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Determination of Some Technological Characteristics of *Streptococcus thermophilus* and *Streptococcus macedonicus* Isolated from Classical White Cheese Production Stages

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ABSTRACT

Lactic acid bacteria (LAB) are classified within the taxonomic family Lactobacillaceae family and are renowned for their pivotal roles in food safety and public health. Additionally, they contribute to the formation of aroma, flavour and texture in fermented foods. Among LAB, *Streptococcus* spp. is considered at the generally recognized as safe (GRAS) level, particularly for use as starter cultures in the dairy industry. The aim of this study was to ascertain the technological properties of *S. thermophilus* BST2001-BST2007 (7) and *S. macedonicus* BSGM2471-BSGM2471 (2). These were isolated from samples obtained at various stages of classical white cheese production, including acid forming capacity, proteolytic activity, viability at different pH, temperature and salt concentrations, and diacetyl production. The findings of the study demonstrated that the acid forming capacities of all isolates were similar during the initial 6th hour. However, at the 12th hour, the *S. thermophilus* BST2006 and BST2007 isolates decreased the pH of the medium to 4.45 and 4.46, respectively, resulting in the formation of a clot. At the end of the 24-hour period, it was observed that all *S. thermophilus* isolates had formed clots, whereas the *S. macedonicus* isolates had demonstrated a markedly reduced capacity for acid production and did not form any clots. In contrast, both species showed high proteolytic activity (zone diameter >10 mm) and were able to growth within a temperature range of 15-45°C. Isolates of *S. macedonicus* were observed to be unable to grow at a salt concentration of 7%. Additionally, diacetyl production was observed in four *S. thermophilus* isolates and one *S. macedonicus* isolate. These results indicate that there may be significant differences in technological properties even among the same LAB species. This highlights the importance of researching and introducing new LAB species to the sector to develop products with specific, targeted properties in the fermented food industry, particularly in cheese production.

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INTRODUCTION

LAB found in fermented foods are an important member of the Lactobacillaceae family. LAB play a significant role in the formation of aroma, flavor, and texture in the production of fermented foods, as well as exerting beneficial effects on food safety and public health (Widyastuti et al., 2014).

Although some species of *Streptococcus* spp. in the LAB group have been demonstrated to exhibit pathogenicity in humans and animals, others have been GRAS for human consumption. The most important species in this genus is *Streptococcus thermophilus*, which is widely used as a starter culture in production of various dairy products (Blaiotta et al., 2011). *S. thermophilus* is a homofermentative lactic acid bacterium that is employed as a starter in the manufacture of fermented milk products, including yoghurt and ripened cheese (Cui et al., 2016).

Another species, *Streptococcus macedonicus*, was first isolated from Greek Kasseri cheese, a naturally fermented product (Tsakalidou et al., 1998). *S. macedonicus*, a member of Streptococcus spp., is a Gram-positive and catalase-negative bacterium. It was isolated during the identification of lactic acid bacteria from different stages of classical cheese production without the addition of starter culture. It has been reported that the optimum temperature for the growth and bacteriocin production of this bacterium is 42.3°C and 20-25°C and the optimum pH is 6.4 and 6.0, respectively (Van den Berghe et al., 2006).

The current study isolated *Streptococccus* spp. from classical cheese production stages and characterized them genotypically using MALDI-TOF. A total of nine isolates belonging to two species were identified, along with some technological characteristics.

MATERIAL and METHOD

The aim of this study was to ascertain the technological properties of two species of *Streptococcus*, namely *S. thermophilus* (7) and *S. macedonicus* (2), isolated from the classical white cheese production steps. These species were evaluated for their acid formation, proteolytic activity, ability to survive at different pH levels, temperatures, and salt concentrations, among other characteristics.

Acid-Forming Capacity

To determine the acidification capacity of LAB strains, the protocol proposed by Terzić-Vidojević et al. (2015) was modified. In brief, the acidification capacity was determined by calculating the percentage of acidity in lactic acid through pH measurement and titration. For this purpose, skimmed UHT milk was incubated at 30°C for 15 days and 55°C for 7 days, and no leakage or bombing of the packaging was observed after incubation. To 10 mL of UHT skimmed milk added into sterile test tubes, 0.1 mL (v/v) of 18-hour-old fresh cultures (0.5 McFarland) were added and incubated at 30°C for 3, 6, 12 and 24 hours. At the end of each incubation period, including the initial 0-hour period, 2 mL samples were aseptically taken from the tubes, and pH was measured with a pH meter (HANNA HI2211). Subsequently, titration was performed.

