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Utilization of microalgae in probiotic white brined cheese

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Abstract

In this study, the effects of *Chlorella vulgaris* and *Arthrospira platensis* fortification on the microbiological, physicochemical and antioxidative properties of probiotic white cheese during storage were investigated. Thereby six groups of white cheese samples were manufactured as follows WC (Control), LAC (*Lactobacillus acidophilus* LA-5), CWC (*C. vulgaris*), SWC (*A. platensis*), CLAC (*C. vulgaris* + *Lb. acidophilus* LA-5) and SLAC (*A. platensis* + *Lb. acidophilus* LA-5). The viability of *Lb. acidophilus* for SLAC sample remained almost constant during storage (>7 log cfu/g) while physicochemical properties of samples showed significant differences ($P<0.01$). The CLAC sample contained increased levels of protein, Ca, P, K, Mg and Zn while the highest Fe values were detected in the SLAC sample. Samples fortified with microalgae showed higher 1,1-diphenyl-2-picrylhydrazyl (DPPH)radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC) and total phenolic content. Consequently, fortification with both microalgae improved the viability of probiotic, nutritional and antioxidative attributes of white cheese.

Key words: microalgae; probiotic cheese; microbiological; antioxidative properties

Introduction

Over the past few decades, international health and nutritional agencies are focused on preventing the occurence of diseases instead of finding optimal treatments of these diseases which are becoming more common. Accordingly, the consumption of functional foods and biologically active compounds has become more important. Functional foods show health-promoting properties such as improving the physical, mental and psychological condition of individuals. The most common biologically active compounds used in functional foods are probiotics, prebiotics, herbals, phytochemicals, polyunsaturated fatty acids, vitamins and microalgae. Their proven health properties and positive lifestyle effects have resulted in greater focus of consumer awareness and scientific studies on foods fortified with these ingredients (Nicoletti 2016; Yilmaz-Ersan et al., 2020; Alongi and Anese, 2021).

Probiotics providing beneficial health effects for the host when consumed in adequate amounts are live microorganisms including *Bifidobacterium* and *Lactobacillus* subsp. (Hill et al., 2014). *Lb. acidophilus* La-5 is the most common probiotic bacteria used in fermented dairy products. The health benefits accredited to *Lb. acidophilus* LA-5 include regulation of intestinal microbiota, inhibition of the growth of intestinal pathogens, antidiarrheal effect, an enhanced immune system, as well as treatment and / prevention of various cancers (Khan and Mahmud, 2015; Matias et al., 2016; Linn et al., 2019; Najarian et al., 2019).

Products such as yoghurt, cheese and ice cream which contain probiotics have also been shown to provide wellbeing effects and are extensively being categorized as nutrient-dense foods by the dairy industry. Cheese has nutritional value, therapeutic attributes and enjoys a broad popularity among consumers. Due to high nutritional components and bioavailability of nutrients, cheese contributes to meeting the necessary daily intake of multiple nutrients (Henriques and Pereira, 2017). Compared to fermented milks, cheese is also a more favorable probiotic carrier during both, storage and gastrointestinal transit due to its higher solid matrix, pH value, buffering capacity, denser matrix of texture and oxygen content (Castro et al., 2015; Tamime et al., 2018). To exert therapeutic effects, viable probiotic bacteria should be able to maintain viability of a product until the end of its shelf life and also reach the gut of the host in sufficient quantity (from 6 to 11 log CFU/g or mL) (Erkmen and Bozoglu, 2016; Terpou et al., 2019; Fiore et al., 2020). The numerous compositional and process factors influencing the viability and survivability of probiotic cultures in cheese are as follows: heat treatment of milk, pH, molecular oxygen, redox potential, types of inoculation, microbial competitions, step-wise/ stage-wise fermentation, salting, ripening and/or storage conditions, growth promoters, food additives, packaging materials and application of novel technologies such as microencapsulation (Champagne and Rastall 2009; Castro et al., 2015; Homayouni et al., 2018). Non-protein nitrogen, sugar sources, vitamins, maltose, dextrin, prebiotics (such as inulin and oligosaccharides) have all been used as growth-promoting substances in probiotic cheese production (Karimi et al., 2011; Gao et al., 2021).

Currently, many studies are focused on enriching cheese with microalgae as a growth promoter for probiotics as well as to increase the functional product characteristics. *Arthrospira platensis* (previously named *Spirulina platensis;* blue-green microalgae) and *Chlorella* (green microalgae) are rich sources of proteins, lipids, carbohydrates, vitamins (A, B_2, B_6, $B_{12,}$ E, H, and K), minerals (Ca, P, Na, Fe, Se), essential amino acids (lysine, leucine, valine and isoleucine), essential fatty acids (omega-3 and omega-6 polyunsaturated fatty acids, gamma linolenic acid), volatile compounds, pigments and various antioxidants (Alsenani et al., 2015; Andrade et al., 2018). As both species are composed of high-quality proteins and well-balanced amino acids, the joint expert consultation of WHO/FAO have recommended them for fulfiiling the daily requirements of essential amino acids (Mahmoud et al., 2018; Pattanaik et al., 2019; Martelli et al., 2020). In addition to their nutritional value, a number of natural antioxidant compounds have been found in both microalgae, in the form of phenolic compounds (caffeic acid, p-coumaric acid, ferulic acid; Zakaria et al., 2020), flavonoid compounds (quercetin, kaempferol, apigenin; Goiris et al., 2014), phytopigments (phycocyanin, chlorophyll, carotenoids, xanthophylls; Silva et al., 2020), vitamins (A, C, E; Del Mondo et al., 2020) and minerals (Se; Radhakrishnan et al., 2017). *Arthrospira platensis* and *Chlorella vulgaris* also exhibit various beneficial health effects in terms of antimicrobial, antiinflammatory, anticarcinogenic, antihypertensive, antioxidant, antiobesity, hypercholesterolemia, hypoglycemic, radiation protection, reduction of hyperlipidemia and a reduction in wound healing time (Deng and Chow, 2011; Wells et al., 2017; Rani et al., 2018; Bellahcen et al., 2020; Menaa et al., 2021; Ramos-Romero et al., 2021). Various studies have shown that they promote growth and acid production of probiotics as potential prebiotic candidates due to the presence of free amino acids, cell wall polysaccharides, adenine, peptone, hypoxanthine and vitamins in their composition (Parada et al., 1998; Varga et al., 1999; De Caire et al., 2000; Varga et al., 2002; Cho et al., 2004; Gyenis et al., 2005; Molnár et al., 2005; Bhowmik et al., 2009; Zare et al., 2012; Beheshtipour et al., 2013; Malik et al., 2013; Mocanu et al., 2013; Hanan et al., 2019).

Considering all the above-mentioned facts, the aim of the present research was to develop a white cheese with the multiple functional effects of probiotic and microalgae and present novel dairy products to the functional food segment. In this study, white cheese was formulated using *Lactobacillus acidophilus* (LA-5) as the probiotic culture, with *Arthrospira platensis* and *Chlorella vulgaris*, and subsequently the microbiological, physicochemical and antioxidative properties of cheese examined during a 90-day storage period.

