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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

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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 (Bafna 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

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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 BSGPs without changing taste acceptability compared to control extract (Kriisa et al., 2022). According to our preliminary study, BSGPs prepared with protease and co-incubation with flavourzyme are responsible for higher ORAC and ABTS antioxidant capabilities and possess higher oil holding capacity and foaming properties compared to BSGPs control (Naibaho et al., 2022c).

By this, the suitability of protease-treated BSGPs in yogurt as protein enrichment might be challenging which has never been reported. Protease-treated BSGPs have been utilized to improve the microstructure and gel formation, flow behavior and syneresis, as well as lactic acid production of coconut-based yogurt-alternatives (Naibaho et al., 2022a). Taking into consideration that peptides availability during the yogurt fermentation influenced its health benefits, BSGPs prepared with protease and co-incubation with flavourzyme might offer a higher potential in protein fortification of yogurt. Current study employed BSGPs prepared by protease incubation as well as its co-incubation with flavourzyme in addition to the control (non-protease treatment) in yogurt fermentation. We hypothesized that BSGPs prepared with proteases and its co-incubation with flavourzyme maintained the growth of LAB, lactic acid production and pH stability during the storage due to the higher amount of available peptides thus influencing the matrix formation of yogurt. Furthermore, enzymatic-prepared BSGPs might preserve rheological behavior, syneresis and consistency, due to its higher ability in oil holding capacity and foaming properties.

2. Materials and methods

2.1. Materials

The BSG samples were collected from a light-type beer-producer brewery in Poland and kept at −20 °C before the extract preparation. Homogenized-pasteurised milk with composition 3.2% fat content; 3% protein; 4.7% carbohydrates and 0.1% salt, was purchased from the commercial market. Yogurt starter was prepared as follows: 2% (*w/w*) of yogurt culture consisted of *Streptococcus thermophilus and Lactobacillus bulgaricus* (Yo-flex, CHR Hansen) was added to a milk and incubated at 43–45 °C until it reached the pH 4.3–4.7 (Naibaho et al., 2022b). The starter then kept at 10 °C for 12 h prior to yogurt preparation.

Dry microbial substrate (MRS and M-17) and microbial agar were purchased from Merck, Germany, cycloheximide was from Applichem, and staining Nile Red and Rhodamine 123 for confocal analysis were purchased from Sigma-Aldrich. All chemicals used for analyses were analytical grade.

2.2. Preparation of BSGPs

Incubated BSGs were collected from Fraunhofer Institute for Process Engineering and Packaging IVV, 85,354 Freising, Germany. Incubation process was done following the procedure described in the previous study (Kriisa et al., 2022). Briefly, BSG and water were mixed with a ratio of 1:10 and the mixture was separated into three group treatments: 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; Szołtysik 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/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 IRSpiritTM, Shimadzu (Shimadzu Europe, Germany, GmbH). The measurement was observed at 4000 and 400 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 (*w:v*), transferred into a glass slide and covered with a coverslip. The image was produced on a confocal microscope using a 20x (NA 0.75) 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 488 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 (Szołtysik et al., 2020). Flow curves were fitted to Power model of Ostwald de Waele with the equation:

$$\eta_{50} = \mathrm{k} \times \gamma^{n-1}$$

η_{50} = apparent viscosity (Pa.s); k = consistency index (Pa.s); γ = 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:

$$Syneresis\ (\%) = \frac{Weight\ of\ supernatant\ (g)}{Weight\ of\ yogurt\ (g)}\ x\ 100$$

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; Szołtysik 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:

$$Lactic\ acid\ (\%) = \frac{volume\ of\ NaOH\ (mL)\ x\ N\ x\ 90}{Sample\ x\ 1000}\ x\ 100$$

2.8. Evaluation of LAB Lactobacillus bulgaricus and Streptococcus thermophilus

LAB was assessed following methods from the previous study (Szołtysik 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

