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FULL PAPER

Characterization of *Caulerpa racemosa* yogurt processed using *Lactobacillus bulgaricus* and *Streptococcus thermophilus*

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Abstract

Lactic acid bacteria such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yogurt maintains the balance of the intestinal microflora by suppressing the growth of harmful bacteria. Dietary fibre and functional compounds in probiotic drinks also have a good effect on health. The modification of low-fat probiotic drinks is a value-added product and can be classified as a healthy drink. *Caulerpa racemosa* is a Chlorophyceae seaweed with high food fibre and functional compounds, including phenolic and chlorophyll as antioxidants. This study aimed to determine the effect of different lactic acid bacteria on the characteristics of the probiotic drink products by combining *C. racemosa* with low-fat cow's milk. *C. racemosa* and low-fat cow's milk with a ratio of 3:4 was fermented with *Lactobacillus bulgaricus* (A), *Streptococcus thermophilus* (B), and a combination of both (C). The products were analysed for protein and fat content, dietary fibre, total phenolic content, antioxidants activity, total lactic acid bacteria, total acid, pH, and with a sensory test for consumer preference. The addition of the different lactic acid bacteria had effects on the fat, antioxidants, dietary fibre, and total acid contents, while protein and total phenolic contents were not significantly different. The combination of *L. bulgaricus* and *S. thermophilus* produced a probiotic drink with the lowest amount of dietary fibre (1.27%) and total acid (0.64%), with antioxidant activity IC_{50} of 183.57 ppm and total phenolic content of 0.11 mg GAE/g. The preference test showed that the panellists preferred yogurt fermented with *L. bulgaricus* and *S. thermophilus* for its aroma, texture, and colour.

1. Introduction

Caulerpa racemosa, also known as sea grape, is a green macroalga seaweed that, for human consumption, is usually served fresh or with salt (Sihono *et al*., 2018). *C. racemosa* contains bioactive components such as protein and polysaccharides, and secondary phenolic and flavonoid metabolites, namely 19.72% protein, 7.65% fat, and 11.51% fibre. It has a bright green colour (Bhuiyan *et al*., 2016; Sihono *et al*., 2018; Yap *et al*., 2019) and is known to have antioxidant properties. However, *C. racemosa* in food products is often either uncommon or sub-optimally used. Pires *et al*. (2013) found that polysaccharides contained in *C. racemosa* function as a catalyst for phospholipase enzymes and have pharmaceutical potential. As such, *C. racemosa* may be applied to the development of healthy foods.

Yogurt is a fermented drink known for its substantial health benefits, particularly in preventing digestive problems, boosting immunity, and increasing lactose tolerance for those who lactose intolerant. It is healthy because bacteria make it easily digestible, create a unique matrix that forms bioavailability and metabolic properties. It is satiating and good for weight control (Weerathilake *et al*., 2014; Rizzoli and Biver, 2017). The health benefits are what drive consumer demand, and the nutritional composition is what consumers consider first. As such, turnoffs for health-conscious consumers are fat and sugar contents, many consumers will prefer yogurt with low sugar and fat content (Miklavec *et al*., 2014).

Yogurt owes its healthy characteristics to bacteria that convert lactose into lactic acid, giving it a unique texture and flavour. Yogurt fermentation with lactic acid bacteria consists of glycolysis, proteolysis, and lipolysis and produces a sour taste and aroma. However, the fermentation and the formation of lactic acid are inherently linked to the quality of the bacteria (Obi *et al*., 2016). *Lactobacillus bulgaricus* and *Streptococcus*

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thermophilus are often used for making yogurt. These are lactic acid bacteria that play a role in converting lactose into secondary metabolite products through the heterofermentative pathway. They are known to produce lactic acid with different isomers, such as D(-) and L(+) (Corrieu and Beal, 2016; Chen *et al*., 2017). Agu *et al*. (2014), Rahman *et al*. (2016), and Sarvari *et al*. (2014) have already studied the characteristics of yogurt made with combination of *L. bulgaricus* and *S. thermophilus*. However, research on yogurt made exclusively with these lactic acid bacteria individually is rarely conducted. Therefore, this is a novel study comparing yogurt made with either a combination of *L. bulgaricus* and *S. thermophilus* or individually.

