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Effect of modified atmosphere packaging on physicochemical and microbiological characteristics of Graviera Agraphon cheese during refrigerated storage

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Research Article

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Abstract

The aim of this work was to examine the effect of modified atmosphere packaging on the physicochemical and microbiological changes of Graviera Agraphon cheese during refrigerated storage. Blocks of Graviera Agraphon cheese weighing around 200 g were packaged under natural (control) or modified atmosphere packaging (MAP) conditions (50% N_2 – 50% CO_2) and stored at 4 °C or 10 °C for up to 85 d. Prior to packaging, groups of cheese blocks were inoculated with one each of the following foodborne pathogens at around 10^4 log cfu/g: *Listeria monocytogenes, Salmonella* Typhimurium, *Escherichia coli* O157:H7 or *Staphylococcus aureus,* whilst further groups of cheese blocks were not inoculated. The protein, fat, moisture and salt contents as well as the pH of control and MAP cheese samples did not change significantly ($P > 0.05$) throughout 4 °C storage, while the pH values of control and MAP cheese samples were significantly ($P < 0.05$) reduced at 10 °C storage. At 10 °C storage, yeasts and molds, psychrotrophs and lactic acid bacteria (LAB) were significantly higher ($P < 0.05$) for the normal atmosphere than the MAP cheese samples after the 4th, 8th and 4th days, respectively. At 4 °C storage, the yeasts and molds or psychrotrophs were significantly higher ($P < 0.05$) than those of control after the 6th and 15th days, respectively at 4 °C storage. All foodborne pathogens showed a higher decrease ($P < 0.05$) at 10 °C than 4 °C storage. *S. aureus* proved more sensitive in inactivation in the MAP conditions than atmospheric conditions. *L. monocytogenes* and *S. aureus* presented a higher decrease than that of *E. coli* O157:H7 and *S.* Typhimurium. In conclusion, MAP proved efficient in retarding the growth of yeasts, molds, psychrotrophs and *E. coli* O157:H7, *L. monocytogenes*, *S. Typhimurium* and *S. aureus* in Graviera Agraphon cheese during refrigerated storage at 4 and 10 °C.

Introduction

Graviera Agraphon cheese is a protected designation of origin (PDO) Greek hard cheese, produced in the mountain of Agrapha, central Greece. The cheese is made out of ewe's milk or mixtures of ewe's and goat's milk, the latter not exceeding a percentage of 30%. Graviera type hard cheeses are also produced in other regions of Greece with different types of milk or different milk mixtures. According to Greek legislation (Greek Codex Alimentarius, 2003), Graviera Agraphon cheese can be sold after three months of ripening. The cheese has been traditionally sold in its initial cylindrical shape (wheel) form. In recent years for commercialization or retail purposes, Graviera Agraphon cheese blocks are also frequently stored under modified atmosphere packaging (MAP) conditions (Fletouris *et al.*, 2015).

E. coli O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* are important food-borne pathogens. The occurrence of these pathogens in sheep and goat cheese products has been verified by several workers (Sara *et al.*, 1999; EFSA, 2008; Farrokh *et al.*, 2013; Pexara *et al.*, 2013). *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* outbreaks associated with consumption of cheese products have been reported in many countries (Espie *et al.*, 2006; Jelastopulu *et al.*, 2006; Pintado *et al.*, 2009; Van Duynhoven *et al.*, 2009).

The microbial stability of cheeses is determined by the combined application of different microbial hurdle factors (low pH, a_w values, NaCl, LAB) during the manufacturing process, ripening and storage (Leistner and Gorris, 1995). However, the fate of several pathogens in cheese products has not been extensively studied during post ripening storage. Modified atmosphere packaging (MAP) is an attractive preservation method for foods and has been extensively used for the refrigerated storage of various foods including cheese (Dermiki *et al.*, 2008). MAP involves the use of O_2 concentrations below atmospheric levels, CO_2 concentrations at relatively high concentrations, usually higher than 20%, and N_2 as an inert filler gas. CO_2 is active against several bacteria including food-borne pathogens.

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The aim of this study was to investigate the physicochemical and microbiological properties of Graviera Agraphon cheese under MAP conditions during refrigerated storage, as well as the fate of the food-borne pathogens *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus*.