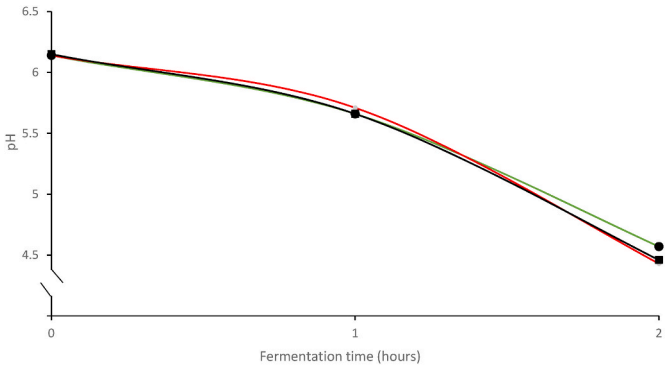

Fig. 1. Influence of BSGPs in pH derivation during fermentation process of yogurt. ((–): YC; (–): YPF; (–): YP).

BSGPs 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 mousse, 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, which shows the 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 González-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 YPF and YP matrix tended to immerse and homogeneously 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.

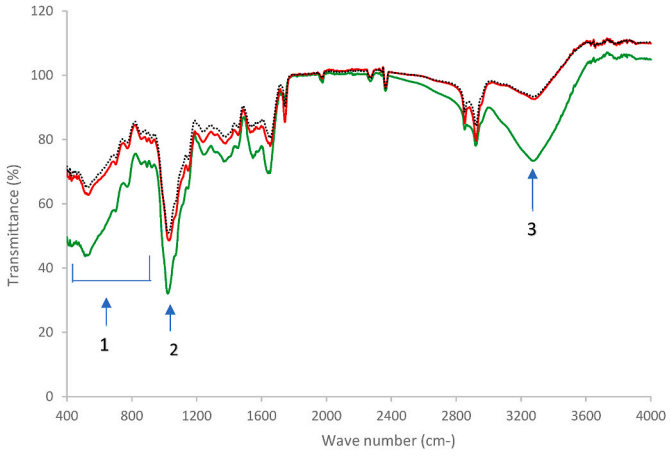

Fig. 2. FTIR spectrum of freeze-dried yogurt enriched with different BSGPs (—): YC; (—): YPF; (—): YP.

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 BSG-PF, respectively (Kriisa 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 (Khubber 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 (Khubber 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