Materials and methods

Materials

Raw cow milk was supplied from a local dairy plant (Ozseymenler Food and Dairy Products Company, Bursa, Turkey). The *Lactobacillus acidophilus* (LA-5) starter culture that was used as the probiotic strain in this study was purchased from Chr. Hansen's (Hoersholm, Denmark). *Chlorella vulgaris* and *Arthrospira platensis* were supplied as a freeze-dried biomas from Akuatik Natural Products (Adana, Turkey). Microbial rennet (Fermento 220; Konya, Turkey) was used, which was produced by biotechnological fermentation of *Rhizomucor miehei* isolated from plants.

Manufacturing of probiotic white cheese fortified with microalgae

Probiotic white cheeses fortified with microalgae were manufactured at Ozseymenler Food and Dairy Products Company (Bursa, Turkey). Six groups of functional white cheeses were manufactured by using probiotic bacteria and different microalgae; WC (conventional white cheese without probiotic culture; control), LAC (white cheese with *Lb. acidophilus* LA-5), CWC (white cheese with *C. vulgaris*), SWC (white cheese with *A. platensis*), CLAC (white cheese with *C. vulgaris* + *Lb. acidophilus* LA-5) and SLAC (white cheese with *A. platensis* + *Lb. acidophilus* LA-5). The milk was pasteurised at 72 °C for 20 min, cooled down to 37 °C and divided into six equal parts. Subsequently, a solution of calcium chloride (0.02 % w/v; Merck, Germany), rennet, probiotic, *C. vulgaris* and *A. platensis* (1 % wt/vol) were added to cooled milk. *Lb. acidophilus* LA-5 was also incorporated into the cheeses in order to achieve at least 10^7 CFU g^{-1}. After coagulation, curd was cut into 1 cm^3 and was left to stand for 20 min (Eren-Vapur and Ozcan, 2012). The surfaces of the curds were covered with cheese cloth and pressed to facilitate whey expulsion for 90 min until the curd reached the appropriate strength, and the resulting pressed cheeses were cut into small rectangular blocks of 400-500 g. The cheese samples were salted (NaCl) in a 12 % (w/v) brine for 4 days then ripened at 8 % (w/v) brine at 4±1 °C for 90 days. Analyses of cheeses were conducted during storage.

Microbiological analysis

Cheese samples were taken for microbiological enumeration during the storage period (1, 30, 60 and 90 days). The total mesophilic aerobic bacteria (TMAB) were counted on a Plate Count Agar (PCA; Merck, Germany) and incubated at 30 °C for 72 h in aerobic conditions (ISO 4833:2003). The non-starter lactic acid bacteria (NSLAB) were determined using the De Man, Rogosa and Sharpe agar (MRS agar; Merck, Germany), incubated at 37 °C for 72 h under anaerobic conditions in jars with Anaero Gen Gas Packs (Oxoid, Basingstoke, UK) (Kasimoglu et al., 2004). The *Lb. acidophilus* count was enumerated on a MRS-Bile (MRS agar with 0.15 % (wt/vol) of bile) and incubated at 37 °C for 72 h in anaerobic conditions (Mortazavian et al., 2007). Plates containing 30-300 colonies were enumerated and recorded as a log of colony forming units (cfu) per g of cheese (log cfu/g).

Physico-chemical analysis

The physico-chemical composition of samples was determined using the following standard methods: dry matter, titratable acidity by the Lactic Acid Method, salt by titration with $AgNO_3$ using the Mohr Method, fat by the Soxhlet Method, protein and ash content (AOAC, 2000). Mineral elements were determined by means of an Inductively Coupled Plasma Optic Emission Spectroscopy (ICP-OES, Perkin Elmer Optima 8000, CT, USA) (Akpinar-Bayizit et al., 2010). Vitamins (C, A and E) were detected by using the high performance liquid chromatography (HPLC, 20ACBM, Shimadzu, Japan) consisting of a Diode-Array Detection (DAD, SPD-M20A) for vitamins A and C and a refractive index detector (Shimadzu, Kyoto, Japan) for vitamin E connected to a recorder (Lampi et al.,1999; Aktas et al., 2005; Michlová et al., 2015).

Preparation of the probiotic cheese extracts for total antioxidant capacity and total phenolic content assays

A water soluble extract of cheese was prepared by modifying according to the method described by Timón et al. (2019). Briefly, 10 g of cheese samples were homogenized in a blender with 5 mL of distilled water and agitated in a dark water bath (35-37 °C for 60 min). Then the samples were centrifuged (11.200 g, 30 min and 4 °C) and the obtained supernatant clarified through a paper filter (75 g m^2, 0.2 mm thickness). The filtered samples were used for antioxidant capacity by DPPH, CUPRAC and total phenolic content assay.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant capacity of samples was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, as described in the literature (Matos et al., 2019). 100 mL of methanol containing 0.01 % DPPH was prepared for the stock solution (0.03949 mg DPPH completed with methanol in a 100 mL volumetric flask). 15 mL of the stock solution was obtained and completed to 250 mL with methanol in a flask. 3900 µL prepared DPPH solution was

added into 100 µL extract. After 30 minutes in the dark, readings were made at 515 nm in the spectrophotometer against methanol. The antioxidant capacity values of extracts for white cheese samples were calculated as "mg trolox equivalent (TE) / g extract" by creating a trolox calibration chart (Matos et al., 2019).

Cupric ion reducing antioxidant capacity(CUPRAC) analysis

Ammonium acetate, copper (II) chloride and neocuproine solutions were prepared. The absorbance values of the samples kept in the dark for 30 minutes by adding 1 mL of copper (II) chloride, 1 mL of neocuproine, 1 mL of ammonium acetate, 0.6 mL of extract and 0.5 mL of distilled water, respectively, were read against pure water in the spectrophotometer at 450 nm (Apak et al., 2004). Calibration graphs were drawn by calculating the results. The antioxidant capacity values of extracts for white cheese samples were calculated using gallic acid calibration charts and the following equation:

Total Antioxidant Capacity (mg GAE/g dry matter) =
A /ε x Vt / Vö x S x Ve/m(1)
A = Sample absorbance measured at 450 nm
ε = Molar absorption coefficient of gallic acid compound in CUPRAC method
Vt = Total volume of CUPRAC measuring solution (4.1 mL)
Vö = Sample volume (mL)
S = Dilution factor (if dilution is not made, this factor is "1")
Ve = Volume of extract prepared (mL)
m = The amount of sample taken in the extraction process (g)

Total phenolic content assay

The total phenolic content was measured according to the method reported in the literature, using a Folin-Ciocalteu reagent (Jayaprakasha et al., 2001; Singh et al., 2002). 1 mL of Folin-Ciocalteu solution (diluted 1:10 with distilled water) was added into 100 µL of extract, waiting for 5 min before 7.5 % sodium carbonate solution was added. The samples were completed to 10 mL with distilled water. The absorbance values of the samples kept in the dark for 30 min. were determined to be at 760 nm in the spectrophotometer. Stock gallic acid solutions of 100 mg L^{-1} in various concentrations were prepared and the absorbance values at 760 nm of the stock solutions were determined by analysis applied to the cheese samples from which the standard curve equation was created. Afterwards, the amount of phenolic compound of white cheese samples in terms of gallic acid were calculated as "mg gallic acid equivalent (GAE) g^{-1} extract" based on the equation.