Determination of Proteolytic Activity

The proteolytic activity was evaluated in accordance with the protocol proposed by Raveschot et al. (2020). In brief, LAB were cultured in M17 broth (Biolife, LB.BL.4017202,) and the final optical density at 600 nm (OD600) were adjusted for each fresh culture. From each prepared cell suspension,

20 µl was added to wells (well diameter 4 mm) in Skim Milk Agar (10%) and incubated at 37°C for 72 h. At the end of the incubation period, the proteolytic activity was evaluated based on the zone diameters formed around the wells: A zone larger than 10 mm was considered to indicate very high activity, a zone between 3 and 10 mm indicated high activity, and a zone smaller than 3 mm indicated low activity (Alapont et al., 2015).

The Ability to Grow at Different Temperatures

To assess the capacity to exhibit growth at varying temperatures, LAB isolates were inoculated into 10 mL M17 broth media at a rate of 1% and incubated at 10˚C, 15˚C and 45˚C for a duration of 48 h. At end of this period, the isolates that showed growth by forming turbidity in the medium were designated as positive, and those that did not form turbidity were designated as negative.

The Ability to Grow at Different NaCl Concentrations

To determine the growth ability of lactic acid bacteria at different NaCl concentrations, 1% of fresh bacterial cultures were inoculated into M17 broth media containing 2% and 6.5% NaCl, respectively, and incubated at 37°C for 24 h. After the incubation period, turbidity formation in the media was considered positive and those without turbidity formation were considered negative.

The Ability to Grow at Different pH

In this study evaluated the ability LAB cultures to grow at two different pH values (3.9 and 9.6). To this end, 10 ml of M17 broth with pH values of 3.9 and 9.6 were inoculated with 1% of active cultures and then to incubation at 30°C for 48 h. The results were considered positive in the tubes with turbidity and negative in the tubes without turbidity.

Diacetyl Production

Diacetyl production was evaluated in accordance with the protocol proposed by Franciosi et al (2009). Briefly, 0.1 mL of fresh cultures of LAB were transferred to 10 mL of sterile skimmed milk (10% w/v) and then incubated at 30°C for 24 h. Following incubation, 1 mL of each bacterial culture was transferred to sterile tubes, 0.5 mL of α-naphthol (1% w/v) and KOH (16% w/v) were added and incubated again at 30°C for 10 minutes. Observation of a red ring at the top of the tubes was considered positive for diacetyl production.

RESULTS

The technological properties of *S. thermophilus* and *S. macedonicus* isolates obtained from different stages of classical white cheese production were determined for their use as natural (autochthonous) starter cultures in some fermented milk products, such as yoghurt and cheese. According to the results of the acid-forming ability test, the pH of the medium changed between 5.82-6.23 at the 6th hour of incubation. Meanwhile, *S. thermophilus* BST2006 and BST2007 isolates decreased the pH of the medium to 4.45-4.46 and formed clots at the 12th hour of incubation, respectively. At the end of the 24th hour of incubation, it was observed that *S. thermophilus* isolates decreased the pH of the medium to 4.31-4.99 and complete coagulation occurred in the medium. On the other hand, the titration acid ratios of the medium in terms of lactic acid were found to vary between 0.18-0.27 at the 3rd and 6th hours of incubation and between 0.25-0.40 at the end of the 12th h. (Table 1).

It was determined that all strains had high proteolytic activity (zone diameter >10 mm) and could grow in the temperature range of 15-45°C. It was observed that isolates of *S. macedonicus* BSGM2471-

BSGM2471 were unable to grow at a salt concentration of 7%. Diacetyl formation was found to be dependent on the strain, rather than the species. A discrepancy in diacetyl production was observed between *S. thermophilus* and *S. macedonicus* strains belonging to the same species (Table 2).

DISCUSSION

LAB are widely used in cheese and yoghurt production due to their contribution to the flavour, texture and nutritional value of fermented products (Mokoena et al., 2021). LAB are well adapted to environmental conditions such as low pH, high NaCl, anaerobiosis and the presence of fermentable carbohydrates. Therefore, significant differences in their technological characteristics may occur between members of the same species. It is of paramount importance to investigate novel species with novel and advantageous characteristics and to disseminate them throughout the industry (Montel et al., 2014; Cui et al., 2016).