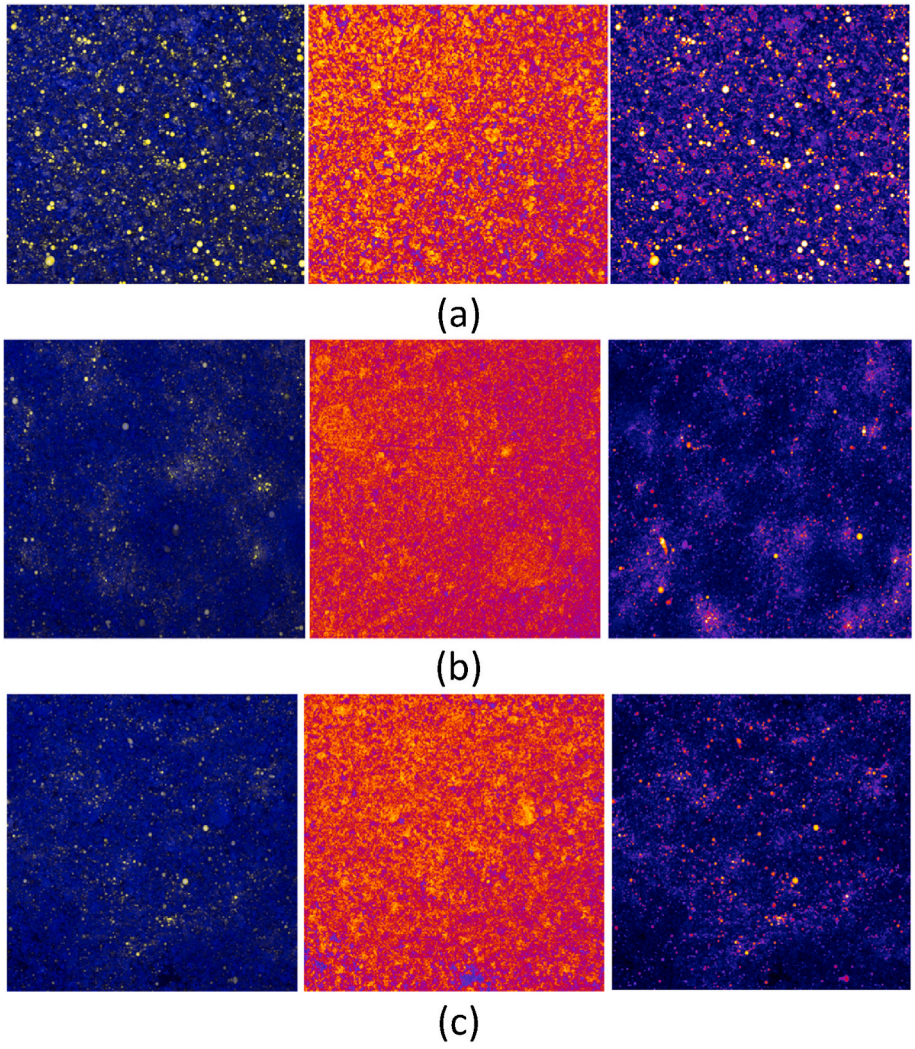

Fig. 3. Confocal laser scanning micrographs of freeze-dried yogurt enriched with different BSGPs stained with Nile red: a. YC; b. YP; c. YPF. The images are presented as maximum intensity projections from confocal Z stacks in a fire intensity scale. (Left: overlay image; middle (yellow channels): fat phase, right (blue channels): yogurt structure visualized with a laser reflection.

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; Szołtysik 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; Szołtysik 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; Szołtysik 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