Yogurt is generally produced with cow's milk as the main ingredient. *C. racemosa* is added to make it rich in antioxidants and dietary fibre, also giving it a green colour. The phenolic components and secondary metabolites in *C. racemosa* survive the heating and cooling process during production (Sihono *et al*., 2018; Nurjanah *et al*., 2019). Currently, *C. racemosa* is rarely used as a raw material besides biscuits (Kumar *et al*., 2017) and jelly candy (Tapotubun *et al*., 2018). It is not yet used in healthy drinks such as yogurt. It was assumed that the nutrient content in *C. racemosa* degrades during the fermentation process when different lactic acid bacteria are included. The goal of this study was to determine the characteristics of low-fat cow's milk made with *C. racemosa* and fermented with different types of lactic acid bacteria.

2. Materials and methods

2.1 Materials

Ultra's low-fat cow's milk was obtained from local supermarkets in Semarang, Indonesia. Fresh *C. racemosa* was harvested in Jepara from the northern Central Java Sea, Indonesia. The commercial strains of lactic acid bacteria *Lactobacillus bulgaricus* FNCC 0041 and *Streptococcus thermophilus* FNCC 0040 were purchased from The Central of Food Research and Nutrition, Gadjah Mada University, Yogyakarta, Indonesia.

2.2 Microbial culture preparation

Lactobacillus bulgaricus and *S. thermophilus* were cultured by inoculating a loop of *L. bulgaricus* and *S. thermophilus* in 15 mL MRS agar media (Oxoid), followed by incubation at 37°C for 72 hrs. The inoculum was made by transferring a loop of pure culture bacteria in a 9 mL milk solution incubated at 37°C for 24 hrs.

2.3 Caulerpa racemosa preparation

Fresh *C. racemosa was* washed and cut into pieces, then chopped, mashed, and blended using a blender (Maspion, Indonesia) until homogenous. The ratio of *C. racemosa* to water was 1:9.

2.4 Yogurt preparation

Yogurt was produced according to Undugoda and Nilmini (2019) with some modifications. A mixture of approximately 30% *C. racemosa*, 40% low-fat milk, 5% sucrose, and 20% water was pasteurized using the HTST method at 72°C for 15 mins. The mixture's temperature was then lowered and 5% *L. bulgaricus* (A), 5% *S. thermophilus* (B), and a mixture of 2.5% *L. bulgaricus* and 2.5% *S. thermophilus* (C) were added. Each treatment was then fermented for 6 hrs at 37°C. Finally, the yogurt was placed into glass bottles, covered with aluminium foil, and stored at 5°C.

2.5 Fat analysis

The analysis was carried out according to AOAC (1992). Briefly, a 1.5 mL of NH_4OH was added to 10 g of the sample and subsequently mixed until becoming homogeneous to remove casein. Next, 10 mL of 95% alcohol was added and the mixture was shaken 15 times. The resulting mixture was added to 25 mL of ethyl ether, shaken for 1 min, then 25 mL of petroleum was added and shaken again for 1 min. Finally, the sample was centrifuged at 600 rpm for 30 secs to separate the ether and water phases (pink coloured) and then dried at ±100°C for 30 mins in a vacuum oven at 70–75°C for 7 mins until a constant weight was measured with the formula:

$$\text{Fat \%} = \frac{(\text{flask weight + fat}) - (\text{Flask weight} - \text{Residual weight})}{\textit{Sample weight}} \times 100$$

2.6 Protein analysis

The 2 g of the sample *was* poured into a Kjeldahl flask and 5 g of K_2SO_4, 200 mg of $CuSO_4$, and 30 mL of concentrated H_2SO_4 were added, homogenized, and distilled. The distillate was filled with a standard solution of 0.1 N HCl in 50 mL and three drops of 1% phenolphthalein indicator were added and titrated with a 0.1 N standard NaOH solution until a constant pink colour appeared (AOAC, 2006). The protein assay was done with the formula:

$$\text{Protein (\% N)} = \frac{(\text{mL titration sample} - \text{mL blank titration})}{\textit{Sample weight}} \times 100\% \times 14.008$$

2.7 Dietary analysis

A 0.5 g sample was combined with 0.1 mL of an alpha-amylase enzyme and heated and stirred at 100°C for 15 min. The sample was then cooled and 20 mL of distilled water and 5 mL of 1 N HCl were added. Next,

1 mL of 1% pepsin enzyme was added and the mixture was warmed in a water bath for 1 hr. After 1 hr, 5 mL of 1 N NaOH and 0.1 mL of a beta-amylase enzyme were added and again incubated in a water bath for 1 hr. Next, the sample was washed with ethanol and acetone, dried in an oven at 105°C for 24 hrs, cooled in a desiccator, and weighed to determine the insoluble dietary fibre weight. The volume was adjusted to 100 mL of filtrate and 400 mL of 95% warm ethanol was added. The filtrate was allowed to settle for 1 hr after which it was filtered with ash-free filter paper and washed again with ethanol and acetone. The filtrate was then dried in an oven at 105°C for 24 h and finally placed in a desiccator to weigh the dissolved dietary fibre.

