Fortification of milk-based yogurt with protein hydrolysates from brewers’ spent grain: Evaluation on microstructural properties, lactic acid bacteria profile, lactic acid forming capability and its physical behavior

Joncer Naibaho a,*, Emir Jonuzi b, Nika Butula c, Małgorzata Korzeniowska a,**, Maike Föst d, Karina Nola Sinamo e, Grzegorz Chodaczek f, Baoru Yang g

a Department of Functional Food Products Development, Faculty of Biotechnology and Food Science, Wrocław University of Environmental and Life Sciences, 51-630, Wrocław, Poland
b Department of Chemistry, Faculty of Natural Sciences and Mathematics, State University of Tetovo, 1200, Tetovo, Macedonia
c Department of Food Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, 10000, Croatia
d Fraunhofer Institute for Process Engineering and Packaging IVV, Freising, Germany
e Department of Food Science and Technology, Faculty of Agriculture, Universitas Sumatera Utara, 20155, Medan, Indonesia
f Bioimaging Laboratory, Łukasiewicz Research Network-PORT Polish Center for Technology Development, 54-066, Wrocław, Poland

g Food Chemistry and Food Development, Department of Life Technologies, University of Turku, 20014, Turku, Finland

** Corresponding author.
E-mail addresses: joncer.naibaho@upwr.edu.pl (J. Naibaho), małgorzata.korzeniowska@upwr.edu.pl (M. Korzeniowska).

Abstract

Current study aimed to evaluate the utilization of protein from brewers’ spent grain (BSGP) on microstructural formation as well as rheological behavior, acidity and lactic acid bacteria (LAB) profile during the refrigerated storage. Three different BSGPs were provided including BSGP-C (extracted without enzymatic hydrolysis), BSGP-P (with protease), and BSGP-PF (with protease co-incubated with flavourzyme). The results demonstrated that BSGPs improved lactic acid forming capability in yogurt production to a higher level than milk-protein based enrichment. BSGPs improved the growth and survival of lactic acid bacteria (LAB), particularly BSGP-P in improving the survival rate of L. bulgaricus. Confocal laser scanning microscopy showed that BSGP-P generated a denser, softer and more homogenous surface appearance as well as showed the tendency to form more compact networks; had a weaker initial gel forming, increased and preserved the consistency of the yogurt during the storage. In conclusion, BSGPs in yogurt improved and preserved the textural properties, consistency, acidity and lactic acid bacteria.

1. Introduction

Yogurt has been well known for its benefits for human health as it contains a high number of macro-and micronutrients including bioactive peptides, vitamins and minerals (Rahmawati and Suntornsuk, 2016; Souza et al., 2018). According to the yogurt market prediction, the value of worldwide yogurt production will increase steadily from 38.7 billion USD in 2018 to 51.2 billion USD in 2024 (Shahbandeh, 2020). This is due to the higher demand as its health benefits. Yogurt have been reported for its ability in preventing of several diseases including cancer, dental caries, irritable bowel syndrome, infection in respiratory and gastrointestinal tract, obesity and weight control, and cardiovascular (Bañà et al., 2018; Barengolts et al., 2019; Bayat et al., 2016; dos Reis et al., 2017; Noorbakhsh et al., 2019; Suzuki et al., 2017), treating some diseases such as diarrhea, antibiotic resistant pathogens, glucose metabolism in type 2 diabetes patients (Hill et al., 2017; Mohamadshahi et al., 2014; Noorbakhsh et al., 2019) as well as improving the immune function (Hummelen et al., 2011). Those biological capabilities of yogurt are due to the presence of bioactive peptides which were formed during the fermentation process (Rahmawati and Suntornsuk, 2016). Protein fortification in yogurt production has been intensively investigated, particularly milk-based protein enrichment (Karam et al., 2013; Lesme et al., 2020). The investigation of plant-based protein yogurt production has also been rapidly growing (Aydar et al., 2020; Mäkinen et al., 2016). However, total replacement of plant-based protein in dairy products cost 3 times higher than milk-based and have significant
consequences to the lack of nutrient intake (Clegg et al., 2021). Partial substitution of plant-based protein into milk-based yogurt production is rarely reported. In fact, incorporating plant-based protein in dairy-based yogurt production is seemingly challenging from the perspective of practical and nutritional. Protein from brewers’ spent grain (BSGPs) has been well studied for its biological properties (Wen et al., 2019) which allows BSGPs suitable for yogurt production. Previously, BSGPs prepared with alcalase had shown anti-inflammatory effects (Crowley et al., 2015).

Current study proposed the utilization of BSGPs which was prepared by three different enzymatic treatments, aiming to evaluate their impact on microstructural formation as well as rheological behavior, acidity and lactic acid bacteria (LAB) profile during the refrigerated storage. Previous study has investigated that protease and flavourzyme were able to solubilize up to 60% of protein from BSG, up to 479 soluble peptides were identified (Kriisa et al., 2022). Furthermore, co-incubation with flavourzyme increased the availability of hydrophobic amino acids. Protease treatments and co-incubation with flavourzyme generated mixture without protease treatment as control (-C), treated with 0.5% protamex (-P) and treated with a combination of 0.5% protamex and 0.1% flavourzyme (-PF). The groups were treated at 50 °C for 3 h at pH 8.5, followed by heating at 90 °C in order to inactivate the enzymes. After that, the treated mixtures were cooled down to room temperature and centrifuged at 4000×g for 15 min to separate the liquid fraction from the sediment. The liquid fraction was dried by a semi-pilot spray dryer (APV Anhydro A/S LAB S1 spray dryer, Denmark). The fraction was evaporated in hot air with an inlet temperature of 160–165 °C and outlet temperature of 82–85 °C. The instrument was operated with an air pressure nozzle at 2 bars and the velocity of the peristaltic pump at 2.5 L/h. BSGP from control treatment was collected as BSGP-C, while from 0.5% protamex as well as 0.5% protamex-0.1% flavourzyme was collected as BSGP-P and BSGP-PF, respectively. Protein content of BSGP-C, BSGP-P, and BSGP-PF was 12.6%, 37.5%, and 31.4%, respectively; with biological and techno-functional properties as previously reported (Naibaho et al., 2022c). Furthermore, BSGPs contained free amino acids at an amount of 1%, 1.5% and 5.3% for BSGP-C, BSGP-P, and BSGP-PF, respectively (Kriisa et al., 2022). The dried extract was packed into an aluminum foil bag, sealed and kept at a chilled temperature (10 °C) for further studies.