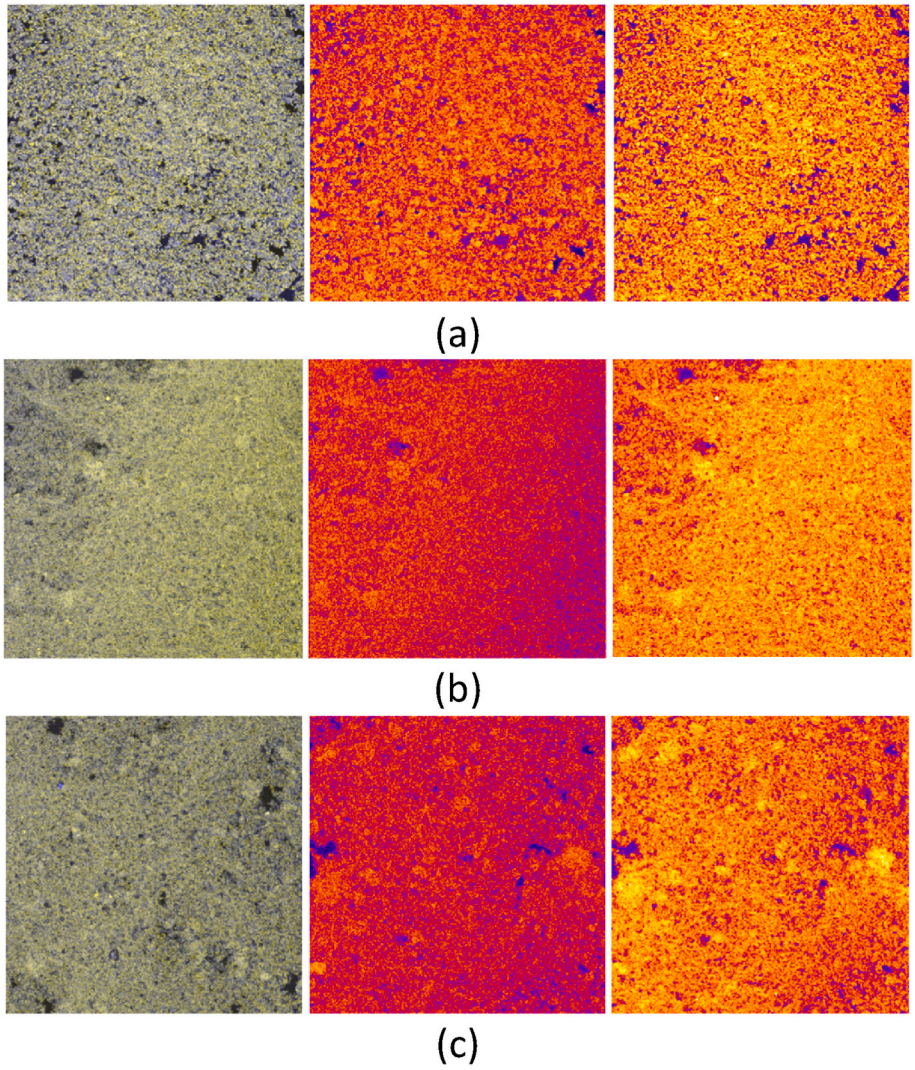

Fig. 4. Confocal laser scanning micrographs of freeze-dried yogurt enriched with different BSGPs stained with Rhodamine 123: a. YC; b. YP; c. YPF. The images are presented as maximum intensity projections from confocal Z stacks in a fire intensity scale. (Left: overlay image; middle (yellow channels): protein phase, right (blue channels): yogurt structure visualized with a laser reflection.

BSGPs to support the growth and adaptation of this strain in yogurt. Because of this, the involvement of protease could enhance the susceptibility of *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 *L. bulgaricus* and thus lowering the ratio of *S. thermophilus* and *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, $\Delta_{(1\text{-}14)}$, in the amount of LAB is higher in the *S. thermophilus* strain while *L. bulgaricus* remains stable. It is reported that *L. bulgaricus* produced a higher amount of lactic acid than *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 *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 ± 0.89^{c}	26.724 ± 0.73^{c}	37.762 ± 3.29^{c}
14	104.710 ± 2.79^{ab}	115.330 ± 7.85^{a}	96.525 ± 0.20^{b}
Flow behavior index – n			
1	0.076 ± 0.00^{ab}	0.084 ± 0.00^{a}	0.071 ± 0.00^{ab}
14	0.064 ± 0.00^{bc}	0.080 ± 0.00^{ab}	0.055 ± 0.00^{c}
Apparent viscosity - n50			
1	0.034 ± 0.01^{b}	0.040 ± 0.00^{b}	0.040 ± 0.00^{b}
14	0.162 ± 0.00^{a}	0.169 ± 0.00^{a}	0.168 ± 0.00^{a}
Syneresis			
1	51.571 ± 1.44^{b}	48.358 ± 0.82^{b}	46.788 ± 0.12^{b}
14	70.752 ± 3.38^{a}	56.038 ± 0.82^{ab}	51.840 ± 0.31^{b}
pH			
1	4.55 ± 0.01^{a}	4.46 ± 0.01^{b}	4.42 ± 0.03^{b}
14	4.02 ± 0.01^{d}	4.12 ± 0.00^{c}	3.97 ± 0.02^{d}
Lactic acid			
1	0.872 ± 0.04^{c}	1.006 ± 0.03^{bc}	0.895 ± 0.00^{c}
14	1.001 ± 0.06^{bc}	1.137 ± 0.04^{ab}	1.176 ± 0.03^{a}
L. bulgaricus			
1	10.129 ± 0.04^{b}	10.133 ± 0.02^{b}	10.439 ± 0.05^{a}
14	9.408 ± 0.01^{d}	9.675 ± 0.07^{c}	9.557 ± 0.07^{cd}
$\Delta_{(1\text{-}14)}$	0.72	0.46	0.88
S. thermophilus			
1	10.303 ± 0.00^{a}	10.171 ± 0.00^{b}	8.214 ± 0.01^{e}
14	8.206 ± 0.01^{e}	9.080 ± 0.04^{c}	8.724 ± 0.03^{d}
$\Delta_{(1\text{-}14)}$	2.1	1.1	+0.51
Total LAB			
1	20.432 ± 0.05^{a}	20.303 ± 0.02^{a}	18.653 ± 0.06^{b}
14	17.614 ± 0.00^{d}	18.755 ± 0.03^{b}	18.282 ± 0.10^{c}
Ratio			
1	1.017 ± 0.00^{a}	1.004 ± 0.00^{a}	0.787 ± 0.00^{e}
14	0.872 ± 0.00^{d}	0.939 ± 0.01^{b}	0.913 ± 0.00^{c}

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. $\Delta_{(1\text{-}14)}$: the declining in the amount of LAB during the 14 days of storage. +: shows an increase of LAB after 14 days of storage.

free amino acids and the amount of available peptides in BSGPs might have altered the lactic acid forming ability of the yogurts.