2.8 Total phenolic content analysis

The total phenolic assay was performed using the Folin-Ciocalteau method and the gallic acid standard. The data obtained were expressed in milligrams of gallic acid equivalent to 1 g of dry extract (Ivanov and Dimitrova, 2019).

2.9 Antioxidant activity analysis

Antioxidant activity was analysed following the DPPH radical scavenging method and expressed as an IC_{50} value. The sample was prepared in several concentrations. Each concentration consisted of 0.1 mL added with 5 mL of DPPH solution (0.00039 g in 1 L of methanol), followed by homogenization. The mixtures were then incubated at room temperature in a dark room for 30 mins. The colour of the mixture would change due to the reaction with the DPPH reagent. The samples were then spectrophotometrically measured at a 517 nm wavelength. The inhibition values obtained were then calculated as the IC_{50} value (Srivastava *et al*., 2015).

2.10 Total lactic acid bacteria

The total lactic acid assay was performed using MRS agar with a pour plate method. The 1 mL sample was mixed with a 9 mL of sterile 0.85% NaCl solution and homogenized for 1 min, to make a dilution 10^{-1}. Concurrently, similar sample dilution solutions (aliquots) were made for series 10^{-2}–10^{-7}. Samples of 1 mL were taken from each dilution series and placed onto sterile Petri dishes, followed by the addition of 10 mL of MRS agar warmed to 45°C. Homogenization was done by shifting the Petri dish to form a figure eight. After the media solidified, the plates were turned upside down and incubated at 37°C for 48 hrs. The bacteria colonies were counted with a colony counter based on the appearance of clear zones with specific shapes (Daroonpunt *et al*., 2016).

2.11 pH analysis

The pH analysis was performed with a Mettler Toledo (Singapore) pH meter calibrated with a standard buffer solution of pH 4 and pH 7 for 15–30 mins. The electrodes were then rinsed with distilled water and dried. A sample of 5 mL was immersed in electrodes for the pH measurement. The electrodes were left until stable data were obtained and the results were recorded directly from the pH meter display (Ly *et al*., 2020).

2.12 Total acid analysis

A 10 mL sample was poured into an Erlenmeyer tube and three drops of 1% phenolphthalein were added. The sample was then titrated with a standardized 0.1 N NaOH solution, giving it a pink colour. The total acid was calculated with the formula:

$$\text{Total Acid (\%)} = \frac{\text{mL NaOH} \times \text{N NaOH} \times 90 \times 100}{ml\ sample \times 1000}$$

2.13 The preference tests

The preference test was based on 30 semi-trained panellists (Ademosun *et al*., 2019) and based on their preferred colour, aroma, texture, and flavour of the yogurt. A five-point scale were used: 1 - dislike, 2 - moderate dislike, 3 - neutral, 4 - moderate like, 5 - like.

2.14 Statistical analysis

This study was conducted as a triplicate and analysed using ANOVA. If significant discrepancies were found, Tukey's HSD test would be carried out. Data were processed with SPSS 23.

3. Results and discussion

3.1 Protein

All yogurts with *C. racemosa* had a protein content of 2.35–2.60%, as shown in Table 1, similar to the 2.35% protein content of commercial yogurt. The treatment of lactic acid bacteria on fermentation did not affect the protein content of the yogurt, which concurred with the findings reported by other researchers (Obi *et al*., 2016; Tenea and Suarez, 2020), who combined lactic acid bacteria *L. bulgaricus* and *S. thermophilus* with *L. bulgaricus* and *L. fermentum.* During the fermentation process, proteins may break into lactic acids and non-nitrogen proteins, making them undetectable by the n-total protein method. It indicated that yogurt with *C. racemosa* had higher protein contents compared to yogurt moringa leaf fermented with *L. bulgaricus* (Rahmawati, 2017). Yogurt fermentation involves two exponential phases of bacterial growth separated by a transition phase. The first exponential phase indicates the growth of *S. thermophilus* at a neutral pH and requires

