


In vitro assessment of health-promoting benefits of sheep ‘Testouri’ cheese

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ABSTRACT

This study aimed to produce probiotic ‘Testouri’, traditional Tunisian sheep cheese, by direct-to-vat inoculum of probiotic adjuncts.

The potential of Testouri sheep cheeses was evaluated by an assessment of gross composition and proteolytic, antibacterial, antidiabetic and antioxidant activities during storage at 4 °C for 28 days. Results highlighted that no significant differences were observed in compositional parameters of the samples at day 0. Probiotic counts in cheeses remained at 8 log CFU g⁻¹ during storage. Probiotic cheeses exhibited measurable antibacterial activities with the maximum value (diameter of 12 ± 0.07 mm) on *Staphylococcus aureus* strain. Also, α-glucosidase and α-amylase inhibitions ranged from 42 ± 0.77 to 58 ± 0.88% and 20 ± 0.9 to 47 ± 1.3%, respectively, during storage. Additionally, cheeses inoculated with probiotics exhibited significant increases in proteolytic and antioxidant activities compared to the control sample.

Therefore, Testouri cheese can be considered a good carrier of probiotics and can be promoted for commercial uses.

KEYWORDS

sheep milk, Testouri cheese, probiotics, health-promoting benefits

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1. INTRODUCTION

Consumers are more and more aware of the nutritional and quality values of food and seek to improve their state of well-being, which encourages food industries to develop more nutraceuticals and functional foods. Recently, functional foods are being widely developed; especially foods containing probiotic bacteria that have positive impact on health (Mushtaq et al., 2016).

Lactobacillus is one of the probiotic genera, and its species such as *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus fermentum* are widely employed in numerous food applications, including yogurt, cheese, ice cream, chocolate, infant formula, etc. (Reale et al., 2019). They have been reported to release bioactive peptides with profound physiological effects during the fermentation of milk and milk products. These peptides, obtained by proteolysis, possess many health benefits, such as antioxidant, antimicrobial and antihypertensive activities (Mushtaq et al., 2019). In this sense, cheese products are considered as the most consumed functional foods due to the generation of bioactive peptides.

Testouri cheese is the most famous North African cheese, and it is especially consumed in Tunisia at breakfast, after lunch, and at dinner. This traditional product is highly appreciated because of its sensory properties such as low acidity, salty flavour, sweet aroma and compact texture. This cheese has a great importance in the economy of sheep's milk-producing regions. Sheep's milk possesses a high nutritional, important medicinal and therapeutic value, and it can be advantageous in the manufacture of cheese (Renes et al., 2020). In fact, higher protein levels in sheep milk allow the production of cheese with dense matrices, which may confer nutrients essential for microbial survival during fermentation (Balthazar et al., 2017). Inverse relationship between the development of chronic diseases and dairy foods consumption is reported by various studies. Evaluating the nutraceutical properties of unexplored dairy products may pave the way for managing lifestyle diseases like cancers, diabetes, and immune disorders. To the best of our knowledge, none of the above studies provided in vitro investigation of health-promoting benefits (proteolytic, antidiabetic, antioxidant and antibacterial activities) of Testouri cheese. In previous studies, Mahmoudi et al. (2016, 2019) have selected lactic acid bacteria from camel and sheep milks with robust probiotic characteristics. *L. fermentum* CABA16 and *Lactobacillus plantarum* M63 were among the promising probiotics found, and therefore, were used in the current study.

Therefore, the aim of this study was to investigate the potential of *L. fermentum* and *L. plantarum*, as adjunct cultures in Testouri sheep cheese and to examine their health-promoting benefits in cheese.

2. MATERIALS AND METHODS

2.1. Bacterial cultures

The probiotic strains *L. plantarum* M63 and *L. fermentum* CABA16 were stored in MRS broth (Biokar Diagnostics, France) with 50% glycerol at -80°C . For culture activation, two successive culture transfers were carried out in MRS broth, and a third transfer was carried out in sterilised reconstituted skim sheep milk (10% wt/vol) and incubated at 37°C for 24 h.