2.3. Yogurt preparation

The preparation of the yogurt was carried out following the methods from previous studies with slight modification (Naibaho et al., 2022b; Szoltysek et al., 2020). Based on the pre-study experiment, concentration of 10% (w/w) of each extract was added into the milk and mixed properly. In total, 6 different mixtures were obtained. After that, 2% of skim milk powder was added to each mixture in order to intensify the texture. The mixtures were heated at 90 °C in a laboratory water bath for 15 min and then cooled down to 43 ± 1 °C. An amount of 0.05% of microbial yogurt starter was added, mixed properly and incubated at 43 °C in a laboratory water bath to reach pH between 4.3 and 4.8. The pH was recorded during the incubation and the mixtures were homogenized slowly using a laboratory scale mixer (260 rounds/min; 4 cm gap) during the pH observation. The fermentation was ended by homogenizing the mixture using a laboratory scale mixer (380 rounds/s/min; 4 cm gap) once the targeted pH was achieved. The obtained yogurt was cooled down to 15 °C, removed into a cup for storage at refrigeration temperature (4 °C) for 18 h before the analysis on the first day. Yogurts were prepared in duplicate and all the analyses were performed at least in duplicate. The yogurt prepared with BSGP-C represented yogurt control (YC), while yogurt prepared with BSGP-P and BSGP-PF represented yogurt protamex-prepared (YP) and yogurt protamex-flavourzyme prepared (YPF), respectively.

2.4. Microstructural analysis

Microstructural characterisation was carried out in order to evaluate the impact of protein-rich extracts from BSG in the network and matrix of the yogurt. The yogurts were dried using a freeze dryer (Labconco Corp., MO, USA) and kept in an aluminum foil bag at 4 °C for the analysis of fourier transform infrared spectroscopy and confocal laser image scanning microscopy as described previously (Naibaho et al., 2022b).

2.4.1. Fourier transform infrared spectroscopy (FTIR)

FTIR were conducted following the instruction of the instrument using IRRISpiritTM, Shimadzu (Shimadzu Europe, Germany, GmbH). The measurement was observed at 4000 and 4000 cm⁻¹.
2.4.2. Confocal laser scanning microscopy (CLSM)

CLSM analysis was conducted by using Leica SP8 MP Confocal Microscope BADD-002030 (Germany). The samples were stained with Nile Red (72,485, Sigma-Aldrich) and Rhodamine 123 (R8004, Sigma-Aldrich) with concentration of 10 μg/ml in water. The sample (9–30 mg) was suspended in a staining solution at 1:4 ratio (v:v) air objective. The structure of the sample was visualized using a reflectant of laser light. The excitement of Nile Red and Rhodamine 123 was done with 561 and 649 nm laser. The reflected light channel was generated with a 488 and 638 nm laser for Nile Red and Rhodamine 123 respectively. For each sample, the image was scanned in three representative fields of view in the Z axis (10–80 μm thick, 0.68 μm intervals).

2.5. Analysis of rheological behavior

Rheological behavior was conducted using a rotational Haake RheoStress 6000 rheometer following the method as described in a previous study (Naibaho et al., 2022b). The sample was left at room temperature for 30 min and mixed properly by using a laboratory scale mixer (260 rounds/min; 4 cm gap) before the measurement. The instrument was equipped with a thermostatic bath (Haake A10) and a UTM Controller (Thermo Electron GmbH, Karlsruhe, Germany). The measurement was done in a constant temperature at 20 °C using a cone/plate (C60/1° Ti L no.222-1868/stainless steel plate TMP60 no.222-1891) with a gap of 1 mm for all samples in the geometry system. Approximately 1 mL of sample was added into the plate surface and the measurement was recorded at shear rate from 0 to 2000 s⁻¹. Shear stress and viscosity were recorded as the increasing of shear rate (Szoltyšik et al., 2020). Flow curves were fitted to Power model of Ostwald de Waele with the equation:

\[ \eta = k \cdot \gamma^{n - 1} \]

\( \eta_{90} = \) apparent viscosity (Pa.s); \( k = \) consistency index (Pa.s); \( \gamma = \) shear rate (s⁻¹); \( n = \) flow behavior index.

2.6. Syneresis

Syneresis describes the amount of water loss after centrifugation with the methods following previous studies (Bouaziz et al., 2021; Khubber et al., 2021). Briefly, 5 g of the yogurt was weighed and centrifuged at 4500 rpm and 10 °C for 15 min. After that, the sedimentation was weighed and the syneresis was calculated with the equation:

\[ \text{Syneresis (\%)} = \left( \frac{\text{Weight of supernatant (g)}}{\text{Weight of yogurt (g)}} \right) \times 100 \]

2.7. The measurement of pH and acidity

The measurement of pH was conducted by using pH-meter (INOlab pH-meter) with the instrument instruction. The acidity analysis was done by titration method as previously described (Naibaho et al., 2022b; Szoltyšik et al., 2020) with 0.25 N NaOH. Briefly, distilled water was added to the yogurt (1:1) and maximum 3 drops of indicator phenolphthalein was then added. The acidity is presented as the total acid which was calculated following the equation:

\[ \text{Lactic acid (\%)} = \left( \frac{\text{volume of NaOH (ml)} \times N \times 90}{\text{Sample} \times 1000} \right) \times 100 \]

2.8. Evaluation of LAB Lactobacillus bulgaricus and Streptococcus thermophilus

LAB was assessed following methods from the previous study (Szoltyšik et al., 2020) by pour-plate method with several dilutions. Lactobacillus bulgaricus was counted in MRS (deMan, Regosa and Sharpe) while Streptococcus thermophilus was counted in M-17 agar. The incubation was done for 48 h at 37 °C and bacterial counts were performed in a log CFU/g sample. The synergism effect was performed by assessing the total of LAB and the ratio of LAB. The total of LAB was counted as the summary of Lactobacillus bulgaricus and Streptococcus thermophilus, the ratio between Streptococcus thermophilus and Lactobacillus bulgaricus was calculated.