Table 1. Chemical characteristics of yogurt

Sample	Protein (%)	Fat (%)	Dietary Fibre (%)	Total phenolic content (mg GAE/g)	Antioxidant activity (ppm)
A	2.52±0.30[a]	0.60±0.03[b]	2.57±0.25[d]	0.10±0.01[b]	191.79±2.80[c]
B	2.60±0.22[a]	0.46±0.02[a]	1.78±0.06[c]	0.10±0.01[b]	175.07±3.28[b]
C	2.35±0.12[a]	0.45±0.02[a]	1.27±0.04[b]	0.11±0.01[b]	183.58±10.19[c]
Commercial	2.35±0.12[a]	0.87±0.02[c]	0.93±0.12[a]	0.04±0.00[a]	138.69±6.36[a]

Values are presented as mean±SD. Values with different superscripts in the same column are significantly different (P<0.05).

more availability of free amino acids than *L. bulgaricus*. In this phase, *S. thermophilus* uses more oxygen, thus supporting *L. bulgaricus* unable to grow at high oxygen conditions. In the transition phase, *S. thermophilus* slows due to the lack of free amino acids. In this phase, *S. thermophilus* cannot break down milk proteins to produce amino acids, so the role of *L. bulgaricus* is significant. *L. bulgaricus* can break down milk protein to produce amino acids that can support the growth of *S. thermophilus* in the second exponential phase (Sieuwert, 2016). The process leads to lower protein value in yogurt fermented with the combination of *L. bulgaricus* and *S. thermophilus* than the other treatments even though it is not statistically significant.

3.2 Fat

Table 1 shows that yogurt in this study contained fat ranging from 0.45 to 0.60%, which is lower than the 0.87% in commercial yogurt. The low-fat content in yogurts A, B, and C was due to the use of low-fat milk as the raw material. These results are in accordance with Singh *et al*. (2016) who reported that the fat content of low-fat yogurt with added fruit extracts ranges from 0.44 to 1.6%. Yogurts B and C contained less fat than yogurt A because lipolysis by lactic acid bacteria during the fermentation process converted fat into fatty acids, decreasing the fat content. The lactic acid bacteria strains used affected the outcome, especially *S. thermophilus* which plays a role in the long-chain fatty acid formation, and *Lactobacillus* requiring a longer time to degrade it (Widyastuti *et al*., 2014; Sieuwert, 2016; Chen *et al*., 2017). Thus, yogurt fermented with *S. thermophilus* contained less fat than with *L. bulgaricus*.

3.3 Dietary fibre

Table 1 shows the different lactic acid bacteria affect the dietary fibre content of yogurt. In this study, yogurt A had a higher dietary fibre content than yogurts B, C, and commercial yogurt. *Lactobacillus bulgaricus* is a superior starter culture for the fermentation of carbohydrates into dietary fibre than the other bacteria used in this study. Sorensen *et al.* (2016) demonstrated *L. bulgaricus* and *S. thermophilus*'s inability to ferment sugars, mainly glucose and galactose. Its combination produces low glucose, and lactic acid bacteria alone can optimize their activity in fermenting lactose. During the fermentation process, pH decreased due to organic acid formation, mainly in the form of lactic and acetic acids. An increase in organic acid may lead to the activation of endogenous enzymes that play a role in the degradation process of biopolymer components. The fermentation process may also activate the β-glucosidase enzyme, which breaks down starch into glucose and dextrin. Finally, lactic acid bacteria may activate extracellular enzymes, such as amylase and pullulanase, which can break down starch into oligosaccharides and undigested starch components (Sari *et al*., 2017; Tsafrakidou *et al*., 2020). *C. racemosa* is a source of carbohydrate and contains 48.97% total carbohydrate and 11.51% fibre content (Bhuiyan *et al*., 2016). The low dietary fibre content of yogurt is presumably related to the combination of two starter cultures, such as *S. thermophilus* and *L. bulgaricus*. Tsafrakidou *et al*. (2020) reported that two Gram-positive thermophilic organisms did not properly ferment sugar into a simple compound. Dietary fibre is good for our health and especially beneficial to the digestive system. The consumption of dietary fibres is not linked to increased blood sugar levels, so it can be consumed without increasing the risk of contracting diabetes mellitus (Tsafrakidou *et al*., 2020). Furthermore, Beretta *et al*. (2018) noted that dietary fibre consumption reduces blood sugar levels in patients with diabetes mellitus type one.

