. The results of
426 sensory evaluation of myzithra cheese samples are presented in Figure 4. In most
427 cases, no significant differences were observed between myzithra cheeses produced
428 by adjunct *P. terebinthus* resin with either free or encapsulated probiotic cells.
429 However, samples prepared without the present of the resin (C, F) presented
430 significantly ($P < 0.05$) lower values in all cases compared to myzithra cheese
431 samples in which *P. terebinthus* resin was added as an adjunct. These findings
432 indicated the high industrialization potential of the proposed technology since sensory
433 evaluation showed high consumers preference referring to all novel myzithra cheeses
434 with incorporated *Pistacia terebinthus* resin.

435

436 **4. Conclusions**

437 The production of a novel functional whey cheese by the adjunct encapsulated *L.*
438 *casei* in *Pistacia terebinthus* resin (pissa Paphos) was assessed in the present study.
439 The probiotic cells were successfully encapsulated in the viscus matric of the resin
440 retaining its viability despite the resins' antimicrobial properties. The obtained results
441 showed that encapsulation favored the viability of *L. casei* in refrigerated storage

while the antimicrobial properties of the resin resulted in shelf-life extension of the produced myzithra cheeses. In the present study, was highlighted the potential use of pissa Paphos as a lactobacilli microencapsulation support, as an antimicrobial additive and as an aromatic enhancement material indicating its future use in nutraceutical and food industry.

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Figure captions

Figure 1. Electron micrographs of *Pistacia terebinthus* resin surface (A), *L. casei* ATCC 393 cells in *Pistacia terebinthus* encapsulating matrix (B, C).

Figure 2. *L. casei* % viability during refrigerated storage (4^{o}C) for 30 days.

Figure 3. SPME GC–MS spectra (full scan mode chromatogram) of myzithra cheese with adjunct *L. casei* cells encapsulated in *Pistacia terebinthus* resin (ME).

Figure 4. Sensory evaluation of produced myzithra chesses from the 1^{st} day of storage at 4^{o}C.

659 **Table 1.** Myzithra cheese microbial population (log CFU g^{-1} ±) during 30 days of

660 storage (4^{o}C).