Materials and methods

Graviera Agraphon cheese

Graviera Agraphon cheese was taken from a cheese manufacturing plant (Kissas Bros, Mouzaki, Greece), 3 months after its production. The loaves of Graviera Agraphon cheese (ca 13 Kg), obtained from the same production batch, were cut in blocks sized 11 × 9 × 1.7 cm with a weight of ca 200 g and used for the MAP tests.

Modified atmosphere packaging (MAP) and experimental design

The cheese blocks were packaged in plastic trays of 16 × 20.5 × 4.8 cm (Cryovac UBRT®, Cryovac, Sealed Air S.r.l., Passirana di Rho, Italy), and sealed by a sealing machine (model TSM105 Foodtech, Minipack-torreS. p.A., Dalmine, Italy) with a film (OPET, Cryovac) of 39 μm thickness, under MAP conditions (50% N_2 – 50% CO_2), or control (atmospheric air). The gas mixtures were obtained from Air Liquid SA, Athens, Greece.

Control and MAP cheese samples were stored at 4 and 10 °C, simulating proper or improper refrigerated storage, respectively. Control cheese samples were stored at 4 °C or 10 °C for 35 and 16 d, respectively. MAP cheese samples were stored at 4 °C or 10 °C for 85 and 28 d, respectively. The above storage times of control and MAP cheese samples at refrigerated temperatures were selected since preliminary tests showed visual spoilage by yeasts or molds by those days.

Headspace gas determination

Prior to opening, CO_2 and O_2 of the headspace of all packages were determined by using a gas analyser Checkmate O_2/CO_2 (PBI-Dansensor A/S, Ringsted, Denmark) equipped with a needle for penetrating through the package. A rubber septum (Syntech Ltd, Glasgow, UK) was glued on the upper surface of the package and pierce with a 23gauge needle connected to the gas analyzer.

Bacterial strains and inoculation procedure of the cheese blocks

The *L. monocytogenes* Scott A and Lmk strains, and *S. aureus* LHA and LHB strains *S.* Typhimurium ST1 and ST2 strains from our Laboratory stock were used. *E. coli* O157:H7 toxigenic strains EDL-932 and EDL-933 were obtained from Prof. Genigeorgis University of California, California, USA. Pathogen inocula were made as previously described (Govaris *et al.*, 2011). In brief, each strain of the pathogens was grown separately in 50 ml brain heart infusion broth (Oxoid, Basingstoke, UK) for 24 h at 37 °C, with two consecutive transfers. The bacterial cells were pelleted by centrifugation at 5000 g for 15 min at 5 °C and washed twice with 10 ml of 0.1 mol/ml phosphate buffer saline (PBS, Oxoid), pH 7.0, and diluted to 1.0×10^8 cfu/ml in PBS. The two strains of each pathogen were combined at equal concentrations for the inoculum of cocktail strains. Cell densities of the final suspension of the inoculum were determined by serial dilution and subsequent enumeration on tryptone soy agar TSA (Oxoid).

Prior to packaging, four groups of the cheese blocks were inoculated with a single pathogen inoculum of *L. monocytogenes, S.* Typhimurium, *E. coli* O157:H7 or *S. aureus,* while one group of cheese blocks served as control (no inoculation). Inoculation was carried out, by spreading on the upper and lower surface of these blocks the pathogen inocula using a sterile bent glass rod to an initial population of ca 10^4 log cfu/g, according to the procedure described by Govaris *et al.* (2011). Prior to inoculation, the cheese has been examined for contamination by the examined pathogens as described in the microbiological analysis section.

Microbiological analysis

Samples for microbiological analysis of Graviera Agraphon cheese packaged under MAP condition or control were taken at 0 d, 2-d intervals up to 10^{th} day and 5-d intervals up to the end of 4 °C storage, while 2-d intervals up to 4^{th} day and 4-d intervals up to the end of 10 °C storage. At each sampling time, duplicate cheese samples (25 g) were placed in the stomacher bags and aseptically filled with 225 ml of peptone water 0.1%. The content was macerated in the stomacher for 2 min at room temperature. Resulting slurries were serially diluted (1 : 10) in 0.1% sterile peptone water and plated (0.1 ml) on appropriate growth media. Populations of *L. monocytogenes* were determined on Palcam Listeria Selective Agar (Merck, Darmstadt, Germany) supplemented with Palcam Listeria Selective Supplement (No.1.1175, Merck) at 30 °C for 48 h. *S. aureus* populations were counted on Baird-Parker agar (Merck, Darmstadt, Germany) supplemented with egg yolk tellurite emulsion (Merck) (BPA + EYT) according to ISO 6888-1 (ISO, 1999), at 37 °C for 24 h. *S.* Typhimurium populations were estimated on selective XLD agar (Merck) at 37 °C for 24 h. *E. coli* O157:H7 counts were estimated on sorbitol MacConkey agar (Oxoid) at 37 °C for 48 h.