3.4 Total phenolic content

Table 1 shows the total phenolic content in yogurt with added *C. racemosa* as a raw material ranged from 0.10 to 0.11 mg GAE/g, higher than the 0.04 mg GAE/g of commercial yogurt. Yap *et al*. (2019) explained that *C. racemosa* extracted with methanol has a total phenolic content of 10.33 mg GAE/g. According to Taneva and Zlatev (2020) and Adebo and Medina-Meza (2020), goji berries yogurt had high total phenolic contents. Lactic acid bacteria did not affect the total phenolic content, Nisa *et al*. (2019) also found that fermentation using different lactic acid bacteria did not affect the total phenolic content in fermented rice bran.

3.5 Antioxidant activity

Table 1 shows antioxidant activity expressed as an IC_{50} value. Lactic acid bacteria affected the IC_{50} of *C.*

racemosa yogurt. The antioxidant activity of yogurt fermented with *S. thermophilus* was higher than yogurt fermented with *L. bulgaricus* and yogurt fermented with a combination of *L. bulgaricus* and *S. thermophilus*. Milk fermented with lactic acid bacteria had higher antioxidant activity than non-fermented milk. During the fermentation process, proteins, carbohydrates, and fats were broken down into amino acids, peptides, organic acids, and other compounds that are beneficial to our health. Amino acids and peptides have anti-hypertensive properties, increase antioxidant activity, and inhibit fat peroxidation (Gjorgievski *et al*., 2014). The high antioxidant activity in yogurt B was due to *S. thermophilus* more effectively degrading amino acids than *L. bulgaricus* (Sieuwerts, 2016). Antioxidant activity is proportional to the total phenol content in yogurt. Fermentation is known to increase the total phenol content due to the change in temperature, pH, fermentation time, and the presence of lactic acid bacteria which can break phenolic bonds and increase antioxidant activity (Tsafrakidou *et al*., 2020). This study's results were in line with Gjorgievski *et al*. (2014) who found that milk fermented with monocultural lactic acid bacteria strains produced higher antioxidant activity than milk fermented with multicultural lactic acid bacterial strains.

3.6 Total lactic acid bacteria and pH

C. racemosa yogurt with different strains of lactic acid bacteria had a significant effect on growth. Yogurt fermented with *S. thermophilus* contained more lactic acid bacteria than yogurt fermented with *L. bulgaricus* (Figure 1). However, combining *S. thermophilus* and *L. bulgaricus* produced a symbiotic mutualism where *S. thermophilus* was more active in the first phase of the fermentation process to degrade amino acids and trace elements than *L. bulgaricus*. Furthermore, *S. thermophilus* used more oxygen, which is beneficial for *L. bulgaricus* as it cannot withstand the presence of high amounts of oxygen (Sieuwerts, 2016). The growth of lactic acid bacteria is influenced by the fermentation duration (Taleghani *et al.,* 2016) and the pH of yogurt. Optimal conditions for *S. thermophilus* range from 4 to 4.5 pH. Decreasing acidity would inhibit the growth of *S. thermophilus* (Chramostová *et al.,* 2014). Yogurts B and C had 4.83 and 5.04 pH respectively, similar to Chramostová *et al*. (2014) who found that the total lactic acid bacteria ranging from 7 to 8 log CFU/mL had a pH of 4.26–4.32. The low pH of yogurt is caused by organic acids, mainly lactic acid, and acetic acid during the fermentation process (Chen *et al*., 2017).

3.7 Total acid

Fermentation by lactic acid bacteria converted carbohydrates into organic acids, which were summed up as the total acid (Figure 1). Lactic acid bacteria convert carbohydrates in milk, such as lactose, into lactic acid. Lactic acid production was affected by lactic acid bacteria strains. *L. bulgaricus* and *S. thermophilus* are known to produce lactic acid with different isomers, such as D(-) and L(+) lactic acid, through heterofermentative pathways. This process also generates acetic acid (Widyastuti *et al*., 2014; Gezginc *et al*., 2015; Chen *et al*., 2017). Table 2 shows that yogurt in this study had a total acid range of 0.64–0.71%. Yogurt with *C. racemosa* produced less acid than commercial yogurt (0.85%). Gezginc *et al*. (2015) explained the optimal conditions for *L. bulgaricus* and *S. thermophilus* to produce lactic acid through fermentation, such as an incubation temperature of 42°C. Incubation at 37°C is optimal for both bacteria strains to produce acetaldehyde, which plays a role in giving yogurt its flavour. In this study, incubation and fermentation took place at 37°C, making the total acid content in yogurt with *C. racemosa* being lower than commercial yogurt to be expected.