Myzithra cheese	Storage time (days)	Total aerobic counts	Lactococci	Lactobacilli	Yeasts & fungi	Staphylococci
C	1	$3.78^{\pm0.19}$	$2.80^{\pm0.14}$	$2.97^{\pm0.15}$	$2.68^{\pm0.08}$	$1.98^{\pm0.10}$
	5	$4.30^{\pm0.21}$	$2.85^{\pm0.15}$	$2.66^{\pm0.13}$	$3.30^{\pm0.12}$	$1.92^{\pm0.09}$
	7	$4.65^{\pm0.23}$	$2.70^{\pm0.14}$	$2.57^{\pm0.13}$	$3.62^{\pm0.13}$	$2.14^{\pm0.11}$
	14	$4.75^{\pm0.24}$	$2.75^{\pm0.14}$	$2.85^{\pm0.15}$	$4.42^{\pm0.12}$	$2.21^{\pm0.11}$
	30	$5.78^{\pm0.29}$	$2.78^{\pm0.14}$	$2.74^{\pm0.19}$	$4.73^{\pm0.12}$	$2.25^{\pm0.11}$
F	1	$3.30^{\pm0.17}$	$2.83^{\pm0.15}$	$9.97^{\pm0.17}$	$2.60^{\pm0.08}$	$2.11^{\pm0.11}$
	5	$4.26^{\pm0.21}$	$2.85^{\pm0.19}$	$9.28^{\pm0.16}$	$2.90^{\pm0.10}$	$1.70^{\pm0.09}$
	7	$4.83^{\pm0.24}$	$2.18^{\pm0.16}$	$9.81^{\pm0.18}$	$2.04^{\pm0.10}$	nd
	14	$4.50^{\pm0.23}$	$2.23^{\pm0.16}$	$9.32^{\pm0.17}$	$2.95^{\pm0.10}$	nd
	30	$5.00^{\pm0.25}$	$2.58^{\pm0.18}$	$9.16^{\pm0.12}$	$2.80^{\pm0.09}$	nd
ME	1	$3.78^{\pm0.19}$	$2.90^{\pm0.15}$	$9.96^{\pm0.10}$	$2.74^{\pm0.09}$	$1.98^{\pm0.10}$
	5	$4.99^{\pm0.24}$	$2.52^{\pm0.17}$	$9.87^{\pm0.19}$	$1.80^{\pm0.09}$	$1.02^{\pm0.07}$
	7	$5.00^{\pm0.25}$	$2.48^{\pm0.18}$	$10.8^{\pm0.12}$	$1.63^{\pm0.09}$	nd
	14	$5.12^{\pm0.25}$	$2.20^{\pm0.16}$	$10.71^{\pm0.10}$	$1.26^{\pm0.08}$	nd
	30	$5.21^{\pm0.26}$	$2.85^{\pm0.14}$	$10.78^{\pm0.17}$	$1.00^{\pm0.05}$	nd
MF	1	$3.30^{\pm0.17}$	$2.69^{\pm0.14}$	$9.85^{\pm0.18}$	$2.70^{\pm0.09}$	$2.01^{\pm0.10}$
	5	$4.22^{\pm0.21}$	$2.20^{\pm0.16}$	$9.42^{\pm0.17}$	$1.84^{\pm0.12}$	$1.00^{\pm0.09}$
	7	$5.00^{\pm0.25}$	$2.11^{\pm0.20}$	$9.34^{\pm0.15}$	$1.36^{\pm0.11}$	nd
	14	$5.02^{\pm0.24}$	$2.06^{\pm0.15}$	$9.54^{\pm0.18}$	$1.11^{\pm0.11}$	nd
	30	$5.30^{\pm0.27}$	$2.01^{\pm0.15}$	$9.46^{\pm0.14}$	$1.05^{\pm0.07}$	nd

661 **Variation within treatments is not greater than 10% in all cases***.**

662 **Table 2.** pH, total acidity and lactose content of myzithra whey cheeses during
663 refrigerated (4°C) storage for 30 days.

myzithra cheese	Storage time (days)	pH	Total acidity (g lactic acid/ 100 g cheese)	Lactose (g /100 g cheese)
C	1	$6.47^{\pm0.04}$	$0.19^{\pm0.01}$	$3.78^{\pm0,08}$
	5	$6.45^{\pm0.05}$	$0.20^{\pm0.01}$	$3.80^{\pm0,08}$
	7	$6.47^{\pm0.05}$	$0.20^{\pm0.01}$	$3.79^{\pm0,07}$
	14	$6.44^{\pm0.04}$	$0.21^{\pm0.01}$	$3.80^{\pm0,07}$
	30	$6.43^{\pm0.04}$	$0.21^{\pm0.01}$	$3.78^{\pm0,04}$
F	1	$6.34^{\pm0.04}$	$0.30^{\pm0.02}$	$3.79^{\pm0,05}$
	5	$6.22^{\pm0.03}$	$0.38^{\pm0.02}$	$3.66^{\pm0,06}$
	7	$6.16^{\pm0.04}$	$0.40^{\pm0.02}$	$3.63^{\pm0,07}$
	14	$6.08^{\pm0.04}$	$0.48^{\pm0.03}$	$3.60^{\pm0,06}$
	30	$6.00^{\pm0.04}$	$0.48^{\pm0.03}$	$3.57^{\pm0,04}$
ME	1	$6.37^{\pm0.04}$	$0.31^{\pm0.02}$	$3.78^{\pm0,07}$
	5	$6.17^{\pm0.04}$	$0.50^{\pm0.03}$	$3.67^{\pm0,06}$
	7	$6.00^{\pm0.03}$	$0.58^{\pm0.04}$	$3.55^{\pm0,05}$
	14	$5.94^{\pm0.03}$	$0.60^{\pm0.04}$	$3.53^{\pm0,07}$
	30	$5.92^{\pm0.04}$	$0.63^{\pm0.04}$	$3.51^{\pm0,07}$
MF	1	$6.33^{\pm0.04}$	$0.29^{\pm0.02}$	$3.78^{\pm0,04}$
	5	$6.19^{\pm0.04}$	$0.28^{\pm0.02}$	$3.67^{\pm0,04}$
	7	$6.21^{\pm0.04}$	$0.40^{\pm0.03}$	$3.63^{\pm0,07}$
	14	$6.04^{\pm0.03}$	$0.44^{\pm0.03}$	$3.61^{\pm0,06}$
	30	$6.03^{\pm0.03}$	$0.47^{\pm0.03}$	$3.59^{\pm0,06}$