2.9. Statistical analysis

Statistical analysis was conducted for quantitative analysis including LAB, pH and acidity, syneresis and flow behavior, in Two-ways analysis of variance (ANOVA) followed by Tukey post-hoc test. The factors were type of BSGP and storage period. The statistical assessment was done using Statistica software version 13.5.0.17.

3. Results and discussion

3.1. Fermentation time

The pH derivation was observed during the fermentation process until a range of 4.3–4.7 was reached, and the changes in the pH are shown in Fig. 1. In general, the correct pH range was achieved after 2 h of fermentation regardless of BSGP types. The pH dropped significantly during the second hour from a range of 5.9–5.6 to reach a pH range between 4.9 and 4.4. Using this method, the pH dropped by about 1.0–1.3, while in the first hour of fermentation, the pH decreased by about 0.3–0.5. The significant drop in pH during the second hour might be due to the isoelectric point of the extract, which was predicted to be below 5 (Vieira et al., 2017). This might be also due to the pH of the incubation during the extraction process (pH at 8.5), which would have allowed for higher pH exposure during yogurt fermentation.

The decrease in pH during fermentation is the result of the impact of lactic acid production and occurred due to LAB growth. In the current study, two strains of LAB: L. bulgaricus and S. thermophilus, were present. During fermentation, those two strains grew synergically. As previously reported (Chandan and O’Rell, 2013), S. thermophilus grew during the first stage of fermentation, lowering the pH of the mixture via free amino acids. This is due to the increased peptide availability, as peptides are needed for L. bulgaricus growth. L. bulgaricus growth generated higher amounts of lactic acid, thus lowering the pH significantly (Chandan and O’Rell, 2013). Because of this, the significant drop in the pH during the second hour of fermentation might be due to the growth of L. bulgaricus in addition to the buffering capacity of the protein and the isoelectric point of the BSG protein, as mentioned previously.

Compared to our previous report, the pH range of control yogurt can be achieved at 4 h fermentation (Naibaho et al., 2022b). By this, the
BSGs that were used in the current study reduced the fermentation time. The addition of BSG flour into the yogurt samples resulted in a fermentation time of 3–4 h (Naibaho et al., 2022b). The incorporation of plant-based ingredients such as moringa leaf powder, sea buckthorn mouse, and other anthocyanin-rich plants into the yogurt increased the fermentation time required to reach a range of pH 4.5–4.6 (Brodziak et al., 2021). Because of this, it was determined that the addition of the BSGPs allowed faster LAB growth, thus reducing the pH. The increase in the fermentation rate seen in this study might be due to the high level of protein availability. The same phenomenon was observed in the yogurt enriched with protein (Giacometti Cavalheiro et al., 2020; Mehrinejad Choobari et al., 2021). It has been reported that the presence of amino acids supported the growth of LAB and thus increased the fermentation rate (Giacometti Cavalheiro et al., 2020; Mehrinejad Choobari et al., 2021). This phenomenon shows the importance of amino acids and protein from BSG extracts in reducing the fermentation period.

3.2. Microstructural characteristics of BSGP-added yogurts

3.2.1. Functional group evaluation by FTIR

The FTIR spectrum of the freeze-dried yogurt samples is shown in Fig. 2. Remarkably, YC had a significant trend in terms of functional group transmittance compared to that in YP and YPF. There were three band areas that showed a lower transmittance (higher absorbance) trend, including 500-800 cm⁻¹, 1100-1000 cm⁻¹, and 3600-3200 cm⁻¹ (1, 2, and 3, respectively, which can be seen in Fig. 2). The band region at 800-500 cm⁻¹ shows the presence of α-glycosidic bonds. The band region at 1100-1000 cm⁻¹ is due to C–O–C stretching, proving the presence of functional groups of the aliphatic ethers. Finally, the region at 3600-3200 cm⁻¹ is responsible for hydroxyl stretching, proving the presence of hydroxyl and amine (Brodziak et al., 2021; Patrignani and Gonzalez-Forte, 2021; Ravindran et al., 2018). These differences might be due to the different amounts of protein content, dry matters and polyphenolic compounds in BSGP. It has been reported that matrix formation in yogurt depends on the structural features of the hydrocolloid backbone and side chains of the added-ingredient molecules (Huang et al., 2021). Band stretching could be observed during FTIR in this study and revealed that the incorporation of protease during the extraction process might have impacted the microstructural surface of the yogurt.

3.2.2. Analysis of matrix distribution and network formation by CLSM

The microstructure evaluation of the yogurt was determined by CLSM, which was performed in order to evaluate the network formation and matrix distribution of the protein–fat and yogurt matrix. Fig. 3 shows the fat structure (stained with Nile red) and Fig. 4 demonstrates the protein structure (stained with Rhodamine 123) of yogurt enriched with BSGPs. The results demonstrated the fat phase of the yogurt (yellow channel) highly influenced by BSGPs. BSGP-C generates a rougher surface, bigger and denser particles and the particles tend to spread and to be separated. However, BSGP-P generated a softer surface appearance, smaller particle size and distribution (Fig. 3b and c). Yogurt structure visualized by a laser reflection revealed that yellow particles in the YP and YP matrix tended to be more homogeneous and mixed in yogurt structure, compared to that in YC. Furthermore, the particles tend to gather and form matrices, thus marking empty spaces. In a comprehensive surface visualization, particle size, particle distribution, density and rough levels were observed to be higher at YC followed by YPF and finally YP. The same phenomenon was observed in the protein matrix of BSGP-enriched yogurt (Fig. 4). Laser reflection on yogurt structure identified that BSGP-C (Fig. 4a) showed an agglomeration of protein in the yogurt structure compared to that in BSGP-P and BSGP-PF (Fig. 4b and c, respectively). The tendency to form network interaction was higher on YP, followed by YPF and YC; meanwhile YC tended to have an agglomerated matrix. By this, protease-treated BSGP showed a better performance in microstructural surface appearance.