Sensory analysis

Sensorial properties of samples were evaluated by a panel of six semi-trained panelists. Each panelist was selected on the basis of interest in sensory description of milk products from the Bursa Uludag University. Cheese samples were removed from a refrigerator (4 °C) 1 h prior to sensory analysis, kept at 20±2 °C and presented in three digit blinding codes. Samples were served simultaneously with a glass of water to clean the oral cavity between sampling. A standard five-point hedonic scale from 1 (the lowest grade=I really disliked it) to 5 (the highest grade=I really liked it) was used to evaluate sensorial attributes of cheese samples. All samples were evaluated for sensory properties such as color and appearance, structure, odor, taste and overall acceptability.

Statistical analysis

The results of this study were analysed by two-way (cheese type and storage time) ANOVA (Minitab Inc., State College, PA, USA). Fischer's multiple range test was performed to estimate the significance of differences between mean values at P≤0.01. Principal component analysis (PCA) was performed on microbiological, physicochemical and antioxidant data. Hierarchical cluster analysis (HCA; clustering) was used to discriminate probiotic cheese samples (Statistica Software Version 10.0 StatSoft Inc, France).

Results and discussion

Microbiological properties of white cheese samples

Results of the total mesophilic aerobic bacteria counts are presented in Figure 1a. Statistical analysis showed that cheese type and storage time had a significant (p≤0.01) effect on the TMAB counts. During storage, SWC (*A. platensis*) had higher counts of TMAB compared to other samples, followed by WC (control) and CWC (*C. vulgaris*) samples. It could be obsereved that the storage period significantly affected (p≤0.01) the TMAB counts which also increased in all cheeses except for LAC sample during storage. Generally, it may be considered that the microbial load of microalgae caused higher total mesophilic aerobic bacteria counts of SWC and CWC samples. However, samples (CLAC and SLAC) containing *Lb. acidophilus* had lower counts due to antimicrobial components such as organic acids, bacteriocins and hydrogen peroxide produced by this probiotic. Raw milk, inadequate pasteurization, contamination after pasteurization, biofilms formed in milk processing equipment and cheese production environment are sources for non-starter lactic acid bacteria (NSLAB) composed of *Lactobacillus, Pediococcus* and *Micrococcus* species (Ong and Shah, 2009; Settanni and Moschetti, 2010). The results of the NSLAB counts are presented in

Figure 1b. There were significant differences (p≤0.01) within these of samples throughout storage. It was determined that generally, the CLAC sample (*C. vulgaris* and *Lb. acidophilus*) on 1, 30 and 60 days of storage had a higher count of NSLAB compared to the other samples, which may be related to the higher *Lb. acidophilus* counts at same storage periods in the CLAC sample. Same trends were also observed for SLAC (*A. platensis* and *Lb. acidophilus*). During the storage period, NSLAB counts decreased except for WC (control) and SWC (*A. platensis*) samples. Some previous studies have reported that the low pH, the high salt content, the hydrolytic activity of enzymes and low ripening temperature have caused such a decrease in NSLAB counts (Diezhandino et al., 2015). Parada et al., (1998) stated that extracellular products obtained from *S. platensis* in the exponential development stage supported the development of *Lactobacillus bulgaricus, Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus casei* and *Streptococcus thermophilus* in vitro. Molnár et al., (2005) stated that *S. platensis* biomass significantly increased the acid production of various mesophilic lactic acid bacteria. They reported that *S. platensis* had an important buffering capacity due to its alkaline character, thus it has an effect on acid formation and promotes bacterial viability. However, Minervini et al., (2012) and Patrignani et al., (2019) described that probiotics in cheese promote the growth of cheese starter culture. Generally, it was thought that the synergistic interaction between *Lb. acidophilus* and microalgae might result in higher NSLAB counts of CLAC and SLAC after storage of 60 days. The viable probiotic counts during storage is the most important qualitative parameter for beneficial health effects. Figure 1c. shows *Lb. acidophilus* counts during storage. Accordingly, bacterial counts in samples had statistically significant changes during storage (p≤0.01). At 60 days, viable counts of *Lb. acidophilus* among all samples did not differ significantly (P>0.05). A decrease was observed in the *Lb. acidophilus* count for LAC (*Lb. acidophilus*) during storage. The viability of *Lb. acidophilus* for CLAC (*C. vulgaris* and *Lb. acidophilus*) sample varied from 7.13 to 6.76 log cfu/g while those of SLAC (*A. platensis* and *Lb. acidophilus*) sample ranged from 7.91 to 7.28 log cfu/g during storage. CLAC sample (*C. vulgaris* and *Lb. acidophilus*) had a higher count of NSLAB compared to the other samples during storage. Thus, *Lb. acidophilus* counts in the CLAC sample are decreased during storage potentially due to inhibitors such as lactic acid, hydrogen peroxide, bacteriocins and alcohol produced by NSLAB. Similarly, Mazinani et al., (2016) found that the *Lb. acidophilus* count in probiotic white cheese produced by using *Mentha longifolia* (0.5 % and 1 %), *S. platensis* (0, 0.3, 0.5 and 0.8 %) with *Lb. acidophilus* varied from 7.14 to 7.64 log cfu/g. Results obtained from this study are generally consistent with researchers who suggested that the addition of *Arthrospira platensis* has a positive effect on the growth of probiotic bacteria in fermented milk (Varga et al., 1999, 2002; Beheshtipour et al., 2013; Mocanu et al., 2013), yoghurt (Guldas and Irkin, 2010), and cheese during storage (Golmakani et al., 2019; Terpou et al., 2020). Researchers have reported that the probiotic promoter effect of *A. platensis* may be due to the presence of vitamins, amino acids, minerals, peptone, adenine and hypoxanthine in its composition. Food matrix incorporated probiotic and microalgae, type and/or concentration of probiotic and microalgae have caused different results. Although there is no universally accepted probiotic microorganism number to achieve beneficial health effects, it is recommended that fermented products contain 6 to 11 log cfu/g of a probiotic culture (Erkmen and Bozoglu, 2016; Terpou et al., 2019; Fiore et al., 2020). IDF (International Dairy Federation) recommends that 100 g of cheese should be consumed daily for functional effects, with a range between 8 and 12 log cfu/day depending on the probiotic strain (Patrignani et al., 2019). Generally, cheese as a probiotic carrier should have two main criteria mainly: i) the compatibility with eventual starter and/or non starter culture during manufacturing and ii) content of a viable cell of at least 6 log cfu/g during the shelf life (Patrignani et al., 2019). The results of this study show that cheese fortified with *A. platensis* and *C. vulgaris* can be a good vehicle for delivery of *Lb. acidophilus* LA-5 (>6 log cfu/g). In addition, the present study showed that *Lb. acidophilus* in white cheese production could be used as the sole starter culture.