664 **Variation within treatments is not greater than 10% in all cases.*

Table 3. Terpenoid content (%) of myzithra cheese samples (C, F, ME) from the 1st day of storage (4°C) identified by SPME GC/MS.

Compound name	ID*	KI	KI from literature	C	F	ME
Monoterpens						
α-pinene	KI, MS	1025	1017[c] 1020[f] 1019[b]	Nd	Nd	$83.5^{\pm 0.17}$
camphene	KI, MS	1063	1053[c] 1080[h] 1063[a]	Nd	Nd	$0.8^{\pm 0.05}$
β-pinene	KI, MS	1105	1113[d] 1108[b]	Nd	Nd	$2.2^{\pm 0.13}$
3-carene	KI, MS	1138	1114[e] 1141[f]	Nd	Nd	$0.6^{\pm 0.10}$
β-myrcene	KI, MS	1152	1157[c] 1152[a] 1158[h]	Nd	Nd	$0.6^{\pm 0.11}$
2-carene	KI, MS	1164	1164[a]	Nd	Nd	$0.2^{\pm 0.05}$
D-limonen	KI, MS	1180	1188[e] 1198[b]	Nd	Nd	$1.3^{\pm 0.14}$
Beta-phellandrene	KI, MS	1188	1188[a]	Nd	Nd	$0.1^{\pm 0.03}$
o-cymene	KI, MS	1250	1250[a]	Nd	Nd	$0.7^{\pm 0.14}$
Oxygenated Monoterpens						
Eucalyptol	KI, MS	1196	1196[a]	Nd	Nd	$0.3^{\pm 0.12}$
Terpinolene	KI, MS	1259	1271[g]	Nd	Nd	$3.5^{\pm 0.13}$
Camphelnol	KI, MS	1482	1482[a]	Nd	Nd	$0.1^{\pm 0.05}$
Linalool	KI, MS	1531	1531[a]	Nd	Nd	$0.2^{\pm 0.05}$
Bornyl acetate	KI, MS	1571	1574[g]	Nd	Nd	$0.8^{\pm 0.12}$
4-terpineol	KI, MS	1593	1602[b] 1593[d]	Nd	Nd	$0.3^{\pm 0.14}$
Pinocarveol	KI, MS	1651	1651[a]	Nd	Nd	$0.4^{\pm 0.14}$
Verbenol	KI, MS	1671	1671[a]	Nd	Nd	$0.3^{\pm 0.05}$
α-terpineol	KI, MS	1688	1691[g]	Nd	Nd	$1.3^{\pm 0.12}$
Melilotal	KI, MS	1783	1783[a]	Nd	Nd	$0.2^{\pm 0.03}$
Myrtenol	KI, MS	1787	1789[g]	Nd	Nd	$0.2^{\pm 0.05}$
p-cymene-8-ol	KI, MS	1842	1846[g]	Nd	Nd	$0.7^{\pm 0.13}$