A rough surface and less dense structure in YC seem to be the result of the lower protein content and dry matters as well as higher phenolic compounds, thus resulting in more complex link-ed networks. This also might be aligned with the FTIR spectrum results in the previous section (Section 3.4.1), which show a lower band stretching transmittance (higher absorbance) in certain functional groups. Hydrolysates using protamex and flavourzyme had better performance in terms of functional properties (Fathollahy et al., 2021), which is aligned with the surface distribution observed in this study. Moreover, the structure formation observed in this study could be the result of the amount of amino acids contained in the extracts. The utilization of protamex and flavourzyme has been reported due to its ability to reduce the molecular weight and increase protein decomposition, thus enhancing the amounts of amino acids and peptides (Rocha Camargo et al., 2021; Ryan et al., 2020).

The ability of BSGP-P in generating a more compact structure (Fig. 4) demonstrated the structure formation ability of BSGP-P. Matrix formation in yogurt begins during the fermentation process, which is mainly influenced by protein interaction. The fermentation process is essential for LAB growth as well as for gel formation in yogurt (Meybodi et al., 2020). Free amino acids content in BSGP was 1%, 1.5% and 5.3% for BSGP-C, BSGP-P and BSGP-PF, respectively (Krisi et al., 2022). By this, better S. thermophilus growth could be expected during the initial fermentation stage, meaning that there would be more peptides available, increasing L. bulgaricus growth. Consequently, better performance in matrix formation can be expected. As the pH decreased, the casein destabilized at pH 5.3–5.2 followed by denaturation and precipitation at pH 4.7 (Das et al., 2019). At a pH below 4.5, casein and protein milk were acidified (Khuber et al., 2021). The acidification phenomenon is responsible for coagulation and gel formation (Das et al., 2019). At the acidification stage, the casein micelles from milk acted as though they were positively charged with an electrostatic interaction and then formed a dense protein gel structure and aggregated particles (Khuber et al., 2021; Luo et al., 2019). Because of this, the BSGPs might have influenced the electrostatic interactions and acidification process depending on the complexity of the obtained extracts. Moreover, the complexity of the yogurt matrix was also influenced by the structural features of the hydrocolloid backbone and side chain of the added-ingredient molecules (Huang et al., 2021).

3.3. The survival of LAB during the storage

In general, a significant difference (p < 0.05) was observed in the number of both S. thermophilus and L. bulgaricus. From the perspective of survival level, decreased amounts of S. thermophilus during the storage were observed through the study period, except for in the YPF which
resulted in there being higher levels after 14 days of storage. The amount of *L. bulgaricus* also decreased during storage. All of the observed groups had a decline in the total LAB. Remarkably, YC had a lower survival rate during the storage period compared to that observed in the YP and YPF, as shown by the highest derivation level. This might be due to the higher amount of free amino acids mentioned earlier, which benefits the LAB growth.

Compared to previous studies, the utilization of protein extracts from BSG generated a higher amount of *S. thermophilus* in yogurt. The incorporation of plant-based ingredients in yogurt resulted approximately 7.0–9.5 log CFU/mL of *S. thermophilus* (Bouaziz et al., 2021; Gürbüz et al., 2021; Szoltysik et al., 2020), while current study had a range of 8.2–11.48 log CFU/mL. Whey protein enrichment in yogurt resulted in *S. thermophilus* in a range of 8.0–8.3 log CFU/mL (Atallah et al., 2020) and 7–8 log CFU/mL of *S. thermophilus* in high-protein goat milk yogurt (Gursel et al., 2016). The amount of *L. bulgaricus* in this study is also higher than that in other studies reporting a range between 5.9 and 5.8 log CFU/mL of *L. bulgaricus* (Bouaziz et al., 2021; Mehrinejad Choobari et al., 2021). Whey protein enrichment in yogurt generated *L. bulgaricus* in a range of 8.1–8.5 log CFU/mL (Atallah et al., 2020), and high-protein yogurt from goat milk was demonstrated to have *L. bulgaricus* present in a range of 7–8 log CFU/mL of (Gursel et al., 2016), which are still lower than the numbers in the current study.

Meanwhile, the addition of BSG flour (which is dominated by dietary fibre) during yogurt production had levels of 8.3–10.4 log CFU/mL and 5.3–7.4 log CFU/mL of *S. thermophilus* and *L. bulgaricus*, respectively (Naibaho et al., 2022b). In this study, a higher number (9.4–10.5 log CFU/mL) of *L. bulgaricus* was generated.

A decline in the amount of LAB in yogurt during the storage period has been observed previously (Bouaziz et al., 2021; Gürbüz et al., 2021; Mehrinejad Choobari et al., 2021; Naibaho et al., 2022b; Szoltysik et al., 2020). However, the amount of LAB in this study was considerably high although it had decreased from the initial amount observed on the first day. The ratio between *S. thermophilus* and *L. bulgaricus* on the first day showed that the majority of the studied groups had higher levels of *S. thermophilus* than *L. bulgaricus*, except with the addition of BSGP-PF. The same phenomenon has been reported previously (Bouaziz et al., 2021; Gürbüz et al., 2021; Mehrinejad Choobari et al., 2021; Szoltysik et al., 2020). This phenomenon occurred due to the higher proteolytic activity of *S. thermophilus* and the resistance of the strain to the acidic and cold conditions during the storage (Nguyen et al., 2014). After 14 days of storage, the amount of *S. thermophilus* was lower than the amount of *L. bulgaricus*, showing that the BSG protein enhanced the survival rate of the *L. bulgaricus* strain in yogurt and consequently improved lactic acid production, as mentioned in the previous section. The higher amount of *L. bulgaricus* can be attributed to the ability of...
BSGPs to support the growth and adaptation of this strain in yogurt. Because of this, the involvement of protease could enhance the susceptibility of \textit{L. bulgaricus} during yogurt storage. Another possible reason is that there might be a synergistic effect between both strains, which improved the survival rate of \textit{L. bulgaricus} and thus lowering the ratio of \textit{S. thermophilus} and \textit{L. bulgaricus}.