Before the inoculation trials, the absence of the pathogen in the cheese was verified by an initial enrichment. Duplicate cheese samples (25 g) were placed in sterile stomacher bags, diluted in 225 ml of modified *E. coli* broth (CM 990, Oxoid) supplemented with novobiocin (SR 181, Oxoid), Listeria enrichment broth (Merck), Rappaport–Vassiliadis broth (Merck), Baird broth (Merck) and incubated (24 h) at 37, 30, 42, 37 °C for *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, and *S. aureus,* respectively. Enriched samples were plated (0.1 ml) in duplicate on appropriate agar media for each pathogen, as described above.

LAB were estimated on de Man Rogosa Sharpe agar (Oxoid), as described by Govaris *et al.* (2002) at 28 °C. Psychrotrophs were enumerated on Plate Count Agar (Oxoid), after incubation at 7 °C for 10 d. Yeasts and molds were enumerated on the Yeast-extract-glucose chloramphenicol agar (YGC) (Merck) according to ISO 6611/IDF (2004). Microbiological analyses were performed in triplicate.

Physicochemical analysis

Standard methods were used for the determination of protein, fat, moisture, and sodium chloride in Graviera Agraphon cheese samples (APHA, 2004). The samples for physicochemical analysis were taken at the same time of microbiological analysis. At each sampling time, pH values of cheese samples were also determined with a pH meter (WTW, type 525, Wissennchaftlich –

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TechnischeWerkstatten, GmbH, D 82362 Weilheim, Germany). Physicochemical analyses were performed in triplicate.

Statistical analysis

Data were subjected to analysis of variance in the general linear model using the SPSS 10.05 statistical program (SPSS Ltd. Woking, UK). When significant treatment effects were disclosed, Duncan's multiple range tests with examination for significant differences at each storage interval for individual treatment were employed. A probability level of $P \leq 0.05$ was used in testing the statistical significance of all experimental data.

Results and discussion

Physicochemical analysis

Chemical analysis of cheese samples showed mean values of 27.1 ± 0.1% for protein, 32.50 ± 0.2% for fat, 34.2 ± 0.4% for moisture, and 2.54 ± 0.1% for sodium chloride. Results showed that protein, fat, moisture and salt contents did not change significantly ($P > 0.05$) among control or MAP cheese samples throughout storage at 4 °C or 10 °C. The cheese used in this study was in compliance with the Greek Codex Alimentarius (2003), which requires that Graviera Agraphon cheese should contain not less than 40% fat in dry matter but no more than 38% moisture.

The initial mean pH value (5.52 ± 0.2) of the MAP cheese samples was not significantly different ($P > 0.05$) than that of the control cheese samples and remained unchanged ($P > 0.05$) throughout the storage at 4 °C. However, the pH values of control and MAP cheese samples were significantly ($P < 0.05$) reduced to 5.10 ± 0.3 and 4.95 ± 0.3 by the end of 16 and 24 d storage at 10 °C, respectively. The decrease in pH of control or MAP cheese samples during storage at 10 °C may be due to further activity and growth of LAB as compared to lower growth at 4 °C. Similar pH values for other Graviera cheese types after repining in Greece were also observed by other workers (Moatsou *et al.*, 2004; Samelis *et al.*, 2010). In agreement to present findings, no important changes of pH values (ca 5.90) of a grated Graviera cheese type of Northern Greece packaged under aerobic or 100% nitrogen atmosphere during storage at 4 °C for 70 d (Mexis *et al.*, 2011). Other workers also observed a similar stability in pH value of other cheese types such as Stracciatella cheese (Gammariello *et al.*, 2009) or white cheese (Kirkin *et al.*, 2013) packaged under various MAP conditions during refrigerated storage.