Physico-chemical properties of white cheese samples

The physico-chemical properties of cheese samples as a function of storage time are presented in Table 1. There were significant differences (p≤0.01) between cheese samples and storage period. The titratable acidity values of WC (control), CWC (*C. vulgaris*) and CLAC (*C. vulgaris* and *Lb. acidophilus*) samples were almost the same (0.23 % and 0.24 %, respectively) while the acidity of SWC (*A. platensis*) cheese was somewhat higher (0.37 %). The titratable acidity values were in agreement with Darwish (2017) who reported that Kareish cheese containing *S. platensis* had higher titratable acidity values because microalgae stimulated the growth of cheese microbiota. The highest salt values (6.47 and 6.37 %) were determined in SLAC (*A. platensis* and *Lb. acidophilus*) and WC (control) samples. The dry matter content of LAC (*Lb. acidophilus*) and CLAC (*C. vulgaris* and *Lb. acidophilus*) was almost the same (32.27 and 31.19 %) while the dry matter content of control cheese was slightly lower. This result was in agreement with the results found by Ortakci et al. (2012) and Minervini et al. (2012) who studied the properties of probiotic Mozzarella cheese. The highest titratable acidity, salt and dry matter values of cheese samples were determined at 60 days of storage, which resulted from absorption and/or diffusion of salt and some water-soluble components between brine and cheese. The increase in titratable acidity values may be due to the residual lactose in cheese converting into lactic acid and the formation of free fatty acids and amino acids via lipolysis and proteolysis (Sulejmani et al., 2021).

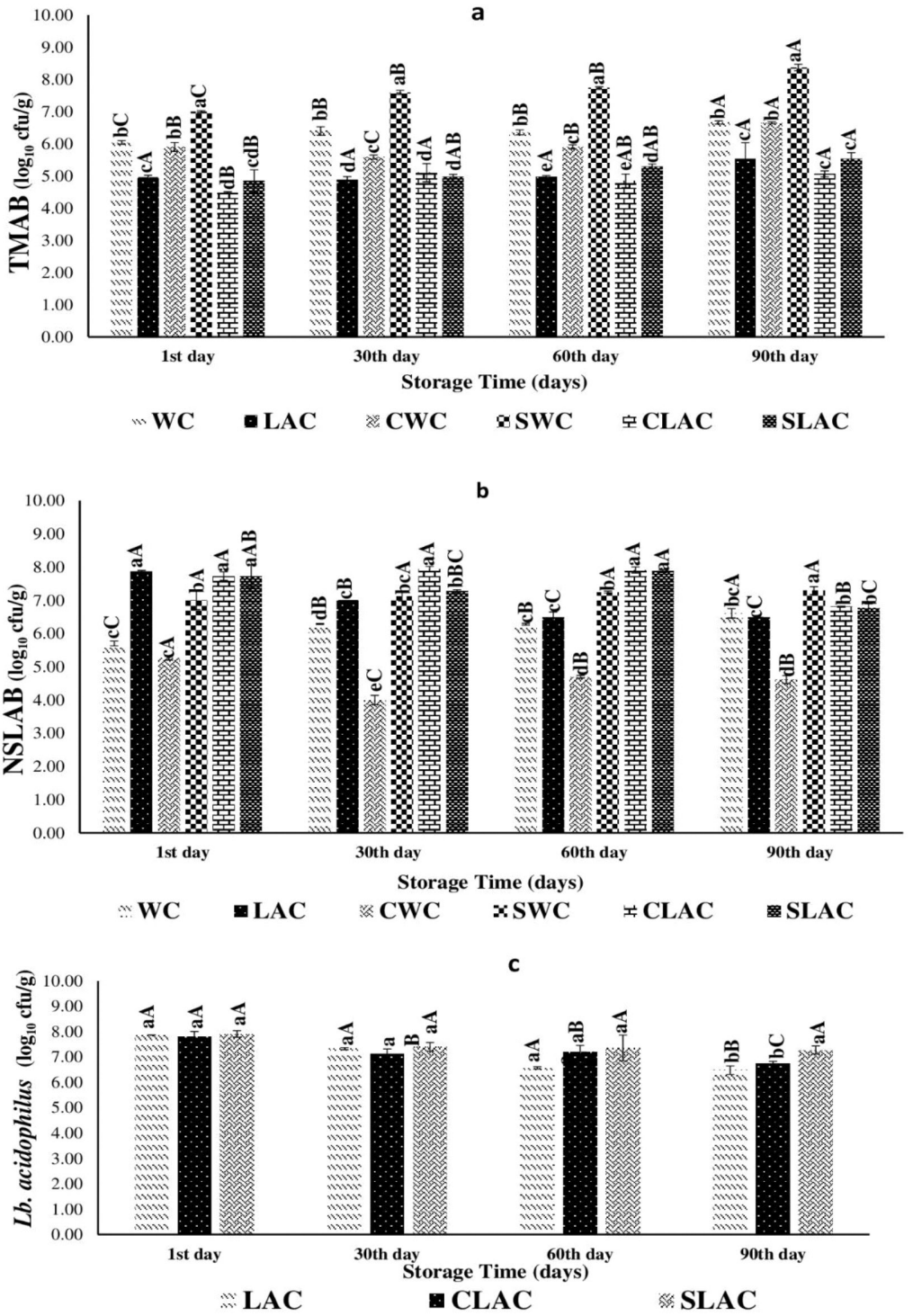

Figure 1. a) Total mesophilic aerobic bacteria (TMAB) counts b) Non-starter lactic acid bacteria (NSLAB) counts c) Lb. acidophilus counts of white cheese fortified with microalgae during storage;

(WC - conventional white cheese without probiotic culture; LAC - white cheese with Lb. acidophilus LA-5; CWC - white cheese with C. vulgaris; SWC - white cheese with A. platensis; CLAC - white cheese with C. vulgaris + Lb. acidophilus LA-5; SLAC - white cheese with A. platensis + Lb. acidophilus LA-5)

[a]; Lower case letters indicate statistically different groups between samples in a storage period (P≤0.01)

[A]; Capital letters indicate statistically different groups of samples in each storage period (P≤0.01)

The control (WC) had higher fat and ash content than other samples. The highest protein content (13.74 %) was in the CLAC sample (*C. vulgaris* and *Lb. acidophilus*), mainly due to the high protein content of *C. vulgaris* (61.22 g/100 g). These results are similar to those of Mohamed et al. (2013), who reported that cheeses including *C. vulgaris* were richer in protein, carbohydrates and fiber contents than control. Mazinani et al. (2016) stated that the addition of *S. platensis* at different rates (0; 0.3; 0.5 and 0.8 %) caused an increase in the protein content of cheese. Golmakani et al. (2019) reported that the values of titratable acidity, dry matter, and protein contents of feta-type cheese with *S. platensis* were higher than the control. Tohamy et al. (2018) stated that protein values in processed cheese were determined as 12 % for control, 12.13 % for cheese with 2 % *C. vulgaris* and 12.25 % for cheese with 4 % *C. vulgaris*. Also, fat content was determined as 19.5 % for the control sample, 19.8 % and 20.5 % for processed cheese with 2 % and 4 % *C. vulgaris*. Agustini et al. (2016) cited that utilization of *S. platensis* powder (0 % to 1.5 %) caused no significant difference in fat content of soft cheese. In this study, fat, protein and ash values tended to decrease during the storage period. The degradation of fat in cheese via lipolysis caused a decrease of fat content, while proteolysis led to the production of water-soluble nitrogen compounds resulting in reduction of protein content. In this study, the low amount of microalgae addition (1%) resulted in unstable variations on the physico-chemical properties of probiotic cheese. In addition, these variations can be explained that when microalgae are added in dry form and dissolved in cheese brine, their nutritional soluble components pass from the cheese to brine.