**ID: Method of identification, KI = tentative identification by Kovats retention index in accordance with literature [[a]: Schoina et al. (2014) [b]: Shiratsuchi, Shimoda, Minegishi, and Osajima (1993), [c]:Gardeli, Papageorgiou, Mallouchos, Kibouris, and Komaitis (2008), [d]: Högnadóttir and Rouseff (2003), [e]:Mallouchos, Paul, Bekatorou, Koutinas, and Komaitis (2007), [f]: Vichi et al. (2007), [g]: Lee, Umano, Shibamoto, and Lee (2005), [h]: Goodner (2008)], MS = tentative identification by mass spectra obtained from NIST107, NIST21, and SZTERP libraries.*

***Nd.: not detected*

****Variation within treatments is not greater than 10% in all cases.*

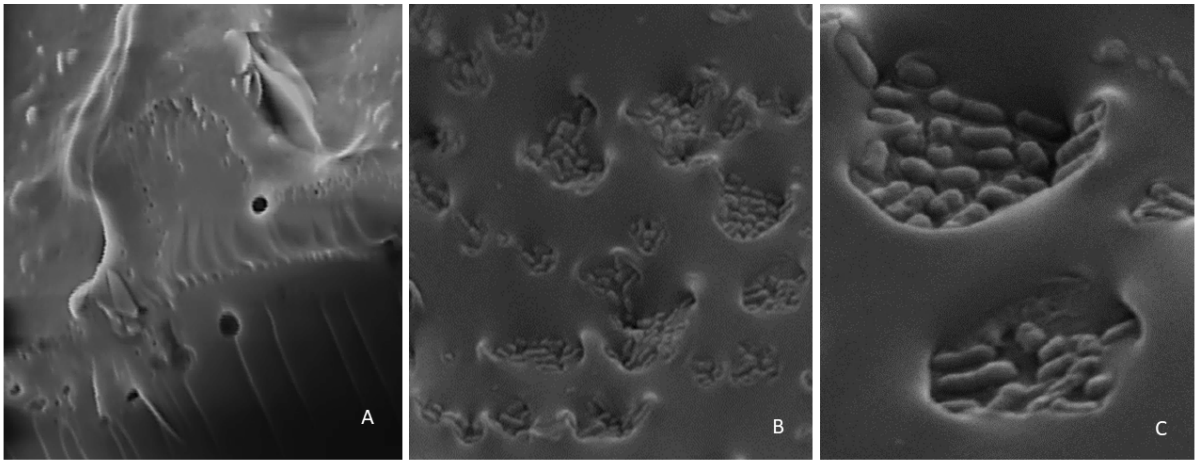
Figure captions

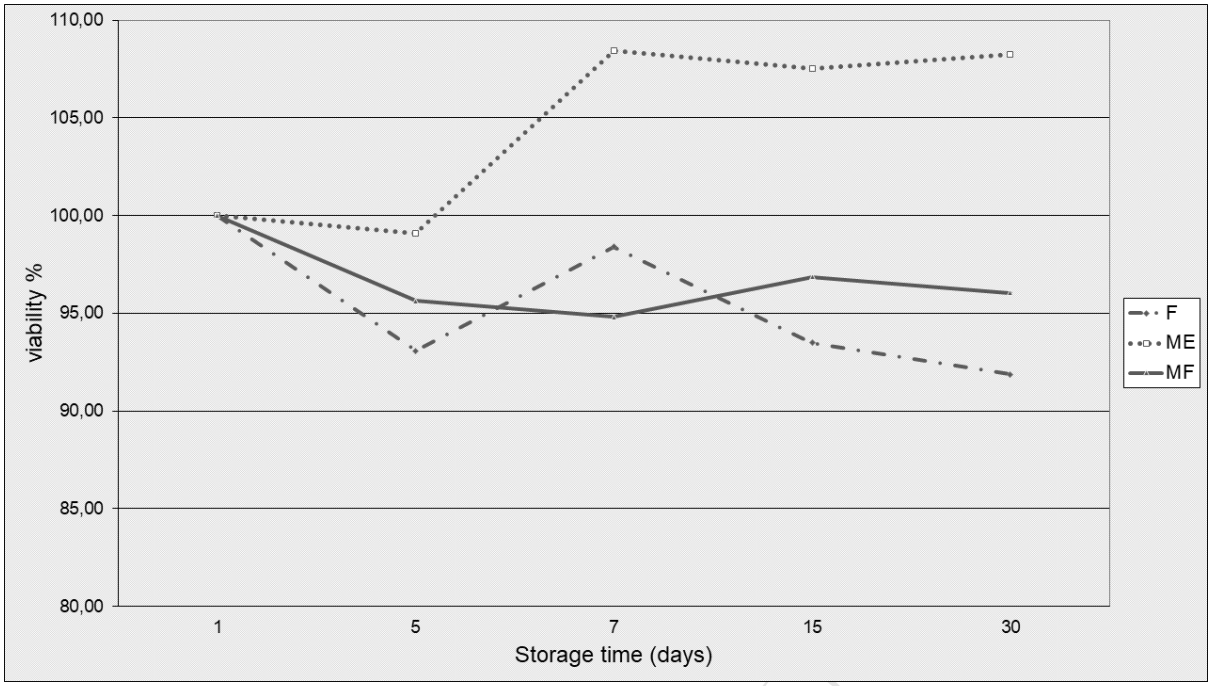
Figure 1. Electron micrographs of *Pistacia terebinthus* resin surface (A) and *L. casei* ATCC 393 cells in *Pistacia terebinthus* encapsulating matrix (B, C).