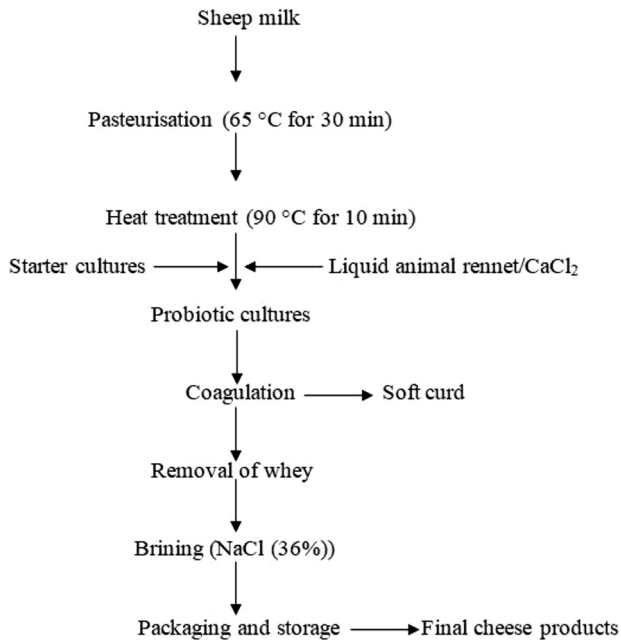


Fig. 1. Cheese manufacturing process

2.2. Cheese making

Testouri cheese production was performed following the standard procedure recommended by the Institute of Vocational Training in Agro-food Industries (Tunis, Tunisia) (Fig. 1). Briefly, refrigerated and pasteurised sheep milks (protein (3%), fat (4%), and lactose (5%)) obtained from a native farm (Mateur, Bizerte, Tunisia) were used. The milk was heat treated at 90 °C for 10 min and then cooled at 37 °C. Calcium chloride, starter cultures, and liquid animal rennet were added at concentrations of 0.3 mL L⁻¹, 10⁸ CFU mL⁻¹, and 300 μL L⁻¹, respectively. The probiotic cultures were added direct-to-vat at a final concentration of about 10⁸ CFU mL⁻¹ to milk. The milk was divided into three equal batches as follows: (1) batch inoculated with *L. plantarum* M63 (SCLP), (2) batch with *L. fermentum* CABA16 (SCLF), and (3) control (SCC). The milk (pH 6.9) coagulated in 40 min, and the resulting curd was cut manually with a knife into 2 cm³ pieces. Subsequently, the whey (pH 6.2) was removed by two repetitive washings with cold water until the pH reached 5.6. The curd was stirred for 20 min at 40 °C until the pH of the whey reached 5.2. Then, the curd cubes were wrapped in cheese cloth in perforated rectangular moulds (≈500 g). Cheese pieces were brined in 36% (w/v) brine solution overnight at 4 °C. Then, they were vacuum-packaged and stored at 4 °C. The cheeses were sampled at 1, 7, 14, 21 and 28 days of storage.

2.3. Gross composition

Cheese samples were analysed for protein content by the Kjeldahl method, fat content by the Gerber method, and moisture according to AOAC (2006).



2.4. Probiotic viability

The counts of probiotics were monitored in cheeses. An aliquot 10 g of minced cheese was placed into a sterile stomacher bag and homogenised with 90 mL of peptone water (Biolife, Milan, Italy) using a Stomacher 80 laboratory blender (Biomaster, Japan). The enumeration of *L. plantarum* was carried out on MRS agar (Biokar Diagnostics, France) supplemented with 4 mg of ciprofloxacin and 20 g of sorbitol (Bujalance et al., 2006). Viable counts of *L. fermentum* were determined on MRS agar with vancomycin (20 mg L⁻¹) (Coeuret et al., 2003). Inoculated plates in duplicate were incubated under anaerobic conditions at 37 °C for 48 h.