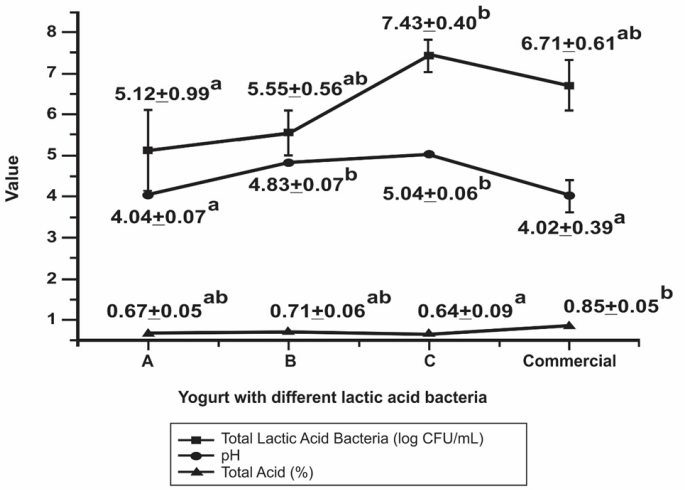

Figure 1. Total lactic acid bacteria, pH and total acid of yogurt sample. Data with different superscripts are significantly different (P<0.05) for each parameter.

3.8 The preference tests

The results of the preference test for *C. racemosa* yogurt are shown in Table 2. The different lactic acid bacteria played a role in the panelists' preferences for colour, aroma, texture, and flavour. Based on colour, the addition of *C. racemosa* was rated from neutral to moderate like. The green colour of *C. racemosa* yogurt is caused by a natural green pigment named chlorophyll (Sihono *et al*., 2018). In terms of aroma, the panellists rated the yogurt as moderate like. The aroma came from the formation of acetaldehyde during the breakdown of threonine during the fermentation process (Krisnaningsih and Yulianti, 2015). It was also related to the effectiveness of the combination of *L. bulgaricus* and *S. thermophilus* at degrading amino acids (Sieuwerts,

Table 2. Preference test of yogurt

Sample	Colour	Aroma	Texture	Taste
A	2.87±0.78[b]	2.50±0.68[a]	2.37±0.61[a]	2.50±0.68[a]
B	3.17±0.53[bc]	3.20±0.55[b]	3.07±0.45[bc]	3.27±0.52[b]
C	3.47±0.68[c]	3.63±0.67[c]	3.33±0.71[c]	3.37±0.49[b]
Commercial	2.40±0.56[a]	2.90±0.66[ab]	2.70±0.60[ab]	3.03±0.67[b]

Values are presented as mean±SD. Values with different superscripts in the same column are significantly different (P<0.05).

2016). The texture and flavour of yogurt C were the most preferred. During yogurt processing, *L. bulgaricus* and *S. thermophilus* play a role in texture production. The texture comes from the total solids and protein content of the yogurt, which gives it firmness. Lactobacilli degrades amino acids in milk into glycine and histidine, which support the growth of streptococci. Combining the two bacteria converted milk sugar to lactic acid, giving it a pleasant flavour and consistency. Acid formation during the fermentation process caused the rearrangement of casein creating a more compact yogurt structure (Mohan *et al*., 2020). Yogurt has a strong sour taste related to the formation of lactic and acetic acid (Chen *et al*., 2017). Nowadays, consumers prefer a tasty and less sour taste, which the low pH resulting from *L. bulgaricus* provides. Combining *L. bulgaricus* and *S. thermophilus* produced a less sour taste. Sieuwert (2016) found that *S. thermophilus* plays a role in aroma formation, so the combination of *L. bulgaricus* and *S. thermophilus* produces yogurt with a mildly sour taste and a desirable aroma.

4. Conclusion

Yogurt with *Caulerpa racemosa* produced a probiotic drink with low fat, tasty, and had functional properties such as total phenolic content, antioxidant IC_{50} and dietary fibre was better than commercial one. Yogurt made with combination *L. bulgaricus* and *S. thermophilus* (yogurt C) was the most preferred by panellists.

Conflict of interest

The authors declare no conflict of interest.

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