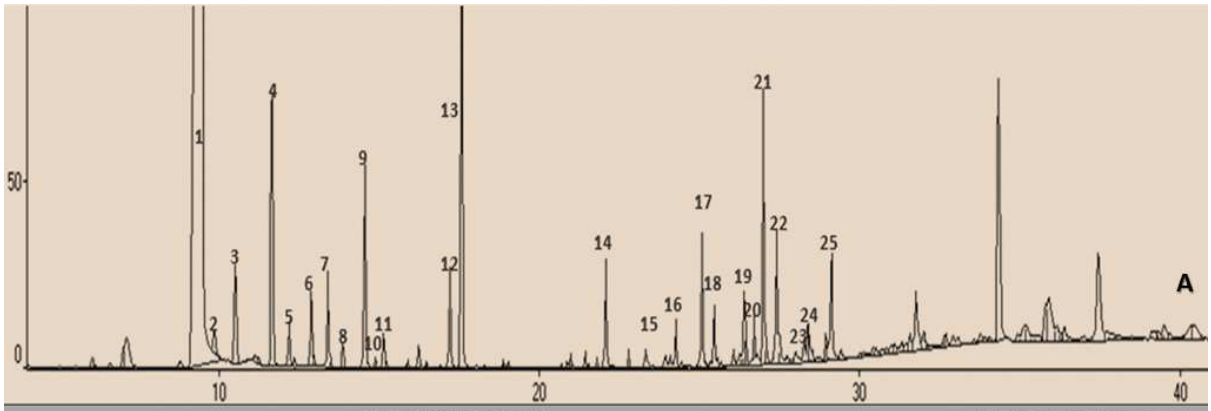
Figure 2. % Survival of *L. casei* in myzithra cheeses during refrigerated storage (4^{o}C) for 30 days.

Figure 3. SPME GC–MS spectra (full scan mode chromatogram - A) presenting the detected terpenes (% total area of hydrocarbons - B) of myzithra cheese with *L. casei* encapsulated cells within *Pistacia terebinthus* resin (ME) from the 1^{st} day of storage at 4^{o}C.

Figure 4. Sensory evaluation of produced myzithra chesses from the 1^{st} day of storage at 4^{o}C.