2.5. Proteolytic activity

For each determination, 10 g of sample was mixed with 50 mL of 10% Phosphate Buffer Saline. After that, the mixture was homogenised for approximately 1 h and then kept at 45 °C in shaking water bath for 1 h. Also, the mixture was centrifuged (10,000 r.p.m., 15 min, 4 °C), then filtered using Whatman filter paper, and the supernatant was stored at -20 °C (Van Ba et al., 2017). For each assay, the water soluble extracts (WSEs) were vortexed for 1 min followed by centrifugation at 10,000 r.p.m. for 5 min.

The *o*-phthalaldehyde (OPA) of WSEs was determined as described by Al-Dhaheri et al. (2017). Proteolysis results are presented as absorbance at 340 nm.

2.6. Antibacterial activity

Antibacterial ability of WSEs against pathogens such as *Salmonella* Typhimurium (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 070 101 121), and *Escherichia coli* (DH5 α , Institute Pasteur of Tunisia) was determined using agar disc diffusion method previously described by Mushtaq et al. (2019). Briefly, 0.1 mL (10⁶ CFU mL⁻¹) of each overnight pathogen culture was spread onto soft nutrient agar (Biokar Diagnostics, France). From each WSE, 100 μ L was transferred onto sterile disks already placed onto the agar surface, and plates were then kept at room temperature to facilitate diffusion of WSE extracts, which were then incubated for 24 h at 37 °C. After incubation, the diameter of the clear zone around the discs was measured in millimetres (disc diameter included) as a measure of antibacterial activity.

2.7. Antidiabetic activities

2.7.1. α -Amylase inhibition activity. The α -amylase inhibition activity was determined as described by Kim et al. (2004). The inhibition percentage was determined by measuring absorbance at 540 nm and calculated by the following equation:

$$\text{Inhibition \%} = \left(\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100$$

2.7.2. α -Gglucosidase inhibition activity. The α -glucosidase inhibitory activity was determined as reported by Chen et al. (2014). The inhibition percentage was calculated as follows:



$$\text{Inhibition \%} = \left(1 - \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control}} \right) \times 100$$

2.8. Antioxidant activity

2.8.1. Radical scavenging rate by ABTS. Radical scavenging rate by ABTS radical (ABTS^{•+}) was assayed according to Al-Dhaheri et al. (2017). The radical-scavenging activity was calculated as follows:

$$\text{Scavenging rate (\%)} = \left(\frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}} \right) \times 100$$

2.8.2. Radical scavenging rate by DPPH. Radical scavenging activity by DPPH was determined according to Al-Dhaheri et al. (2017). The radical-scavenging activity was expressed as follows:

$$\text{Scavenging rate (\%)} = \left(1 - \frac{\text{Abs sample}}{\text{Abs blank}} \right) \times 100$$

2.9. Statistical analysis

SPSS statistics 20.0 commercial software was used to perform statistical analysis of the results (ANOVA). Tukey's test ($P < 0.05$) significance level was performed to determine significant differences between the means. Data are presented as mean \pm standard deviation. The experiments were carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1. Gross composition

The chemical compositions of all cheeses at day 0 of storage are presented in Table 1. We found no significant differences ($P > 0.05$) in total proteins, fat, and moisture contents between the probiotic cheeses and the control one. These results are in accordance with those of Mushtaq et al. (2016), who reported similar results in Himalayan cheeses.