The acid-forming capacity of *S. thermophilus* is a determining factor in the duration and quality of dairy production, while its proteolytic activity affects the formation of flavouring substances (Cui et al., 2016). The results of this study indicated that *S. thermophilus* BST2006 and *S. thermophilus* BST2007 isolates reduced the pH to 4.45 and 4.46 by the end of the 12th h, respectively, and coagulation was observed in the medium. This suggests that these two isolates were more effective at acidifying the medium. Additionally, it was observed that these two isolates had high proteolytic activity (Table 1; Table 2). Galia et al. (2019) reported a close correlation between proteolytic activity and acidifying capacity of *S. thermophilus* strains. Some *S. thermophilus* strains cannot lower the pH below 5.2 and are therefore often used in combination with other starter bacteria. *S. thermophilus* proteolytic activity plays a role in releasing peptide sequences from caseins and whey proteins during lactic fermentation (Rodríguez-Serrano et al., 2018). Gaglio et al. (2014) reported that the pH values and diacetyl formation of the *S. thermophilus* strains identified in the Vastedda cheese, a raw sheep's milk product produced without the addition of a starter culture and protected by the European Union in Italy, exhibited strain-specific variations. These findings are like those of the current study.

In the present study, two isolates of *S. macedonicus* were identified from samples obtained at different stages of the production of a classical white cheese. It was determined that both *S. macedonicus* BSGM2470 and *S. macedonicus* BSGM2471 isolates decreased the pH of the medium to 5.06 and 4.69, respectively, by the end of the 24th h. Furthermore, an observation was made that the technological properties of both isolates were like each other (Tables 1; Table 2). Among the *S. macedonicus* (L36-L37) isolates that were isolated from white cheese samples, the L36 isolate was found to have high acid-forming capacity (Meral Aktaş and Erdoğan, 2022). It has been reported that *S. macedonicus* species has weak acidification, low proteolytic and citrate catabolizing activity in milk. However, it was found to have moderate lipolytic activity (Georgalaki et al., 2000). In contrast to the findings of Georgalaki et al. (2000), the *S. macedonicus* BSGM2470 and BSGM2471 strain identified in this study was found to have high both acidification and proteolytic activity. De Vuyst and Tsakalidou (2008) reported that *S. macedonicus* has lipolytic and proteolytic activity, and some strains of *S. macedonicus* were also characterised by exopolysaccharide and bacteriocin production. Gaglio et al. (2014) reported that the pH values of *S. gallolyticus* subsp. *macedonicus* strains identified from Vastedda cheese exhibited variation depending on the strain the end of 8th and 24th h. The findings of this study are like to the findings of our study. The same researchers also found that diacetyl production varied depending on the strain. De Leonardis et al. (2013) stated that diacetyl production may vary depending on the milk structure and the strain of lactic acid bacteria. In their study, they reported that *L. paracasei* and *L. rhamnosus* produced diacetyl better than *S. thermophilus*. Pacini et al. (2006) detected *S. macedonicus* in traditional Italian cheese samples, while Renye et al. (2011) detected the same species in traditional

Mexican cheese samples produced from both raw milk and pasteurized milk. Demir and Kaptan (2025) isolated *S. macedonicus* from Edirne white cheese and reported that further research on the technological properties of this bacterium is needed. In this study, some technological properties of *S. macedonicus* (e.g. acid-forming capacity, proteolytic activity) were revealed. The findings of both studies are like the findings of our study. On the other hand, according to the literature review, it was observed that there are limited number of studies on the technological properties of both bacteria.

CONCLUSION

In the last decades, there has been an increasing focus on scientific research investigating the identification and technological characteristics of indigenous starter culture bacteria in the production of fermented dairy products, especially in Europe. This study showed that the technological properties of *S. thermophilus* and *S. macedonicus* strains isolated during the white cheese production process exhibited species-dependent variations. The findings of this study suggest that the technological properties of these bacteria can be used as autochthonous starter cultures in the production of fermented milk products such as yoghurt and cheese.

Ethical Statement

According to Article 8(k) of the Regulation on the Working Procedures and Principles of the Ethics Committees on Animal Experiments, Ethics Committee Approval is not required.

Author Contributions

Research Design (CRediT 1) Author 1 (%100)
Data Collection (CRediT 2) Author 1 (%100)
Research - Data analysis - Validation (CRediT 3-4-6-11) Author 1 (%100)
Writing the Article (CRediT 12-13) Author 1 (%100)
Revision and Improvement of the Text (CRediT 14) Author 1 (%100)

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Conflict of Interest

Author declares that there is no conflict of interest.

Sustainable Development Goals (SDG):

3 Good Health and Well-Being
12 Sustainable consumption and production

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