Determination of headspace gases

The O_2 content of the headspace of MAP packages was estimated at 0.5% on 0 d and remained almost the same throughout the refrigerated storage at 4 °C or 10 °C. The initial CO_2 content of MAP packages was found to be 49.85% at 0 d and declined ($P < 0.05$) to 41.3 and 40% at 10^{th} and 12^{th} day of storage at 4 °C or 10 °C, respectively. No important changes of the CO_2 content were observed during subsequent storage time. The O_2 content of the headspace of control packages was found to be 20.5% on 0 d and decreased to 20.1 and 20.3% by the end of storage at 4 °C or 10 °C, respectively. Trobetas *et al.* (2008) found that the CO_2 content remained rather stable for grated Graviera cheese packaged under various MAP conditions (100% CO_2, 50% CO_2– 50% N_2, 100 N_2) during storage at 4 °C. Consistent with the present findings, a similar pattern of CO_2 changes for grated Graviera Agraphon cheese was observed by Fletouris *et al.* (2015) or other types of cheese products stored under MAP conditions, such as feta cheese (Govaris *et al.*, 2011) or white cheese (Kirkin *et al.*, 2013).

Microbiological analysis

Changes in yeasts and molds, psychrotrophs and LAB of control or MAP Graviera Agraphon cheese samples during storage at 4 °C or 10 °C are shown in Figs 1 and 2, respectively. The initial counts of yeasts and molds, psychrotrophs and LAB were 2.5, 5.8 and 7.7 log cfu/g, respectively. Similar populations for yeasts and molds or LAB were also observed for a type of Graviera cheese produced in Northern Greece after the ripening time in a previous work (Mexis *et al.*, 2011). Yeasts and molds, psychrotrophs and LAB of control or MAP cheese samples were significantly increased ($P < 0.05$) during storage at 4 °C or 10 °C. The yeasts and molds, psychrotrophs and LAB were significantly higher ($P < 0.05$) for control than MAP cheese samples after the 4^{th}, 8^{th}, and 4^{th} days, respectively, at 10 °C storage. During storage at 4 °C, the comparison of control samples to MAP cheese samples showed that LAB were not significantly different ($P > 0.05$) throughout storage, but yeasts and molds or psychrotrophs were significant higher ($P < 0.05$) after 6^{th} and 15^{th} days, respectively.

LAB can grow under aerobic or anaerobic conditions in various food products. No important differences in growth of LAB were found in grated Graviera cheese (Mexis *et al.*, 2011) or shredded Mozarella cheese (Eliot *et al.*, 1998) packed under aerobic and nitrogen atmosphere conditions during storage at 4 °C. Whitley *et al.* (2000) reported lower counts in LAB packed under various MAP conditions as compared to control samples packed under aerobic conditions. However, Kirkin *et al.* (2013) observed a higher decrease in LAB in white cheese packed under various MAP conditions as compared to packs under aerobic conditions during storage at 4 °C for 13 weeks. In agreement to present findings, the growth of psychrotrophs was retarded in cheese products stored under high CO_2 concentrations MAP conditions, such as Stracciatella cheese (Gammariello *et al.*, 2009), Cameros cheese (Gonzalez-Fandos *et al.*, 2000) or Mozzarella cheese (Alves *et al.*, 1996; Eliot *et al.*, 1998; Alam and Goyal, 2011). However, other workers did not observe any inhibitory effect of CO_2 against psychrotrophs in cottage cheese (Chen and Hotchkiss, 1991). The different behaviour of growth of the psychrotrophs in the presence of CO_2 may be due to the different nature of psychrotrophic microflora in the cheese products.

It is important to note that visible spots of spoilage were observed when populations of yeasts and molds reached ca 7 log cfu/g. The growth of yeasts and molds requires the presence of O_2, while an atmosphere with high levels of CO_2 acts against this growth (Singh *et al.*, 2012). The inhibitory action of CO_2 against yeasts and molds was demonstrated in previous studies for other cheese products stored under MAP conditions, such as Mozzarella cheese (Alves *et al.*, 1996; Eliot *et al.*, 1998), white cheese (Kirkin *et al.*, 2013), or Stracciatella cheese (Gammariello *et al.*, 2009).

