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Development of *Arthrospira platensis* fortified spiced buttermilk

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Abstract

An incredible surge in demand for fermented dairy products has been experienced recently due to its nutraceutical benefits and lifestyle preferences. As a traditional fermented dairy beverage, cultured buttermilk is an ideal product choice in this context. This study introduces a novel cultured buttermilk fortified with the high protein microalgae Arthrospira platensis, making it beneficial and appealing due to its preferable mouthfeel and antioxidant characteristics. The goal of this study was to extensively evaluate and determine the optimum amount of Spirulina powder to be mixed into buttermilk, while also analyzing the alterations in the physicochemical and sensory properties of the resulting product. The pH, titratable acidity, DPPH inhibition activity, Phenolic content, and sensory properties of newly developed cultured buttermilk with varying concentrations of Spirulina 0.25, 0.45, 0.65, 0.85 and 1.5% were compared with that of the control buttermilk sample with no added Spirulina. Based on the findings derived from the conducted study, it has been concluded that the incorporation of 0.25% Spirulina, can potentially enhance the nutritional composition and functional health attributes of cultured buttermilk without affecting sensory score.

Keywords: *Butter milk, fortification, Arthospira platensis, antioxidant activity, total phenolic content, sensory evaluation*

The consumption of fermented foods has the potential to confer many health benefits to individuals due to their provision of readily metabolizable nutrients and helpful bacteria (González *et al*.,2019). In the present context, buttermilk, alternatively referred to as mattha, chaas, or chaach, has achieved increased popularity as a refreshing milk-based beverage (Gandhi *et al*.,2018).

Buttermilk, a dairy-based beverage, is known for its nutritional composition, which includes essential vitamins, such as vitamins A and D, as well as minerals like potassium, calcium, and traces of phosphorus. These nutrients make buttermilk a healthy alternative for relieving thirst, especially in summer. The culinary specimen under research has a great palatability, with complex flavors and mild to prominent acidity. This composition includes 6-7% milk solids and 1-2% fat. Buttermilk has more low-melting fat than other dairy products. The composition of this particular food item is noteworthy due to its inclusion of milk fat globule membrane, which is abundant in phospholipids such as phosphatidylcholine (lecithin), phosphatidylethanolamine, and sphingomyelin. These phospholipids contribute to the overall nutritional value of the food, providing additional health benefits (Rombaut *et al*.,2006). This particular substance exhibits properties that

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are known to support the digestion process by effectively reducing the level of acidity subsequent to a meal. Its mechanism of action involves the removal of lipids or oily substances that may accumulate on the intestinal lining.

The planktonic blue-green alga *Arthrospira platensis*, known as *Spirulina*, is popular due to its 65% protein content and nutritional benefits. This drug has been FDA-approved as Generally Recognized As Safe since 1981. Protein makes up 70% of spirulina, along with minerals and vitamins like B12, B1 (thiamine), B2 (riboflavin), B3 (niacin) and tocopherol. Spirulina contains antioxidants such as phycocyanin, carotenoids, and phenolic compounds (Beheshtipour *et al.,* 2012). Recent investigations have demonstrated that *Spirulina* possesses the ability to stimulate the proliferation of lactic acid bacteria, not only in synthetic media but also in milk and yoghurt (Debbabi *et al*., 2018). The consumption of *Spirulina* exhibits potential health effects, including combating protein energy malnutrition, immunomodulation, antioxidant properties, anticancer activity, antiviral activity, antibacterial activity, and positive effects against malnutrition, hyperlipidemia, diabetes, obesity, inflammatory allergic reactions, heavy metal/ chemical-induced toxicity, radiation damage and anaemia (Kumar *et al*., 2018).