The mineral elements and vitamins of probiotic cheese analysed at the first day of storage are presented in Table 2. Significant differences were detected in mineral values among samples depending on the probiotic and microalgae used ($p \leq 0.01$). It was determined that the most abundant minerals in cheese samples were Ca, P, Na and K. The CLAC (*C. vulgaris* and *Lb. acidophilus*) sample had higher Ca, P, K, Mg and Zn than other samples, which could be due to increased mineral content of *C. vulgaris*. The highest Fe value was detected in the SLAC *(A. platensis* and *Lb. acidophilus*) sample. Similarly, Mazinani et al. (2016) reported that as the concentration of *S. platensis* increased, Fe values of samples increased in cheese containing *S. platensis, Lb. acidophilus* and *Mentha longifolia L*. Darwish (2017) stated that as the concentration (0.5; 1; 1.5 %) of *S. platensis* in Kareish cheese increased, the Fe content of cheese samples also increased from 1.33 mg/100 g to 2.05 g/100 g. Puyfoulhoux et al. (2001) stated that Fe bioavailability from *S. platensis* was better than other stuffs, thus it is thought to be an important source of daily Fe intake, especially for women. Tahomy et al. (2018) determined that Fe, Mg and K values were 52.26 mg/kg, 38.53 mg/kg and 62.69 mg/kg for processed cheese with 4 % *C. vulgaris* and 6.938 mg/kg, 17.27 mg/kg and 46.39 mg/kg for the control, respectively. When vitamin E values were compared, SWC, LAC, CLAC and SLAC samples contained higher ($P \leq 0.01$) levels, while the CWC and WC samples contained less. Vitamin A levels ranged from 0.85 mg/100 g for the WC sample to

Table 1. Physico-chemical properties of white cheese fortified with microalgae

Samples	N	Titratable acidity (%)	Salt (g/100 g)	Dry matter (g/100 g)	Fat (g/100 g)	Protein (g/100 g)	Ash (g/100 g)
WC	8	0.23±0.11^{b}	6.37±0.12^{a}	31.33±0.46^{b}	10.74±1.87^{a}	11.40±0.31^{f}	7.36±0.43^{a}
LAC	8	0.26±0.07^{b}	6.16±0.22^{b}	32.27±1.27^{a}	10.68±2.57^{a}	12.65±0.97^{c}	6.73±0.55^{c}
CWC	8	0.24±0.18^{b}	5.89±0.41^{c}	30.83±1.44^{b}	7.17±2.51^{e}	11.88±1.46^{e}	6.94±0.26^{b}
SWC	8	0.37±0.14^{a}	5.96±0.56^{c}	30.69±0.60^{b}	7.31±2.12^{d}	12.57±0.77^{d}	6.30±0.21^{e}
CLAC	8	0.24±0.05^{b}	5.89±0.17^{c}	32.11±0.59^{a}	8.82±0.95^{c}	13.74±0.59^{a}	6.51±0.28^{d}
SLAC	8	0.27±0.04^{b}	6.47±0.63^{a}	31.19±1.33^{b}	10.36±0.74^{b}	13.19±0.46^{b}	7.10±0.70^{b}
Storage days							
1. day	12	0.29±0.10^{b}	6.23±0.78^{ab}	31.49±1.37^{ab}	10.18±0.74^{a}	13.13±1.55^{a}	7.36±0.74^{a}
30. day	12	0.20±0.06^{c}	5.94±0.36^{c}	31.37±1.38^{ab}	9.73±1.10^{b}	12.76±1.90^{b}	6.76±0.41^{b}
60. day	12	0.36±0.15^{a}	6.29±0.29^{a}	31.87±0.63^{a}	9.33±0.97^{c}	12.61±2.61^{c}	6.59±0.34^{c}
90. day	12	0.29±0.06^{b}	6.21±0.13^{b}	30.98±0.77^{b}	6.93±0.99^{d}	11.60±1.96^{d}	6.66±0.25^{bc}
ANOVA							
Samples		**	**	**	**	**	**
Storage days		**	**	**	**	**	**
Samples x Storage days		**	**	**	**	**	**

***P≤0.01; [a]; Lower case letters indicate statistically different groups between samples*
WC - conventional white cheese without probiotic culture; LAC - white cheese with Lb. acidophilus LA-5; CWC - white cheese with C. vulgaris; SWC - white cheese with A. platensis; CLAC - white cheese with C. vulgaris + Lb. acidophilus LA-5; SLAC - white cheese with A. platensis + Lb. acidophilus LA-5

3.48 mg/100 g for the LAC sample while Vit C was 26.42 mg/100 g for the CLAC sample and up to 40.55 mg/100 g for the WC sample. Tahomy et al. (2018) stated that processed cheese with 4 % *C. vulgaris* had higher A, D, B6 and B2 vitamins than control cheese. In a recent study by Del Mondo et al. (2020) Vitamin A and C values in cheeses without microalgae were higher than other cheeses. This result can be explained because vitamin contents of microalgae are affected by temperature, salinity, light, nutrient or metal concentrations. In addition, the use of the dried form and low concentration of microalgae in cheese production resulted in lower Vitamin A and C.