3.4. pH and acidity

In the first day, YP and YPF had a lower pH compared to that in YC, showing a higher LAB’s growth. After 14 days, the pH decreased significantly (\(p < 0.05\)) in all observed groups. The difference in pH might be aligned with the lactic acid production. The amount of lactic acid was lower on the first day compared to that after 14 days of storage. The decrease in the pH and increase in lactic acid production after 14 days of storage might be due to the synergic LAB growth that took place during the storage period. As shown in Table 1, the change, \(\Delta_{1-14}\), in the amount of LAB is higher in the \textit{S. thermophilus} strain while \textit{L. bulgaricus} remains stable. It is reported that \textit{L. bulgaricus} produced a higher amount of lactic acid than \textit{S. thermophilus} (Chandan and O’Rell, 2013). Because of this, the higher amount of lactic acid production might be associated with the higher stability of \textit{L. bulgaricus} during the storage. Initially, the addition of the BSGPs before the fermentation process had no impact on the pH of the mixture. pH derivation began during the incubation period, thus resulting in different pH levels. Because of this, the different BSGPs had an influence on the pH and lactic acid production in the yogurt. The pH value and lactic acid content influence LAB growth. The incorporation of leaf powder, sea buckthorn mousse, and forsk seed mucilage powder induced LAB growth (Bouaziz et al., 2021; Brodziak et al., 2021; Mehrinejad Choobari et al., 2021), thus increasing the amount of lactic acid and lowering the pH. The addition of dietary fiber from certain by-products generated a stable pH during the storage period due to the stable amount of LAB during the storage (do Espírito Santo et al., 2012).

The amount of lactic acid in this study is higher than that in previous studies, which is reported around 0.8-0.9% in yogurt (Delikanli and Ozcan, 2017; Giacometti Cavalheiro et al., 2020); however, in this study, it ranged between 0.87 and 1.18. However, protein enrichment in the yogurt was able to improve the amount of lactic acid to a range between 1.0 and 1.33 (Delikanli and Ozcan, 2017; Giacometti Cavalheiro et al., 2020), which is in alignment with the values determined in this study. A higher lactic acid content in high-protein goat milk yogurt was reported to be in a range between 1.5 and 1.8% (Gursel et al., 2016). Because of this, BSGPs are comparable to those of the milk-based proteins that generate lactic acid in yogurt. BSG is known for its high protein content (Wen et al., 2019). The different amount of protein content,
Table 1: Physical properties, rheological properties and the acidity of the yogurt enriched with BSGPs during the storage period.

| Storage period (days) | Yogurt | YC | YP | YPF |
|-----------------------|--------|----|----|-----|
| Consistency index - k |        |    |    |     |
| 1                     | 25.448 ± 0.89d | 26.724 ± 0.73c | 37.762 ± 3.29c |
| 14                    | 104.710 ± 2.79ab | 115.330 ± 7.85ab | 96.525 ± 0.20b |
| Flow behavior index - n |        |    |    |     |
| 1                     | 0.076 ± 0.00ab | 0.084 ± 0.00a | 0.071 ± 0.00ab |
| 14                    | 0.064 ± 0.00bc | 0.080 ± 0.00bc | 0.055 ± 0.00c |
| Apparent viscosity - n50 |        |    |    |     |
| 1                     | 0.034 ± 0.01b | 0.040 ± 0.00b | 0.040 ± 0.00b |
| 14                    | 0.162 ± 0.00a | 0.169 ± 0.00a | 0.168 ± 0.00a |
| Syneresis              |        |    |    |     |
| 1                     | 51.571 ± 1.44d | 48.358 ± 0.82bc | 46.788 ± 0.12b |
| 14                    | 70.752 ± 3.38bc | 56.038 ± 0.82bc | 51.840 ± 0.31b |
| pH                    |        |    |    |     |
| 1                     | 4.55 ± 0.01c | 4.46 ± 0.01b | 4.42 ± 0.03b |
| 14                    | 4.02 ± 0.01d | 4.12 ± 0.00a | 3.97 ± 0.02d |
| L. bulgaricus         |        |    |    |     |
| 1                     | 10.129 ± 0.04d | 10.133 ± 0.02b | 10.439 ± 0.05b |
| 14                    | 9.406 ± 0.01ed | 9.675 ± 0.07b | 9.557 ± 0.07ed |
| ΔL(1-14)              | 0.72 | 0.61 | 0.895 | 0.00c |
| S. thermophilus       |        |    |    |     |
| 1                     | 10.303 ± 0.00c | 10.171 ± 0.00b | 8.214 ± 0.01c |
| 14                    | 8.206 ± 0.01c | 9.080 ± 0.04c | 8.724 ± 0.03c |
| ΔS(1-14)              | 2.1 | 1.1 | +0.51 |    |
| Total LAB             |        |    |    |     |
| 1                     | 20.432 ± 0.05c | 20.303 ± 0.02c | 18.653 ± 0.06b |
| 14                    | 17.614 ± 0.00d | 18.755 ± 0.03c | 18.282 ± 0.10c |
| Ratio                 |        |    |    |     |
| 1                     | 1.017 ± 0.00e | 1.004 ± 0.00e | 0.797 ± 0.00e |
| 14                    | 0.872 ± 0.00ed | 0.939 ± 0.01b | 0.913 ± 0.00e |

Note: The data is shown as mean ± standard deviation of triplicate measurement. A different subscription letter shows a significant difference (P < 0.05) in the same observed parameter. ΔL(1-14): the declining in the amount of LAB during the 14 days of storage. ±: shows an increase of LAB after 14 days of storage.

### 3.5. Syneresis

Syneresis level was stable during the 14 days of storage, except for the BSGP-C substitution. It was previously mentioned that the addition of BSGP-C in the yogurt resulted in the formation of a rougher surface and a less dense protein and fat distribution. This result showed that the BSGP-C was less stable in terms of their effects on consistency, although they did result in a higher initial gel formation, as seen in Fig. 5. The result explained that BSGPs showed a better performance in preserving the syneresis of yogurt. The ability of BSGP-P to preserve syneresis during 14 days of refrigerated storage might be related to the strength of the formed protein network. It has previously been mentioned that protein interaction forms an initially weak bond (Pachekrepol et al., 2021). After that, macromolecule hydration occurred, thus strengthening the formed bond during storage (Ramirez-Sucer and Velez-Ruiz, 2013). Because of this, both YP and YPF had a strong yogurt matrix due to the abundance of free amino acids, particularly in the enzyme-treated extracts.