E. coli O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* pathogens were significantly decreased ($P < 0.05$) during refrigerated storage under control or MAP at 4 °C (Fig. 3) or 10 °C (Fig. 4). It is also important to note that no significant differences ($P > 0.05$) were observed between populations of *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium for samples of Graviera Agraphon cheese stored under control or MAP

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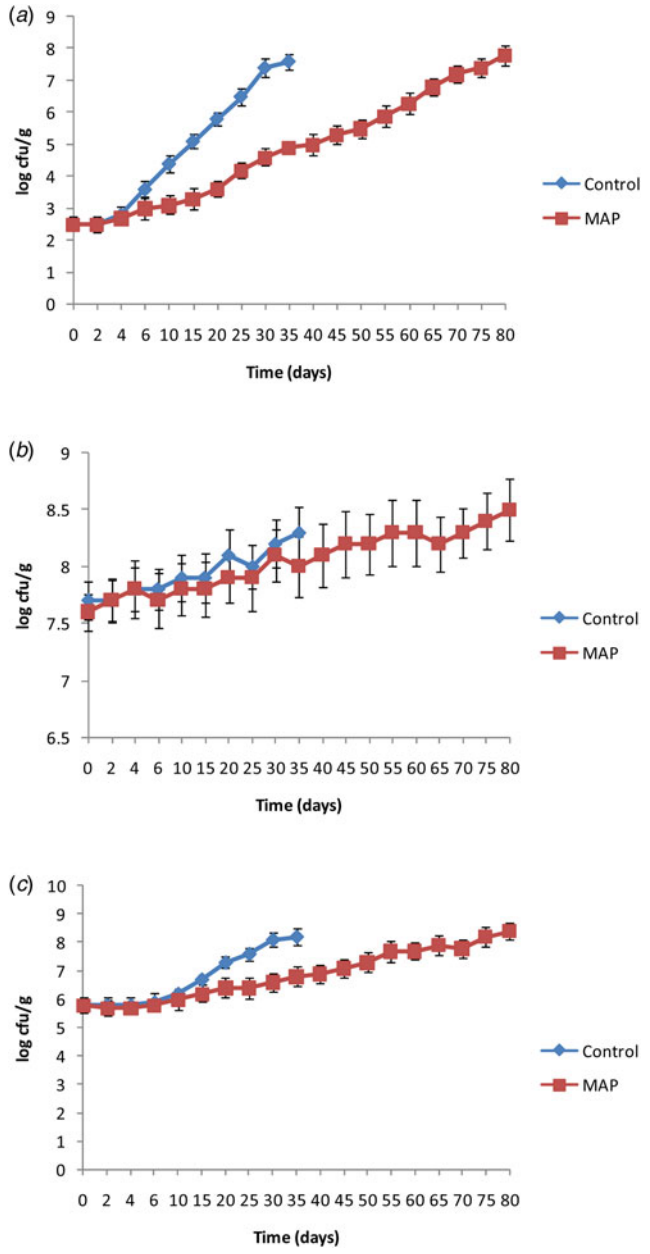

Fig. 1. Changes in yeasts and molds, LAB and psychrotrophs of control or MAP Graviera Agraphon cheese samples during storage at 4 °C (yeasts and molds = a; LAB = b; psychrotrophs = c).