Antioxidant capacity and total phenolic content of white cheese samples

Microalgae act as free radical scavengers due to antioxidant compounds contained in their water-soluble phycocyanin pigments, carotenoids, phenolic components and vitamins (Barka and Blecker 2016). Changes in DPPH, CUPRAC and TPC values of the cheeses analysed at the beginning and end of storage with the results of the statistical analysis are given in Figures 2a, b and c. Statistical analyses reveal that cheese type and storage time were the effective factors on DPPH, CUPRAC and TPC values ($p \leq 0.01$). Significant differences were determined in DPPH values between cheese with and without microalgae ($p \leq 0.01$). Therefore, the addition of microalgae, especially *C. vulgaris,* had positive effects on the DPPH values of cheese. The lowest DPPH value was determined in WC (control) and LAC (*Lb. acidophilus*) samples on day 1 (0.129 and 0.133 mg trolox/g, respectively) and day 90 (0.132 and 0.138 mg trolox/g, respectively). The highest values were in CWC (*C. vulgaris;* 0.164 mg trolox/g) and CLAC (*C. vulgaris* and *Lb. acidophilus;* 0.162 mg trolox/g) samples on day 1; in SWC (*A. platensis;* 0.179 mg trolox/g), CLAC (*C. vulgaris* and *Lb. acidophilus;* 0.178 mg trolox/g) and SLAC (*A. platensis* and *Lb. acidophilus;* 0.182 mg trolox/g) samples on day 90. In addition, it was determined that DPPH values of SWC (*A. platensis*), CLAC (*C. vulgaris* and *Lb. acidophilus*) and SLAC (*A. platensis* and *Lb. acidophilus*) increased during storage. Darwish (2017) reported that as the concentration (0; 0.5; 1; 1.5 %) of *S. platensis* in Kareish cheese increased, the DPPH value increased from 0.199 mg TE/100 g to 4.688 mg TE/100 g, respectively. At the end of the storage period, probiotic cheese (LAC) had higher DPPH value than control cheese (WC), which may result from various peptides formed as a result of the activity of *Lb. acidophilus* in ripening. The steady increase of antioxidant capacity continued until the end of storage. Similar results were reported by Songisepp et al. (2004) who cited increase of total antioxidant activity of probiotic cheese in ripening. Bjekić et al. (2021) reported that DPPH value (0.95 μM TE/g) of fresh cheese produced with kombucha inoculum was higher than for cheese produced with traditional starter culture (0.93 μM TE/g). Statistical analyses revealed that the cheese types and storage time were the effective factors on CUPRAC values. The lowest CUPRAC value was determined in WC (control; 0,039 mg GA/g) and LAC (*Lb. acidophilus*; 0.209 mg GA/g) samples on day 1. The highest values were determined in SWC (*A. platensis*; 0.285 mg GA/g) sample on day 1; in SLAC (*A. platensis* and *Lb. acidophilus*; 0.723 mg GA/g) sample followed by SWC (*A. platensis*; 0.412 mg GA/g) sample at the end of storage. Phenolic compounds (phenols, flavonoids, phenylpropanoids, tannins, phenolic acids) are critical secondary metabolites in determining the antioxidant capacity of foods. The lowest total phenolic amounts were determined in the LAC sample (*Lb. acidophilus*; 1.891 mg GAE/g) at the first day of

Table 2. *The mineral elements and vitamins of probiotic cheese fortified with microalgae*

	Cheese samples						
	WC	**LAC**	**CWC**	**SWC**	**CLAC**	**SLAC**	**P**
Mineral elements (mg/100 g)							
Ca	791.16±0.23^{c}	669.73±0.00^{d}	617.39±0.55^{e}	850.24±0.34^{b}	894.34±0.48^{a}	610.26±0.37^{f}	**
P	302.65±0.073^{c}	268.02±0.03^{d}	252.23±0.04^{e}	314.54±0.06^{b}	363.36±0.51^{a}	248.80±0.00^{f}	**
Na	131.28±0.40^{a}	100.22±0.31^{c}	66.21±0.29^{d}	54.33±0.47^{f}	63.48±0.68^{e}	112.93±0.01	**
K	85.34±0.02^{c}	76.86±0.02^{d}	85.80±0.14^{c}	89.42±0.59^{b}	113.82±0.03^{a}	49.53±0.04^{e}	**
Fe	2.37±0.002^{d}	2.60±0.03^{d}	3.16±0.23^{c}	4.36±0.02^{a}	3.75±0.07^{b}	4.68±0.03^{a}	**
Mg	0.17±0.03^{e}	14.67±0.03^{d}	17.52±0.03^{c}	21.04±0.06^{b}	26.32±0.45^{a}	14.14±0.20^{d}	**
Zn	1.54±0.01^{bc}	1.39±0.06^{c}	1.04±0.02^{d}	1.50±0.0^{bc}	1.75±0.07^{a}	1.62±0.03^{ab}	**
Cu	0.04±0.01^{a}	0.05±0.00^{a}	0.06±0.00^{a}	0.08±0.00^{a}	0.05±0.00^{a}	0.08±0.00^{a}	ns
Vitamins (mg/100 g)							
Vitamin C	40.55±0.07^{a}	30.50±0.011^{c}	26.18±0.26^{d}	27.45±0.64^{d}	26.42±0.03^{d}	31.58±0.00^{b}	**
Vitamin E	22.55±0.00^{d}	44.66±0.09^{b}	22.37±0.10^{d}	51.45±0.07^{a}	30.54±0.06^{c}	30.51±0.01^{c}	**
Vitamin A	0.85±0.07^{f}	3.48±0.03^{a}	1.03±0.04^{b}	1.29±0.01^{b}	1.83±0.02^{b}	1.59±0.00^{b}	**

***P≤0.01; ns non-significant; [a]; Lower case letters indicate statistically different groups between samples,*
WC - conventional white cheese without probiotic culture; LAC - white cheese with Lb. acidophilus LA-5; CWC - white cheese with C. vulgaris; SWC - white cheese with A. platensis; CLAC - white cheese with C. vulgaris + Lb. acidophilus LA-5; SLAC - white cheese with A. platensis + Lb. acidophilus LA-5