3.2. Probiotic counts

The viability of *L. plantarum* and *L. fermentum* strains during storage at 4 °C for 28 days is summarised in Table 2. The viable counts of probiotic cultures ranged from 8 ± 0.15 to 9.01 ± 0.027 log CFU g⁻¹ during storage time ($P > 0.05$). So, the probiotic counts were higher than the threshold number (10^6 CFU g⁻¹) that is suggested to confer probiotic benefits (FAO/WHO, 2002). Our results agree with the findings of Ningtyas et al. (2019), who reported that *L. rhamnosus* maintained its number at $>10^6$ CFU g⁻¹ in reduced-fat cream cheese. The probiotic survival can be attributed to better buffering capacity and growth factors, as peptides, of sheep milk compared to cow milk (Balthazar et al., 2017). Also, *L. fermentum* and *L. plantarum* showed high tolerance even at pH 2 *in vitro* (Mahmoudi et al., 2016; 2019). Their viability in



Table 1. Gross composition of sheep Testouri cheeses (day 0)

Variable (%)	SCC	Cheese samples	
		SCLP	SCLF
Protein	22.66 ± 0.017 ^a	22.65 ± 0.022 ^a	22.66 ± 0.03 ^a
Fat	22.58 ± 0.04 ^a	22.61 ± 0.01 ^a	22.62 ± 0.01 ^a
Moisture	47 ± 0.011 ^a	47.2 ± 0.05 ^a	47.4 ± 0.4 ^a

^a Means values ± SD ($n = 3$) within a column with different superscript letters and within a row with different superscript letters are significantly different ($P < 0.05$).

SCC: Sheep cheese inoculated with starter culture (control); SCLP = Sheep cheese inoculated with *L. plantarum* as adjunct; SCLF: Sheep cheese inoculated with *L. fermentum* as adjunct.

Table 2. Viable probiotics counts in Testouri cheeses during storage at 4 °C

Storage (Days)	Probiotics	Probiotics counts of cheese samples (log CFU g ⁻¹)	
		SCLP	SCLF
1	<i>L. plantarum</i>	9.03 ± 0.047 ^a	–
	<i>L. fermentum</i>	–	9.01 ± 0.027 ^a
7	<i>L. plantarum</i>	8.7 ± 0.32 ^a	–
	<i>L. fermentum</i>	–	8.9 ± 0.03 ^a
14	<i>L. plantarum</i>	8.24 ± 0.5 ^b	–
	<i>L. fermentum</i>	–	8.5 ± 0.05 ^b
21	<i>L. plantarum</i>	8.2 ± 0.05 ^b	–
	<i>L. fermentum</i>	–	8.34 ± 0.019 ^b
28	<i>L. plantarum</i>	8.00 ± 0.15 ^b	–
	<i>L. fermentum</i>	–	8.21 ± 0.022 ^b

^{ab} Mean values ± SD ($n = 3$) within a column with different superscript letters and within a row with different superscript letters are significantly different ($P < 0.05$).

SCLP: Sheep cheese inoculated with *L. plantarum* as adjunct; SCLF: Sheep cheese inoculated with *L. fermentum* as adjunct.

cheese could also be explained by salt tolerance, which is an important characteristic during cheese manufacturing. In particular, sodium chloride is used as a protective for probiotics in cheeses (Carafa et al., 2015). Moreover, the high protein and fat levels in sheep cheese may provide effective protection for probiotics during storage and passage through the gastrointestinal tract (Balthazar et al., 2017).

The shelf life of Testouri cheese is 10 days at 4 °C. Nevertheless, at the end of the study period (28 days), the viable counts of probiotics were still $\geq 10^8$ CFU g⁻¹.