Compared to other studies, the addition of the different BSGPs revealed the same syneresis level as reported previously. The addition of stabilizer ingredients in yogurt generated a range of 35–50% during syneresis (Bouaziz et al., 2021; Mehrinejad Choobari et al., 2021). Because of this, these BSGPs could work as a stabilizer in addition to their biological properties. Plant-based extracts have also been shown to generate a similar effect on the syneresis level of around 35–50% in yogurt, while plant seed mucilage resulted in a yogurt with a syneresis between 70 and 80% (Bouaziz et al., 2021; Mehrinejad Choobari et al., 2021). Moreover, protein enrichment in yogurt resulted in a syneresis level of 50–74% (Atallah et al., 2020; Delikanli and Ozcan, 2017).

### 3.6. The evaluation of rheological behavior

The curves depicting the relationship between the shear rate vs shear stress of BSGPs-enriched yogurt at 1 day of storage (a) and 14 days of storage (b); shear rate vs viscosity at 1 day storage (c) and 14 days of storage (d) ((–): YC (–): YPF, and (–): YP).

![Fig. 5. The relation between shear rate vs shear stress of BSGPs-enriched yogurt at 1 day of storage (a) and 14 days of storage (b); shear rate vs viscosity at 1 day storage (c) and 14 days of storage (d) ((–): YC (–): YPF, and (–): YP).](image-url)
stress and shear rate vs viscosity during storage are presented in Fig. 5. Initially, YP had a similar and stable shear stress trend which is stable with the increasing of shear stress (Fig. 5a). Meanwhile, YC showed different behavior which slightly increased with the increase of shear rate. The shear stress of the YC increased due to the increase in the shear rate, while the yogurt prepared with the YP had the most stable shear stress. Shear stress represents the energy required to damage the structure of the yogurt matrix (Vénica et al., 2020), thus showing the strength of the matrix. Because of this, the addition of BSG-C might have induced gel formation faster than BSGP-P and BSGP-PF. This is in alignment with the FTIR spectrum results, which determined that a lower transmittance was observed, which was determined to be responsible for α-glycosidic bonds, aliphatic ethers, and hydroxyl and amine groups. This might also be related to the CLSM results, where a rough and grainy-looking appearance was observed on the structure of the matrix. BSGP-C contained a lower dry matter and fewer amino acids. Therefore, its gel formation ability is higher at the initial time.

After 14 days, the shear stress behavior increased dramatically compared to that at the initial observation (Fig. 5b). This phenomenon demonstrates that BSGPs resulted in the yogurt having increased gel formation, which could be beneficial for the textural properties as well as for the consistency and for reducing syneresis. Different shear stress trends in yogurt have been reported previously and were found to be dependent on the ingredients that had been added as well as the treatments (Azari-Anpar et al., 2021; Körzendorf et al., 2019; Vénica et al., 2020), which were shown to be related to flow behavior-related properties, microstructural properties, and syneresis.

In general, the viscosity of the yogurt after 14 days (Fig. 5c and d) of storage was higher than that on the first day of storage. As seen from the apparent viscosity (h0 - P.a.s) in Table 1, the results revealed that the viscosity increased significantly (p < 0.05). The increase in viscosity might be aligned with the change in shear stress, as previously mentioned. As can be seen in Table 1, a significant (p < 0.05) increase in the consistency index was observed due to the 14 days of refrigerated storage, although there was no significant difference (p > 0.05) observed in the consistency index on the first day. A different trend was observed in the flow behavior index in which slight decrease was observed after 14 days of storage. All of the samples revealed a flow behavior index below 1 (n < 1), showing non-Newtonian fluid behavior (Vénica et al., 2020). The addition of BSGPs tended to improve gel formation during storage, which did not occur on the first day of storage. This phenomenon can be explained by the interaction between the amino acids and the casein micelles during the fermentation process (Ramírez-Sucre and Vélez-Ruiz, 2013). During the fermentation, the amino acids from BSG interacted with the surface of the casein micelles. Initially, the formed bond was weak due to the shorter fermentation time (Pachekrapapol et al., 2021), and then it increased during the storage period due to the hydration of the macromolecules and the stabilization properties of certain ingredients (Ramírez-Sucre and Vélez-Ruiz, 2013). This phenomenon led to an improvement in the viscosity and consistency of the yogurt. Protein availability in yogurt fermentation impacted the structural formation in the yogurt, thus modifying the physical properties of the yogurt (Gursel et al., 2016; Körzendorf et al., 2019). Furthermore, higher amounts of protein facilitated the acid whey production, which hardened the yogurt structure (Körzendorf et al., 2019).

4. Conclusion

Yogurt prepared with BSGP-P and BSGP-PF had a denser and softer fat and protein microstructure surface. YC had a rough surface structure, a finding that was in alignment with the gel formation ability demonstrated in the initial stage and its instability while maintaining the syneresis level. BSGP-C resulted in faster gel formation in the yogurt; however, its consistency in terms of texture formation was less stable compared with the enzyme-treated BSGPs. Enzyme-treated BSGPs showed a weaker texture in the initial stage, but the texture became stronger during the storage period due to hydration of the macromolecules and the stabilization properties of the added extracts, thus improving the flow behavior. BSGP-P maintained yogurts’ consistency during the storage period, which is shown by a stable syneresis level. It also improved the ability of LAB to grow and to survive during refrigerated storage, particularly in the survival rate of L. bulgaricus. The study presents evidence that yogurt prepared with BSGPs produced a higher amount of lactic acid compared with milk-protein-based enrichment yogurts. Further investigation on consumer perceptions is seemingly important in the near future.