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 (Pachekrepapol et al., 2021). After that, macromolecule hydration occurred, thus strengthening the formed bond during storage (Ramírez-Sucre and Vélez-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, 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

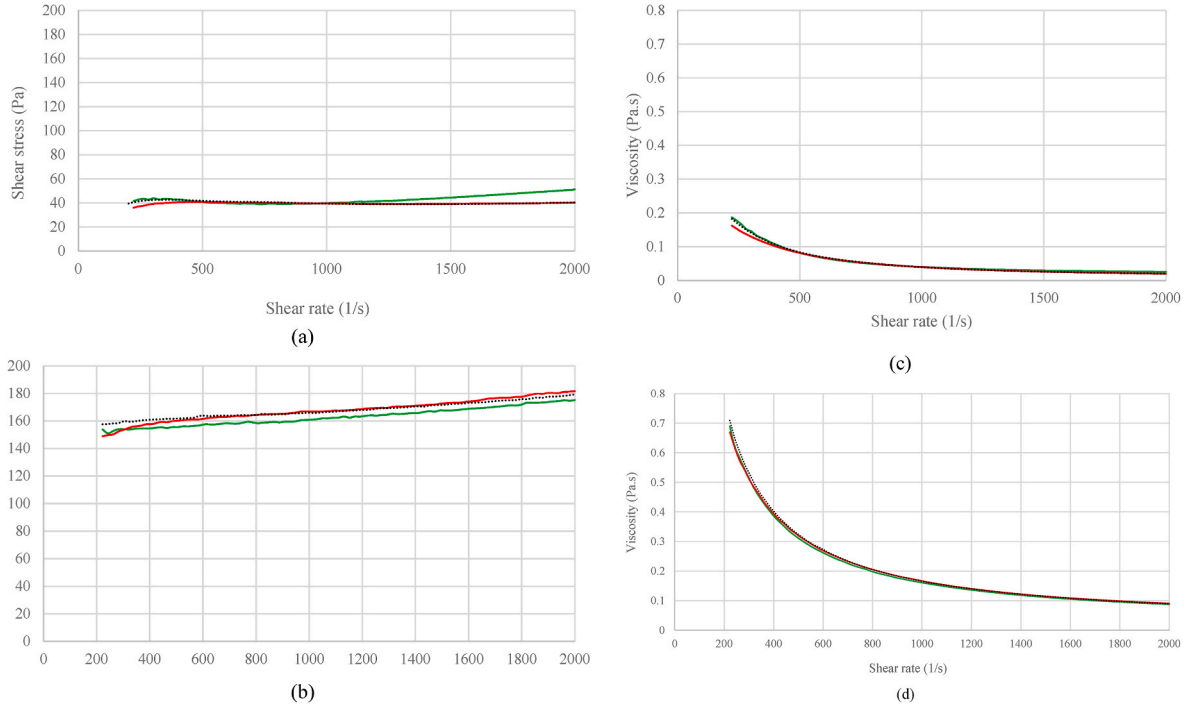

Fig. 5. The relation between shear rate vs shear stress of BSGPs-enriched yogurt at 1 day of storage (a) and 14 days of storage (b); shear rate vs viscosity at 1 day storage (c) and 14 days of storage (d) ((–): YC (–): YPF, and (···): YP).

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 BSGP-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; Körzendörfer 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 (h_{50} - Pa.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 (Pachekrepapol 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; Körzendörfer et al., 2019). Furthermore, higher amounts of protein facilitated the acid whey production, which hardened the yogurt structure (Körzendörfer 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. **Emir 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 Föste:** Methodology, Formal analysis, Writing – review & editing. **Karina Nola Sinamo:** Methodology, Formal analysis, Writing – review & editing. **Grzegorz Chodaczek:** 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

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