3.3. Proteolysis assessment

The OPA assay results are depicted in Fig. 2. The values increased significantly ($P < 0.05$) in all cheeses during storage. Levels in probiotic cheeses were significantly higher ($P < 0.05$) compared to the control during storage with DO_{340nm} final values of 2.1 ± 0.25 , 2.9 ± 0.022 , and 2.9 ± 0.03 for SCC, SCLP, and SCLF samples, respectively. Similarly, Al-Dhaheri et al. (2017) reported that



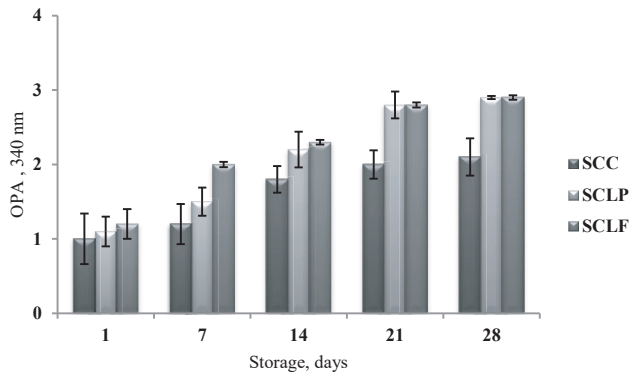


Fig. 2. Proteolytic activity of sheep Testouri cheeses during storage at 4 °C measured by o-phthalaldehyde at 340 nm. SCC: Sheep cheese inoculated with starter culture (control);

SCLP: Sheep cheese inoculated with *L. plantarum* as adjunct; SCLF: Sheep cheese inoculated with *L. fermentum* as adjunct

proteolysis was important in Akawi cheese. The higher values of OPA obtained in probiotic cheeses may be due to accelerated primary proteolysis as well as the high proteolytic activities of probiotic cultures.

3.4. Antibacterial activity

The inhibition ability of the WSEs against pathogens is presented in Table 3. The results show that the WSEs inhibited the growth of *L. monocytogenes*, *S. aureus*, *Salmonella* Typhimurium, and *Escherichia coli*. Antibacterial activities of probiotic WSEs were significantly higher than control ($P < 0.05$) with a maximum inhibition diameter of 12 ± 0.07 mm exhibited by SCLF sample against *S. aureus*. This can be explained by the fact that probiotic cultures improved the bioactivity of the Testouri cheese by generating peptides during fermentation. Similarly, Kar-iyawasam et al. (2019) revealed that *Weissella cibaria* D30 exhibited a protective activity against *L. monocytogenes* in cottage cheese. Besides, Mushtaq et al. (2019) reported that the probiotics in

Table 3. Antibacterial activity expressed as inhibition zone diameter (mm) of WSEs of Testouri cheeses

Pathogen strains	Cheese samples		
	SCC	SCLP	SCLF
<i>Listeria monocytogenes</i>	4.5 ± 0.01^a	11.7 ± 0.05^b	11.8 ± 0.007^b
<i>Staphylococcus aureus</i>	4.8 ± 0.015^a	11.8 ± 0.001^b	12 ± 0.07^b
<i>Salmonella</i> Typhimurium	3.8 ± 0.001^a	8.6 ± 0.002^b	8.6 ± 0.01^b
<i>Escherchia coli</i>	2.6 ± 0.01^a	8.2 ± 0.007^b	8.5 ± 0.019^b

^{a,b}Mean values \pm SD ($n = 3$) within a row with different superscript letters are significantly different ($P < 0.05$).

SCC: Sheep cheese inoculated with starter culture (control);

SCLP: Sheep cheese inoculated with *L. plantarum* as adjunct; SCLF: Sheep cheese inoculated with *L. fermentum* as adjunct.



Kalari cheeses enhanced their antimicrobial activities due to milk-derived peptides and by producing antimicrobial compounds such as acetic acid, lactic acid, and bacteriocin.