CRediT authorship contribution statement

Joncer Naibaho: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, preparation, Writing – review & editing, Funding acquisition. Ėmir Jonuzi: Methodology, Formal analysis, Writing – review & editing. Nika Butula: Methodology, Formal analysis, Writing – review & editing. Małgorzata Korzeniowska: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. Maike Foste: Methodology, Formal analysis, Writing – review & editing. Karina Nola Sinamo: Methodology, Formal analysis, Writing – review & editing. Grzegorz Chodacek: Methodology, Formal analysis, Writing – review & editing. Baoru Yang: Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data is included in the manuscript

This work was supported by UPWR 2.0, international and interdisciplinary programme of development of Wrocław University of Environmental and Life Sciences, co-financed by the European Social Fund under the Operational Programme Knowledge Education Development 2014–2020: Axis III Higher education for the economy and development; Action 3.5. Comprehensive programmes for schools of higher education (POWR.03.05.00-00-Z062/18).

It was also supported by ERA-NET CO-FUND Horizon 2020 - FACCE SURPLUS Sustainable and Resilient Agriculture for Food and Non-Food Systems and PROWASTE Protein-fibre biorefinery for scattered material streams (2019–2021).

References

Atallah, A.A., Morry, O.M., Gemiel, D.G., 2020. Characterization of functional low-fat yogurt enriched with whey protein concentrate, Ca-caseinate and spirulina. Int. J. Food Prop. 23, 1678–1691. https://doi.org/10.1080/10942912.2020.1823409.
Aydar, E.F., Tutuncu, S., Ozcelik, B., 2020. Plant-based milk substitutes: bioactive compounds, conventional and novel processes, bioavailability studies, and health effects. J. Funct. Foods 70, 103975. https://doi.org/10.1016/j.jff.2020.103975.
Azari-Anpar, M., Khomeiri, M., Daraei Garmakhany, A., Lotfi-Shirazi, S., 2021. Development of camel and cow’s milk, low-fat frozen yogurt incorporated with Qodume Shahri (Lepidium perfoliatum) and cress seeds (Lepidium sativum) gum: flow behavior, textural, and sensory attributes’ assessment. Food Sci. Nutr. 9, 1640–1650. https://doi.org/10.1002/fsn3.2139.
Bafna, H., Ajithkrishnan, C., Kalantharakath, T., Singh, R., Kalyan, P., Vathar, J., Patel, H., 2018. Effect of short-term consumption of amul probiotic yogurt containing Lactobacillus acidophilus La5 and Bifidobacterium lactis Bb12 on salivary streptococcus mutants count in high caries risk individuals. Int. J. Basic Med. Res. 8, 111. https://doi.org/10.4103/ijbmr.IJABMR.447.16.
Barendols, E., Smith, E., Reustrakul, S., Tonucci, L., Anothaisintawee, T., 2019. The effect of probiotic yogurt on glycemic control in type 2 diabetes or obesity: a meta-analysis
of nine randomized controlled trials. Nutrients 11, 671. https://doi.org/10.3390/nu11030671.

Bayat, A., Azizi-Soleiman, F., Heidari-Beni, M., Feizi, A., Iraj, B., Ghiavasand, R., Askari, G., 2016. Effect of curcubitacins fiscilola and probiotic consumption on blood glucose, lipid profile, and inflammatory marker in Type 2 Diabetes. Int. J. Prev. Med. 7, 30. https://doi.org/10.4103/2008-7625.157455.

Bouaziz, M.A., Bhir, M., Khodja, H., Sbiti, H., Sebai, H., Karra, S., Smouss, A., Attia, H., Besbes, S., 2021. Techno-functional characterization and biological potential of Agave americana leaves: impact on yogurt qualities. Food Measure 15, 309–326. https://doi.org/10.1016/j.foomea.2020.10.003.

Brodzka, A., Krol, J., Matwijczuk, A., Ceernicki, T., Glikowski, P., Wlazlo, I., Litwiniczuk, A., 2021. Effect of sea buckthorn (hippophae rhamnoides L.) mousse on properties of probiotic yogurt. Appl. Sci. 11, 545. https://doi.org/10.3390/app11020545.

Chand, R.C., O’Rell, K., 2013. Principles of yogurt processing. In: Chandan, R.C., Kilara, A. (Eds.). Manufacturing Yogurt and Fermented Milks. John Wiley & Sons, Oxford, pp. 239-261. https://doi.org/10.1002/9781118481201.ch11.

Clegg, M.E., Tarrado Ribe, A., Reynolds, R., Klem, K., Stergiadi, S., 2021. A comparative assessment of the nutritional composition of dairy and plant-based dairy alternatives available for sale in the UK and the implications for consumers’ dietary intakes. Food Res. Int. 48, 110586 https://doi.org/10.1016/j.foodres.2021.110586.

Crowley, D., O’Callaghan, Y., McCarthy, A., Connolly, A., Piggott, C.O., FitzGerald, R.J., O’Brien, N.M., 2015. Immunomodulatory potential of a brewers’ spent grain protein hydrolysate incorporated into low-fat milk following in vitro gastrointestinal digestion. Int. J. Food Sci. Nutr. 66, 672-676. https://doi.org/10.1080/09637487.2015.1077788.

Das, K., Choudhary, R., Thompson-Witrick, K.A., 2019. Effects of new technologies on the current manufacturing process of yogurt-to-increase the overall marketability of yogurt. Lebensm. Wiss. Technol. 108, 69-80. https://doi.org/10.1016/j.lwt.2019.03.058.

Delikanli, B., Ozcan, T., 2017. Improving the textural properties of yogurt fortified with milk proteins: textural properties of yogurt. J. Food Process. Preserv. 41, e13101 https://doi.org/10.1111/jfpp.13101.

do Espirito Santo, A.P., Cartolano, N.S., Silva, T.F., Soares, F.A.S.M., Giocelli, L.A., Perego, P., Converti, A., Oliveira, M.N., 2012. Fibers from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yogurt. Int. J. Food Microbiol. 154, 135-144. https://doi.org/10.1016/j.ijfoodmicro.2011.12.025.

do Reis, S.A., da Conceição, L.L., Siqueira, N.P., Rosa, D.D., da Silva, L.L., Peluzio, M. do C.G., 2017. Review of the mechanisms of probiotic actions in the prevention of colorectal cancer. Nutr. Res. 37, 1-19. https://doi.org/10.1016/j.nutres.2016.11.009.