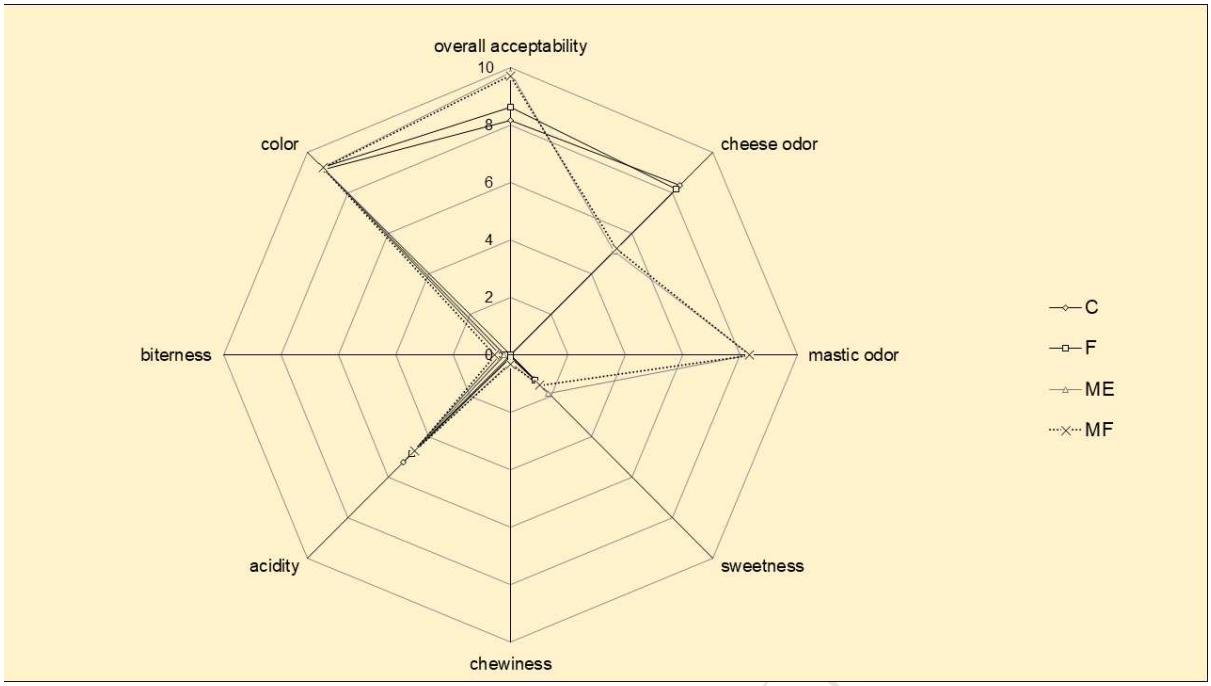






Component	% in the total area of hydrocarbons	Component	% in the total area of hydrocarbons
Monoterpene Hydrocarbons		**Oxygenated Monoterpenes**	
α-pinene **(1)**	83.5	Eucalyptol **(11)**	0.3
Camphene **(3)**	0.8	Terpinolene **(13)**	3.5
beta-pinene **(4)**	2.2	Camphenol **(15)**	0.1
3-carene **(6)**	0.6	Linalool **(16)**	0.2
beta-myrcene **(7)**	0.6	bornyl acetate **(17)**	0.8
2-carene **(8)**	0.2	4 terpineol **(18)**	0.3
D-limonene **(9)**	1.3	Pinocarveol **(19)**	0.4
beta-phellandrene **(10)**	0.1	Verbenol **(20)**	0.3
o-cymene **(12)**	0.7	a- terpineol **(21)**	1.3
total	**89.9**	5.8.10 undecatrien-3-ol **(22)**	0.7
		Melilotal **(23)**	0.2
		Myrtenol (24)	0.2
		p-cymene-8-ol **(25)**	0.7
		total	**9.0**

B



Highlights

- Manufacture of a novel functional myzithra cheese with probiotic characteristics.
- *Pistacia terebinthus* viscus matrix as encapsulation support of probiotic *Lactobacillus* cells.
- Significant reduction of fungi/yeasts in cheeses with incorporated *Pistacia terebinthus* resin.
- SPME GC/MS analysis indicates the upgraded terpenoid profile of cheeses with *P. terebinthus* resin.
- Terpene exceptional aroma and possible antimicrobial/ antioxidant effects to cheese products.