3.5. Antidiabetic activities

The inhibitory effects of Testouri cheeses on α -glucosidase and α -amylase activities through 28 days of storage are presented in Fig. 3A and B, respectively. The percentages of α -glucosidase inhibition increased during storage and ranged from 41 ± 0.08 to $58 \pm 0.08\%$ (Fig. 3A). The probiotic WSEs exhibited significant α -glucosidase inhibition compared to the control from 14 to 28 days of storage ($P < 0.05$). Similarly, α -amylase inhibition was significantly higher ($P < 0.05$) in probiotic cheeses than in control during storage (Fig. 3B). The SCLF sample showed

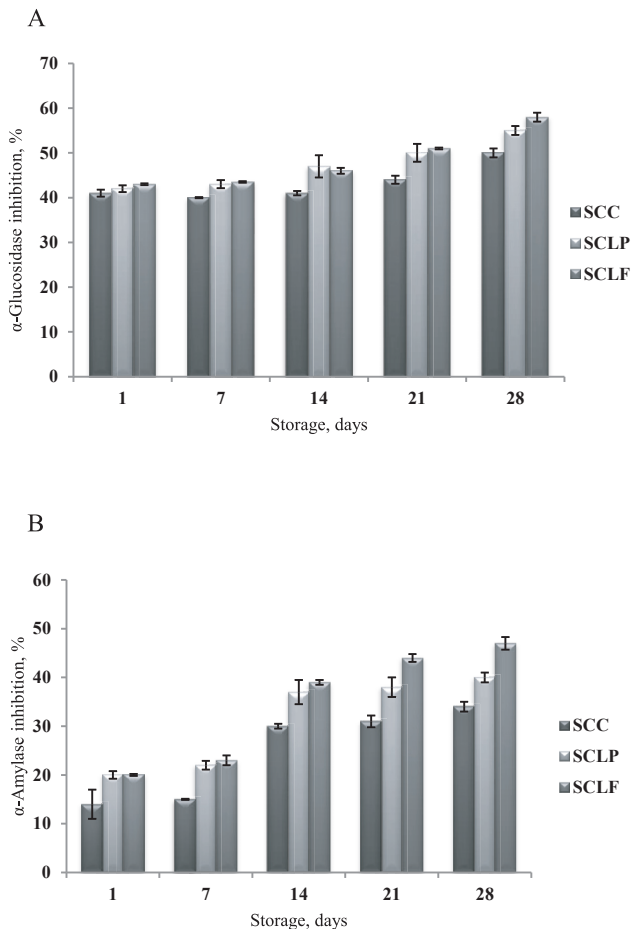


Fig. 3. α -Glucosidase inhibition (A) and α -amylase inhibition (B) of sheep Testouri cheeses during storage at 4 °C. SCC: Sheep cheese inoculated with starter culture (control); SCLP: Sheep cheese inoculated with *L. plantarum* as adjunct; SCLF: Sheep cheese inoculated with *L. fermentum* as adjunct



higher α -amylase inhibition activity than SCLP and SCC samples at the 21st day of storage with percentage of $44 \pm 0.05\%$. The OPA assessment suggests that bioactive peptides might be formed in the Testouri cheeses that correlated with α -amylase and α -glucosidase inhibition rates. Al-Dhaheri et al. (2017) and Mushtaq et al. (2019) revealed that the α -glucosidase and α -amylase activities could be attributed to the presence of higher number of bioactive peptides generated by the proteolytic enzymes of probiotics during fermentation. Anyway, it must be mentioned that α -amylase and α -glucosidase inhibitions are considered an effective approach to control diabetes via reducing carbohydrate hydrolysis (Donkor et al., 2012).

3.6. Antioxidant activities

The scavenging activity, determined by DPPH and ABTS methods, of Testouri cheeses for 28 days of storage are depicted in Fig. 4A and B. DPPH scavenging rates increased significantly ($P <$