Fathiollahy, I., Farmani, J., Kasaei, M.R., Hamishehkar, H., 2021. Characteristics and functional properties of Persian lime (Citrus latifolia) seed protein isolate and enzymatic hydrolysates. Lebensm. Wiss. Technol. 140, 110765 https://doi.org/10.1016/j.foodhyd.2020.106240.

Giacometti Cavalheiro, F., Parra Baptista, D., Domingos Gali, B., Negrão, F., Nogueira Eberlin, M., Lúcia Gigante, M., 2020. High protein yogurt with addition of Lactobacillus helveticus: peptide profile and angiotensin-converting enzyme ACE-inhibitory activity. Food Chem. 333, 127482 https://doi.org/10.1016/j.foodchem.2020.127482.

Gürbüz, Z., Ezkaya-Koton, T., Şengül, M., 2021. Evaluation of physicochemical, microbiological, textural, and structural microstructure characteristics of set-type yogurt supplemented with quince seed mucilage powder as a novel stabilizer. Int. Dairy J. 114, 104938 https://doi.org/10.1016/j.idairyj.2020.104938.

Gursel, A., Gunay, A., Anil, E.A.K., Budak, S.O., Aydemir, S., Durulu-Ozkaya, F., 2016. Role of milk protein hydrolysates in products obtained by partial enzymatic hydrolysis of milk protein. J. Dairy Sci. 99, 2694-2703. https://doi.org/10.3168/jds.2015-10993.

Hill, D., Ross, R.P., Arendt, J., Stanton, C., 2017. Microbiology of yogurt and bio-yogurts containing probiotics and prebiotics. In: Yogurt in Health and Disease Prevention. Elsevier, pp. 69-85. https://doi.org/10.1016/B978-0-12-801034-4.00004-3.

Huang, T., Tu, Z., Zhangxuan, X., Wang, H., Zhang, L., Bansal, N., 2021. Characteristics of fish gelatin-anionic polysaccharides complexes and their applications in yoghurt: rheology and tribology. Food Chem. 343, 128413 https://doi.org/10.1016/j.foodchem.2021.128413.

Hummel, J., Changalucha, J., Butula, N., J. Naibaho et al. then, 2021. Formulation of yogurt-like product from coconut milk and evaluation of physico-chemical, rheological, and sensory properties. Int. J. of Dairy Science and Food Sci. 25, 100393 https://doi.org/10.1016/j.ijds.2021.100393.

Patrignani, M., González-Forte, I., 2015. Characterization of maltooligosaccharides derived from Brewers’ spent grain: new insights into their structure and antioxidant activity. Int. J. of Dairy Science. 25, 384–391. https://doi.org/10.1111/ijds.14653.

Rahman, S., Sultornuw, M., 2016. Effects of fermentation and storage on bioactive activities in milks and yogurts. Procedia Chem. 18, 53-62. https://doi.org/10.1016/j.proche.2016.01.010.

Ramírez-Sucre, M.O., Vachon, D.A., Dolly, P., Mozammal, A.A., 2019. Metalloproteinase analysis revealed metabolic changes in patients with diabete-s predimaninate irritable bowel syndrome and metabolic responses to a synbiotic yogurt intervention. Eur. J. Nutr. 58, 3109-3119. https://doi.org/10.1007/s00394-018-1855-z.

Pachkarpapal, U., Kokhuenhakan, Y., Ongsawat, J., 2021. Formulation of yogurt-like protein hydrolysate incorporated into low-fat milk following in vitro gastrointestinal digestion. Int. J. Food Sci. Nutr. 78, 693-700. https://doi.org/10.1080/09637487.2021.1840888.

Rocchi, M., Avila, D.V.L., Nascimento, C.C., Costa, S.S.L., Metabolomics analysis revealed metabolic changes in patients with diabetes-predimaninate irritable bowel syndrome and metabolic responses to a synbiotic yogurt intervention. Eur. J. Nutr. 58, 3109-3119. https://doi.org/10.1007/s00394-018-1855-z.

Roche Camargo, T., Ramos, P., Monteiro, J., Prentice, C., Fernandes, C.J.C., Zambuzzi, W.F., Valenti, W.C., 2021. Biological activities of the protein hydrolysate obtained from two fishes common in the fisheries bycatch. Food Chem. 342, 128361. https://doi.org/10.1016/j.foodchem.2020.128361.

Ryan, G., O’Regan, J., FitzGerald, R.J., 2020. Emulsification properties of bovine milk protein isolate and associated enzymatic hydrolysates. Int. Dairy J. 110, 104811 https://doi.org/10.1016/j.idairyj.2020.104811.

Shahbandeh, M., 2020. Global Yogurt Market Value Forecast 2018-2024. Forecast. 2017.06.039.

Roche Camargo, T., Ramos, P., Monteiro, J., Prentice, C., Fernandes, C.J.C., Zambuzzi, W.F., Valenti, W.C., 2021. Biological activities of the protein hydrolysate obtained from two fishes common in the fisheries bycatch. Food Chem. 342, 128361. https://doi.org/10.1016/j.foodchem.2020.128361.

Soyuzdik, M., Kucharska, A.Z., Sokol-Lotowska, A., Dąbrowska, A., Bobak, Ł., Grzechnikowa, J., 2020. The effect of rosà spinosissima fruit extract on lactic acid bacteria growth and other yogurt parameters. Foods 9, 1167. https://doi.org/10.3390/foods9061167.
stirred-type yogurt. Int. J. Food Sci. Technol. 55, 1916-1923. https://doi.org/10.1111/ijfs.14415.

Vieira, Elsa F., et al., 2017. Protective ability against oxidative stress of brewers’ spent grain protein hydrolysates. Food Chem. 228, 602-609. https://doi.org/10.1016/j.foodchem.2017.02.050. In this issue.

Wen, C., Zhang, J., Duan, Y., Zhang, H., Ma, H., 2019. A mini-review on brewer’s spent grain protein: isolation, physicochemical properties, application of protein, and functional properties of hydrolysates. J. Food Sci. 84, 3330-3340. https://doi.org/10.1111/1750-3841.14906.