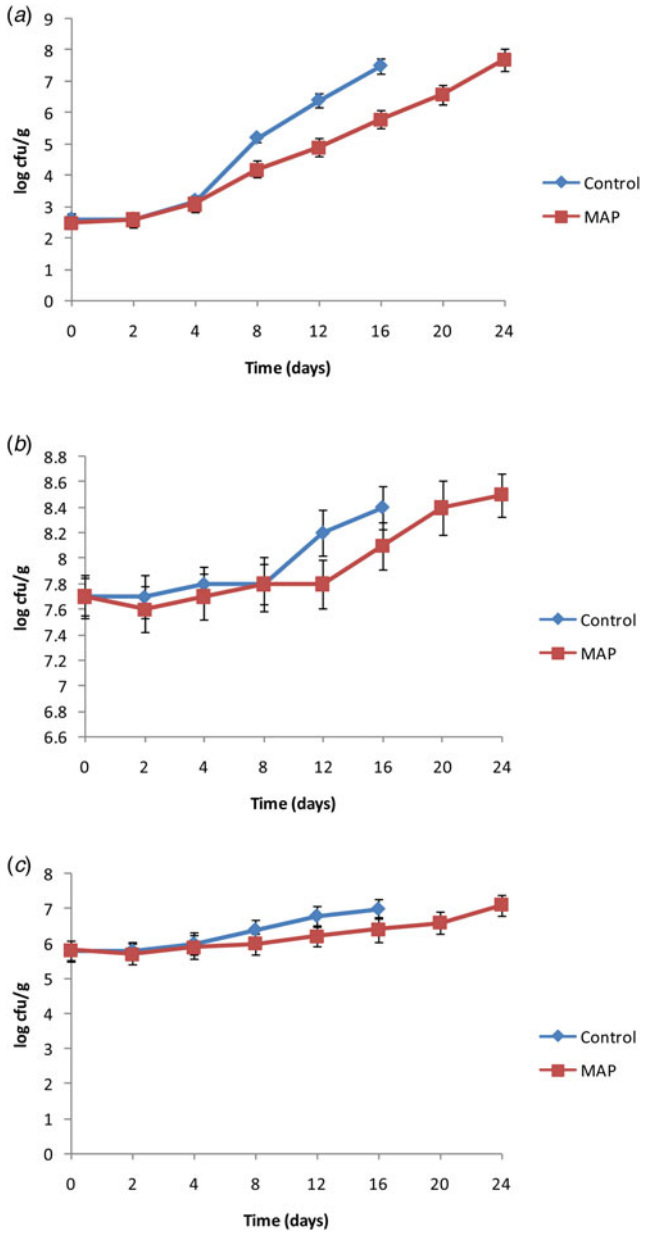

Fig. 2. Changes in yeasts and molds, LAB and psychrotrophs of control or MAP Graviera Agraphon cheese samples during storage at 10 °C (yeasts and molds = a; LAB = b; psychrotrophs = c).

throughout the storage at both refrigerated temperatures. In contrast, *S. aureus* showed populations significantly different ($P < 0.05$) between control and MAP after 10 and 8 d of storage at 4 and 10 °C, respectively.

To the best of our knowledge, there are no studies available on the fate of *E. coli* O157:H7, *S.* Typhimurium and *S. aureus* in cheeses stored under MAP conditions at refrigerated storage. In Cameros cheese, a fresh goat cheese with a pH of 6–6.7 and no starter culture, packed under 80%N_2 – 20% CO_2, 60% N_2- 40% CO_2, and 100% CO_2, *L. monocytogenes* populations increased by 3.4, 3.0 and 2.5 log cfu·g^{-1} by the end of 28 d storage at 4 °C, respectively (Gonzalez-Fandos *et al.*, 2000). In Stilton cheese, a mold ripened cheese with a pH of 6.2–6.6, packed under 80%N_2 – 10% CO_2 – 10% O_2, *L. monocytogenes* showed an initial lag of 2 weeks and a subsequent growth by 1.5 log cfu/g by the end of 6 weeks storage at 2 °C (Whitley *et al.*, 2000). Giannou *et al.* (2009) studied the fate of *L. monocytogenes* in ripened Graviera cheese, produced in the northwest of Greece, packed under air or vacuum and stored at 4, 12 and 25 °C for 90 d. The pathogen declined faster under air packaging than vacuum packaging for all temperature treatments. By the end of storage, the initial populations of *L. monocytogenes* (ca 3 log cfu/g) in Graviera cheese samples reached, 1.48 and 2.43 log cfu/g under air or vacuum packaging at 4 °C, respectively, but to undetectable level regardless of packaging (air or vacuum) at 12 °C or 25 °C.

In the present study, populations of all examined foodborne pathogens showed a higher decrease ($P < 0.05$) at 10 °C than 4 °C storage. Enhanced inactivation of certain foodborne