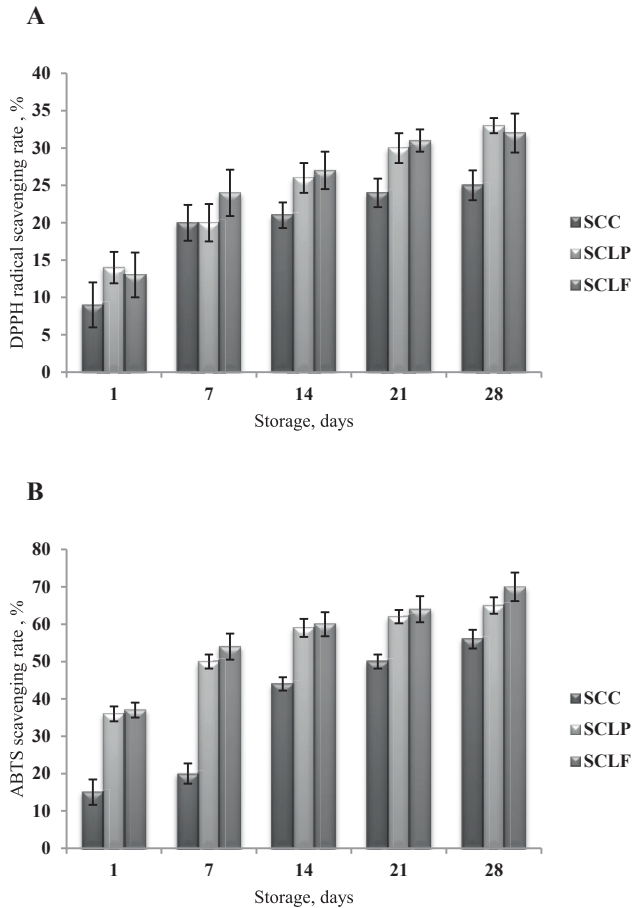


Fig. 4. Antioxidant activity by DPPH (A) and ABTS (B) of sheep Testouri cheeses during storage at 4 °C
SCC: Sheep cheese inoculated with starter culture (control); SCLP: Sheep cheese inoculated with *L. plantarum* as adjunct; SCLF: Sheep cheese inoculated with *L. fermentum* as adjunct



0.05) during storage and ranged from a minimum of 9 ± 2.4 to a maximum of $33 \pm 1\%$ registered by SCC and SCLP samples, respectively (Fig. 4A). Scavenging activities of SCLP and SCLF cheeses were higher ($P < 0.05$) compared to the control. The SCLF sample exhibited comparable antioxidant activity to SCLP one except at seven days of storage. For ABTS method, the rates increased significantly ($P < 0.05$) in all cheeses with storage time (Fig. 4B). The rates increased for a maximum of $70 \pm 3.8\%$ for SCLF sample at the end of storage. Probiotic cheeses exhibited higher antioxidant activity than control ($P < 0.05$). No difference in ABTS rates between probiotic samples was found ($P > 0.05$). The increased proteolysis during storage might result in the generation of more bioactive peptides, including antioxidant peptides that are released from their inactive state in the parent protein structure, thus increasing the antioxidant activity of the Testouri cheeses. These bioactive compounds prevent enzymatic and non-enzymatic peroxidation of essential fatty acids by reducing the effect of reactive oxygen species formed by oxidative stress in cells such as superoxide ($O_2^{\bullet-}$), hydroperoxyl ($^{\bullet}OOH$), hydroxyl ($^{\bullet}OH$), and peroxy (ROO^{\bullet}) radicals (Balthazar et al., 2017) by donating electrons to neutralise free radicals.

4. CONCLUSIONS

The present study was carried out to prove that *L. fermentum* and *L. plantarum* strains have potential for use as adjunct cultures in Testouri cheeses. Their viabilities being $\geq 10^8$ CFU g^{-1} until the end of storage shows their potential to deliver health benefits to the consumers. Also, our findings revealed that probiotic cultures enhance the nutraceutical values of sheep cheeses such as antibacterial, antidiabetic and antioxidant properties. In particular, their incorporation into the cheeses inhibits the growth of pathogens and ensures the microbial safety. According to the results, addition of different probiotics not only increases the functionality of the product but also extends its shelf life without any use of synthetic compounds (preservatives or antioxidants) that would raise consumer concerns.

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