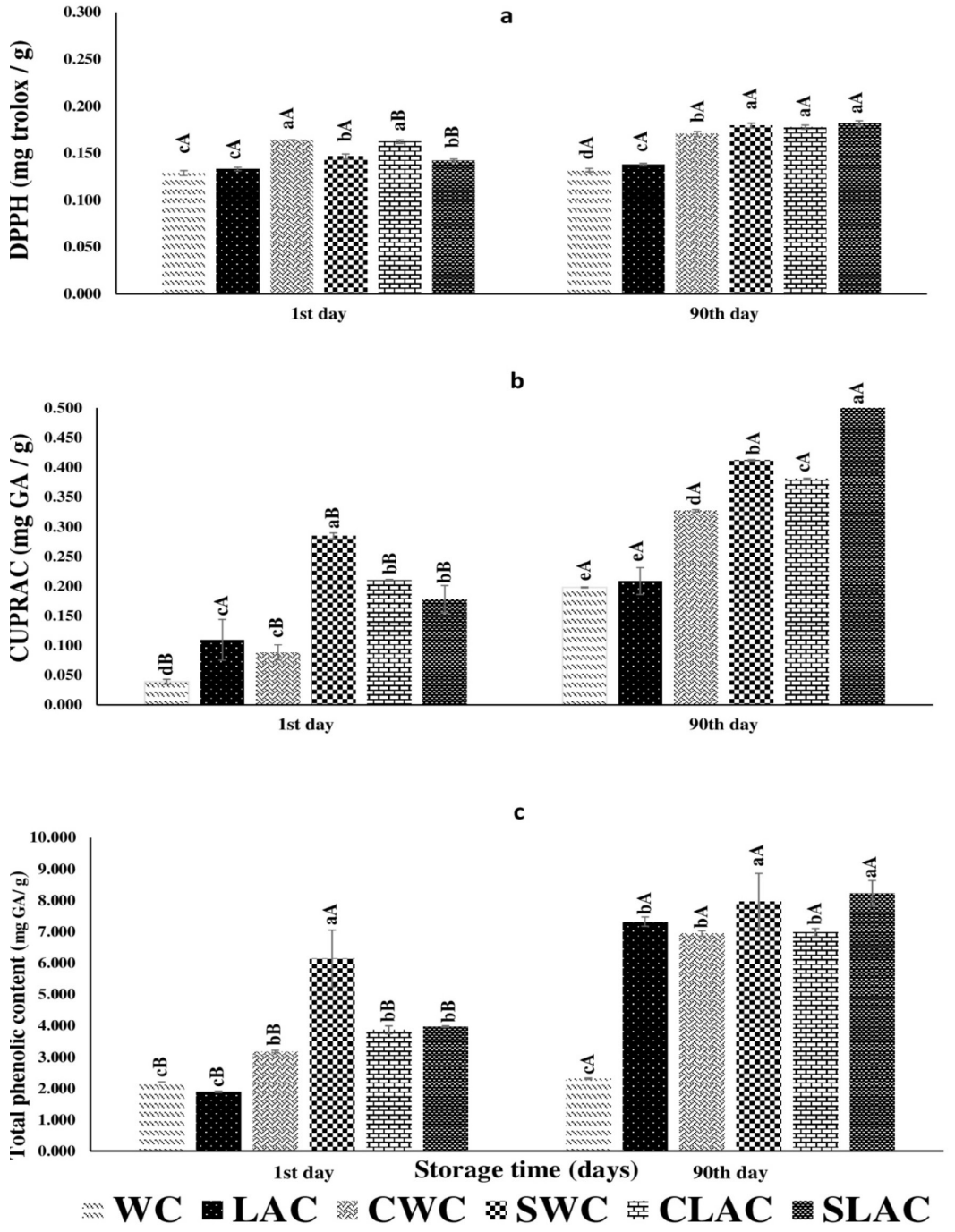

Figure 2. a) DPPH (1-diphenyl-2-picrylhydrazyl radical scavenging activity) values b) CUPRAC (cupric ion reducing antioxidant capacity) values c) TPC (total phenolic content) of probiotic white cheese samples fortified with microalgae during storage

(WC - conventional white cheese without probiotic culture; LAC - white cheese with Lb. acidophilus LA-5; CWC - white cheese with C. vulgaris; SWC - white cheese with A. platensis; CLAC - white cheese with C. vulgaris + Lb. acidophilus LA-5; SLAC - white cheese with A. platensis + Lb. acidophilus LA-5)

[a]; Lower case letters indicate statistically different groups between samples in a storage period (P≤0.01)

[A]; Capital letters indicate statistically different groups of samples in each storage period (P≤0.01)

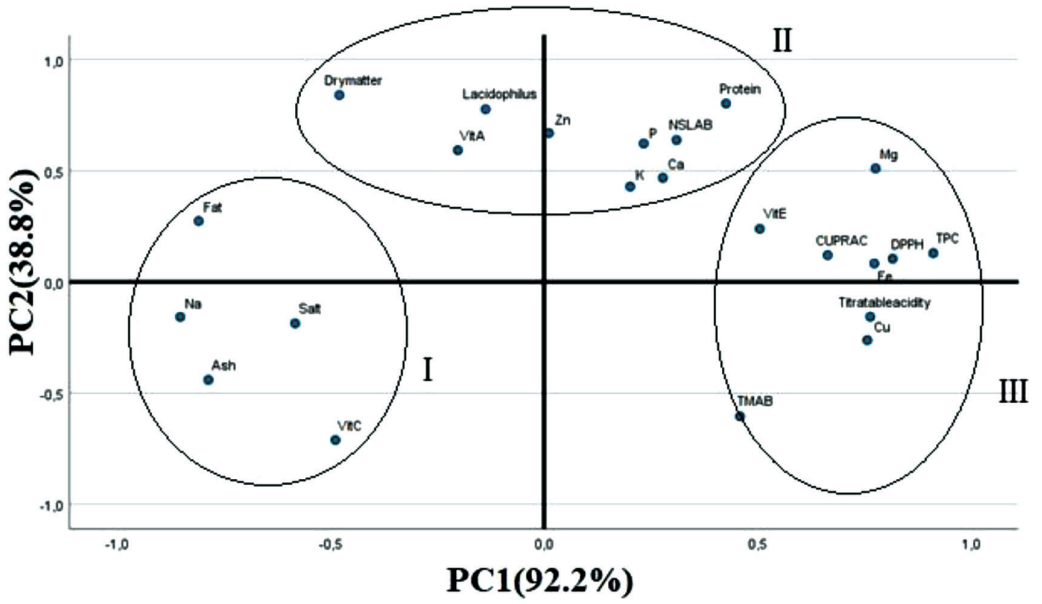

Figure 3. Principal component analysis representing attributes analysed for probiotic white cheese samples fortified with microalgae

storage and in the WC sample (control; 2.313 mg GAE/g) sample at the end of storage. The highest values were determined in SWC sample (*A. platensis*; 6.144 mg GAE/g) at the beginning of storage; in SLAC (*A. platensis* and *Lb. acidophilus*; 8.225 mg GAE/g) and SWC (*A. platensis*; 7.954 mg GAE/g) at the end of storage. Generally, total phenolic contents of cheese samples with *A. platensis* were higher than in other samples. At the end of storage, the total phenolic content of the LAC (*Lb. acidophilus*) sample was similar to CWC (*C. vulgaris*) and CLAC (*C. vulgaris* and *Lb. acidophilus)* due to the metabolic activities of *Lb. acidophilus* which can hydrolyze complex phenolic compounds into free phenolics. Darwish (2017) reported that the total phenolic contents were determined for control Kareish cheese as 14.53±0.230 mg GAE/100 g and for Kareish cheese that contained 1.5 % *A. platensis* as 18.44±0.270 mg GAE/100 g. Tahomy et al. (2018) stated that antioxidant activity of processed cheese with *C. vulgaris* (4 %) and withhout were 68.33 % and 54.85 %, respectively. Because antioxidants have biological functions and beneficial health effects, foods including natural antioxidants have received an increasing interest from both consumers and manufacturers (Vučić et al., 2020; Bjekić et al., 2021). The results of the present study show that fortification of probiotic white cheese with microalgae improved the antioxidant properties of cheese as well as the functional value. It has been thought that this novel cheese may play an important role both in fighting with oxidative stress and meeting consumer demand.

In order to better examine the relationship between microbiological, physico-chemical and antioxidative parameters of cheese samples, principal component analysis (PCA) - the most popular multivariate statistical technique - was performed. PCA was calculated on all these parameters and the relative importance of each variable is shown in Figure 3. The first principal component (PC1; X axis) explained 92.2 % of the total variation in the analysed attributes, while the second-axis (PC2; Y axis) explained 38.8 % of those. Also, after a cluster analysis, three different groups were formed. The first group comprises fat, Na, ash, salt, and Vit C. Dry matter, *Lb. acidophilus* count, Vit A, Zn, P, SSLAB count, protein, P, Ca, Ca and K belong to second group. Finally, third group related to CUPRAC, DPPH, TPC. Mg, Vit E, titratable acidity, Cu and TMAB count.