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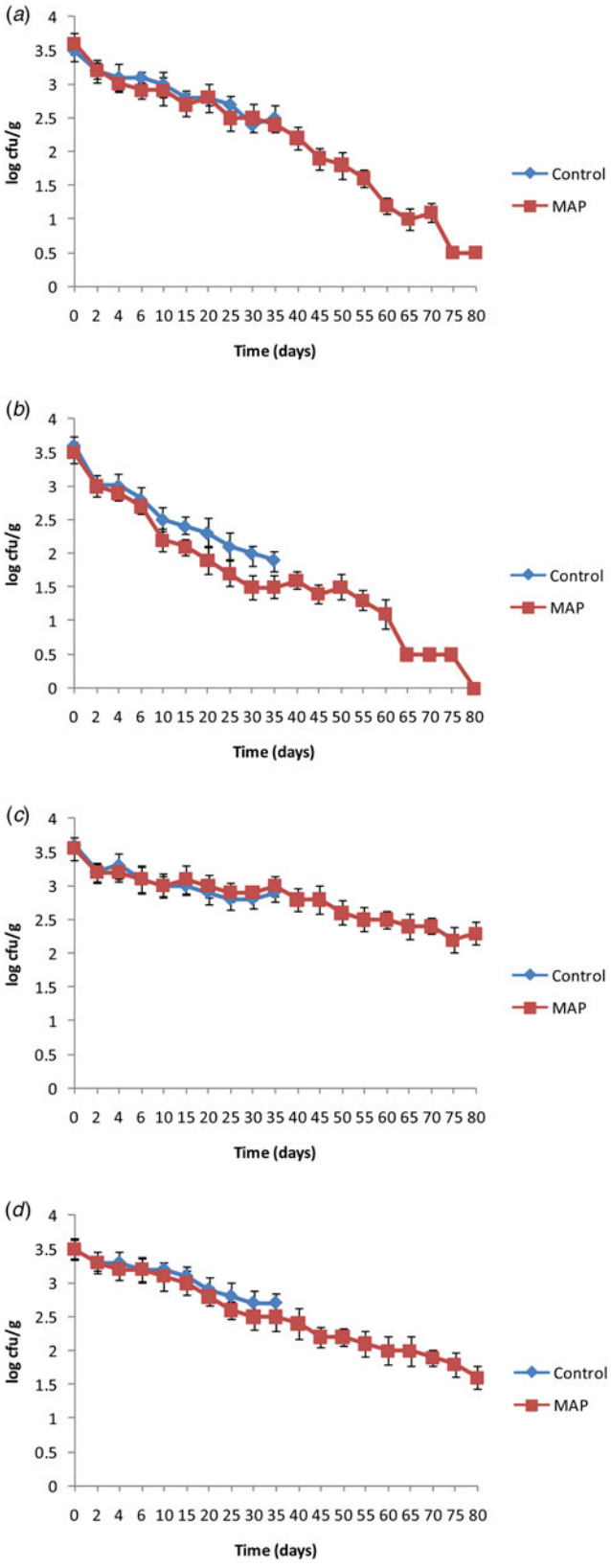

Fig. 3. Changes in *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* of control or MAP Graviera Agraphon cheese samples during storage at 4 °C (*L. monocytogenes* = a; *S. aureus* = b; *S.* Typhimurium = c; *E. coli* O157:H7 = d).

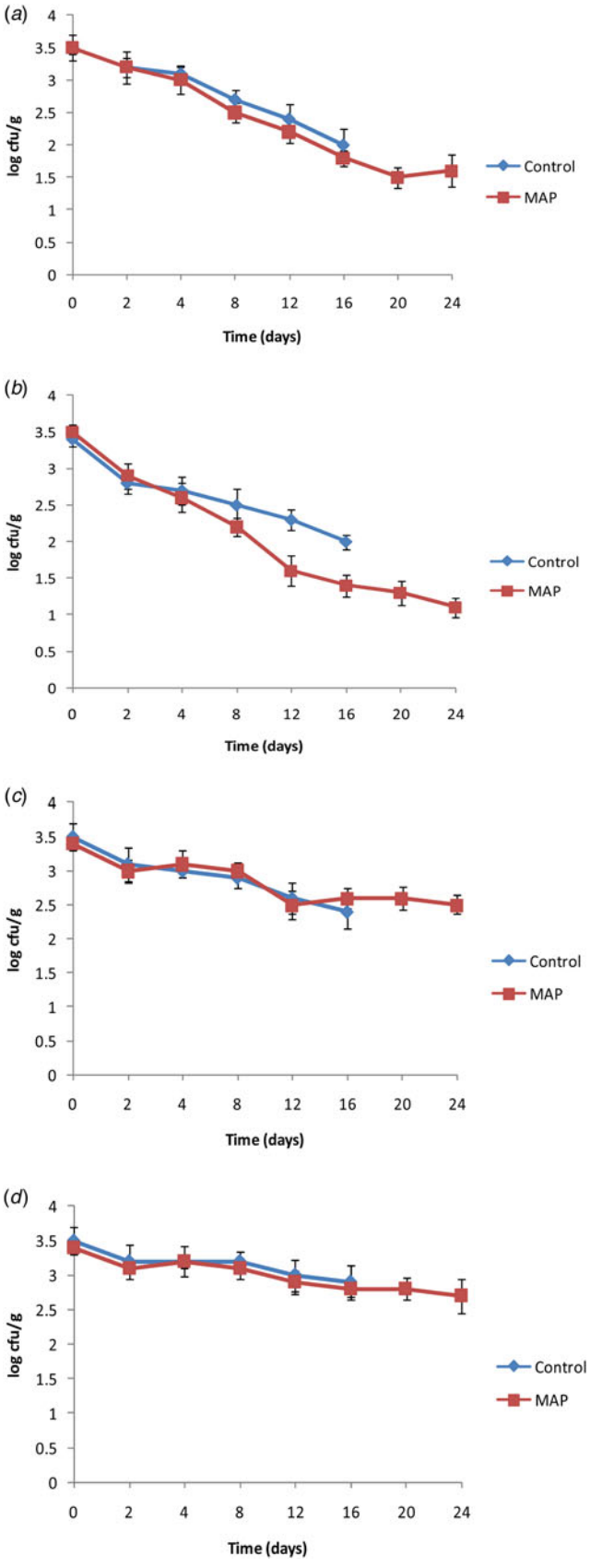

Fig. 4. Changes in *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* of control or MAP Graviera Agraphon cheese samples during storage at 10 °C (*L. monocytogenes* = a; *S. aureus* = b; *S.* Typhimurium = c; *E. coli* O157:H7 = d).

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pathogens at higher temperatures than at refrigerated temperatures was also reported in previous studies in post-aging cheese products. A higher inactivation was reported for *Salmonella* spp. (Shrestha *et al.*, 2011*a*) or *L. monocytogenes* (Shrestha *et al.*, 2011*b*) in cheddar cheese at 21 °C compared to 4 °C storage, and *E. coli* O157:H7 in feta cheese at 12 °C compared to 4 °C storage (Govaris *et al.*, 2002). Giannou *et al.* (2009) observed a higher survival of *L. monocytogenes* in Graviera cheese samples at 4 °C than 12 °C and 25 °C storage, as previously noted. This phenomenon of higher inactivation of foodborne pathogens may be due to a high antagonistic activity of LAB or lower pH values in cheese products at higher temperatures (Farrokh *et al.*, 2013).

According to our results, *S. aureus* proved more sensitive in inactivation in the MAP conditions than the atmospheric conditions in Graviera Agraphon cheese samples. The mechanism of the antimicrobial effect of CO_2 may be due to the displacement of oxygen, decrease in pH, and by cellular penetration (Eklund and Jarmund, 1983). In agreement with our results, previous *in-vitro* studies showed that *S. aureus* had a higher decrease than that of other *Enterobacteriaceae* food borne pathogens (e.g. *E. coli*) in atmospheres with high CO_2 content as compared to atmospheric air (Kimura *et al.*, 1999).

The Gram positive pathogens *L. monocytogenes* and *S. aureus* presented a higher decrease than that of Gram negative pathogens *E. coli* O157:H7 and *S.* Typhimurium. Sims *et al.* (1989) found a decrease in populations of *S. aureus*, but an increase in populations of *S.* Typhimurium in cottage cheese during storage at 10 °C. The lower decrease in populations of certain Gram-negative bacteria, as compared to Gram positive bacteria may be due to the protective role of the outer membrane of Gram negative bacteria against antimicrobial compounds such as lactic acid produced by LAB in fermented milk or cheese products (Sims *et al.*, 1989; Millette *et al.*, 2007).

In conclusion, MAP (50% N_2 – 50% CO_2) proved efficient in retarding the growth of yeasts, molds and psychrotrophs in Graviera Agraphon cheese during refrigerated storage 4 and 10 °C as compared to atmospheric packaging conditions. *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* populations were decreased either in MAP or atmospheric conditions, but *S. aureus* decreased faster in the MAP conditions than the atmospheric conditions.

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