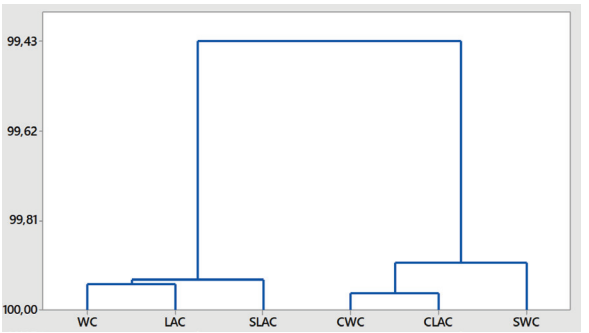

Figure 4. Cluster analysis of probiotic white cheese samples fortified with microalgae

(WC - conventional white cheese without probiotic culture; LAC - white cheese with Lb. acidophilus LA-5; CWC - white cheese with C. vulgaris; SWC - white cheese with A. platensis; CLAC - white cheese with C. vulgaris + Lb. acidophilus LA-5; SLAC - white cheese with A. platensis + Lb. acidophilus LA-5)

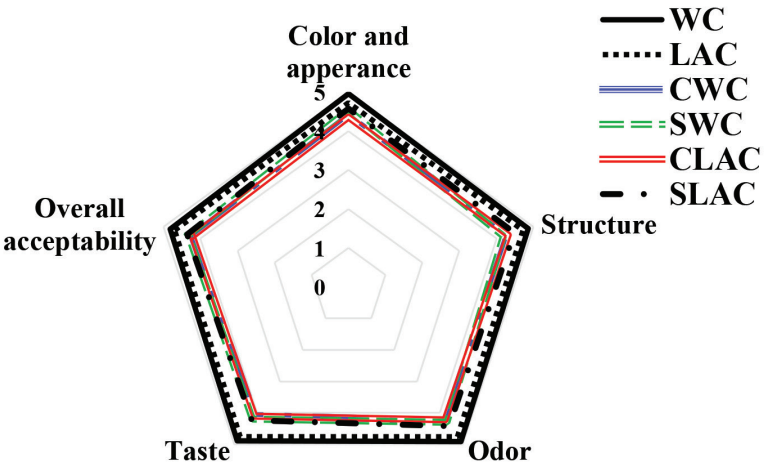

Figure 5. Sensorial properties of probiotic white cheese samples fortified with microalgae

Cluster analysis was performed using the hierarchical clustering method with Ward's linkage based on similar microbiological, physico-chemical, minerals, vitamins, antioxidant capacities and TPC values (Figure 4). Six cheese samples were grouped into two big clusters based on the mean values generated from the unweighted pair group mean average method of analysis. The results showed that the WC sample was very similar first to LAC and later SLAC on the measured variables, which means these samples have the similar compositional properties. The other cluster is composed of CWC and CLAC samples which showed high similarity in their composition. Furthemore, it was determined that SWC was the nearest in this group.

Sensorial properties of white cheese samples

The average scores of the sensorial attributes evaluated by panelists were statistically calculated during the entire storage period (1, 30, 60 and 90 days). The average scores obtained from statistical analysis are represented in Figure 5. Concerning sensorial scores there were significant differences among samples ($p<0.05$). Although *A. platensis* and *C. vulgaris* are more often used in various products, they have restricted sensorial acceptability in the fortification of white cheese. According to the results of the sensorial analysis, WC was the most appreciated sample. However, the appearance, structure and odor scores of cheese with *Lb. acidophilus* were similar to those of samples with *A. platensis*. In the hedonic scale evaluation, the overall acceptability of cheese with microalgae in the range of 4.23 ± 0.005 (for *C. vulgaris*) and 4.35 ± 0.023 (for *A. platensis*). The seaweed aroma and green colour of *A. platensis* was found as acceptable and innovative by panelists. Jeon (2006) reported that processed cheese including 0.5 % *Chlorella* was more acceptable than the control and 1.0 % *Chlorella* cheeses. Terpou et al. (2020) stated that the green colour of feta type cheese with *Spirulina* received high scores by panelists. In another study, increasing microalgae concentration in Kareish cheese production resulted in low sensorial scores (Darwish, 2017). Tohamy et al. (2018) reported that processed cheese analogue with 2 % and 4 % *C. vulgaris* was preferred by panelists over cheese with 4 % alg.

Conclusion

In this research, it was observed that microalgae could serve as growth promoting factors for probiotic bacteria since they contain vitamins, amino acids, minerals, peptone, adenine and hypoxanthine. In addition, probiotic cheese fortified with microalgae had relatively higher amount of protein, Ca, P, K, Mg, Zn, Fe, vitamin E, antioxidant capacity and total phenolic content. The results indicate that microalgae could be used to produce probiotic white cheese to increase functional and health-promoting effects, as well as to enhance nutritional attributes. Also, when sensorial scores of this study are taken into consideration, the utilization of microalgae in probiotic cheese was found by panelists as an attractive and innovative approach. Data in this study can be used to develop probiotic milk products fortified with microalgae with consistent quality on a commercial scale. However, the functional effects of the microalgae addition into dairy products should be proven by clinical studies such as in vivo viability of probiotics.

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Primjena mikroalgi u proizvodnji probiotičkih sireva u salamuri

Sažetak

U ovom istraživanju ispitivan je utjecaj dodatka mikroalgi vrsta *Chlorella vulgaris* i *Arthrospira platensis* na mikrobiološka, fizikalno kemijska i antioksidativna svojstva probiotičkog sira u salamuri. Pri tom je proizvedeno šest različitih uzoraka sireva - WC (kontrolni uzorak), LAC (*Lactobacillus acidophilus* LA-5), CWC (*C. vulgaris*), SWC (*A. platensis*), CLAC (*C. vulgaris* + *Lb. acidophilus* LA-5) i SLAC (*A. platensis* + *Lb. acidophilus* LA-5). U uzrocima oznake SLAC je tijekom cijelog perioda skladištenja broj živih bakterija soja *Lb. acidophilus* bio gotovo nepromijenjen (>7 log cfu/g), dok su za fizikalno-kemijske parametre utvrđene statistički značajne razlike (P<0.01). U uzorku oznake CLAC utvrđene su povišene vrijednosti za koncentraciju proteina, Ca, P, K, Mg i Zn, no najviše vrijednosti koncentracije Fe su utvrđene u uzorcima oznake SLAC. Uzorci sireva obogaćenih mikroalgama imali su više vrijednosti antioksidacijskog kapaciteta određenog pomoću DPPH 1,1-difenil-2-pikrilhidrazil (DPPH) radikala i CUPRAC (sposobnost redukcije iona bakra) metodom, kao i veće udjele ukupnih fenola. U skladu sa svim navedenim, obogaćivanje sireva u salamuri s oba soja ispitivanih mikroalgi rezultiralo je poboljšanim nutritivnim i antioksidativnim svojstvima sireva, te povećalo preživljavanje probiotičkih bakterija.

Ključne riječi: mikroalge; probiotički sir; mikrobiološki parametri; antioksidativna svojstva

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