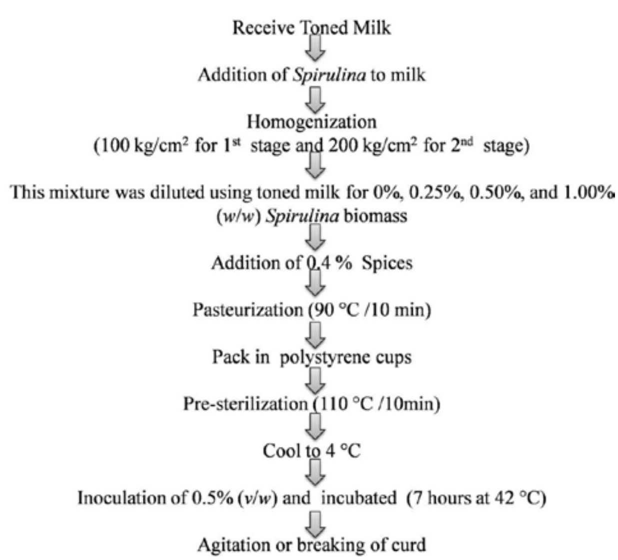
The primary objective of this research endeavour was to thoroughly examine and ascertain the optimal proportion of *Spirulina* powder to be incorporated into buttermilk, while simultaneously evaluating its consequential impact on the physicochemical attributes as well as the sensory characteristics of the resultant product.

Materials and methods

Double-toned, pasteurized milk with 1.5% fat and 9.0% SNF was purchased from Amul Milk outlet in Varanasi, India and refrigerated at 4 °C until use. Freeze-dried direct vat-set (DVS) yoghurt culture (S*. thermophilus* and *L. bulgaricus)* and NCDC-167 culture (*Lactococcus lactis ssp. lactis, Lactococcus lactis ssp. cremoris and Lactococcus lactis ssp. lactis biovar. diactetylactis* in 1:1:1 ratio) were procured from Dairy Microbiology Division, National Collection of Dairy Cultures, ICAR-National Dairy Research Institute, Karnal (Haryana)-132001. The culture was kept at -18 °C until use. *Spirulina* was purchased from Heilen Biopharm Pvt. Ltd., India and was refrigerated until usage.

Method for preparation of Spirulina fortified spiced buttermilk

The optimisation of probiotic buttermilk preparation was conducted by Rodas *et al*. (2002). The present study employed a similar methodology for the formulation of fortified spiced buttermilk, with a few changes made to the protocol. The *Spirulina*-milk blend was prepared by incorporating *Spirulina* biomass, comprising 85-90% of the total mixture, into toned milk at a concentration of 2% (w/w) at a temperature of 27°C. The initial investigation revealed that *Spirulina* exhibited perfect solubility at a concentration of 2% w/w. The inclusion of *Spirulina* directly following the synthesis of buttermilk was deemed unfavourable as a result of the presence of insoluble particles of *Spirulina* within the sample. Consequently, a consistent concentration of the blend including *Spirulina* and milk was produced. Subsequently, in order to get an ideal combination of *Spirulina* and milk, the blend underwent homogenisation via a homogenizer, applying pressures of 100 kg/cm^2 for the initial stage and 200 kg/cm^2 for the subsequent stage. In order to acquire aliquots of *Spirulina* biomass with concentrations of 0%, 0.25%, 0.45%, 0.85 and 1.50% (w/w), the combination was diluted utilising toned milk. Spices were incorporated into the control milk and the *Spirulina*-milk blend at a concentration of 0.4% w/w. The mixture was then subjected to pasteurization using a water bath for 10 minutes at a temperature of 90°C. The samples, each measuring 100 mL, were subjected to pasteurisation and afterwards transferred into polystyrene cups that had been pre-sterilized. The cups were then allowed to cool to a temperature of 4°C. The aliquots were inoculated with NCDC-167 stock cultures at a concentration of 0.5% (v/w). All samples were enveloped in aluminum foil, placed in low density polyethylene (LDPE) bags, and incubated at 42°C for seven hours in a temperature-controlled incubator. The samples were monitored on an hourly basis until they reached a pH range of 4.6–4.7 and an acidity level of 0.8–0.9%. The curd setting phenomenon was noticed over a period of 7 hours of total fermentation. Subsequently, the process of agitation or curd disruption was carried out utilizing a laboratory blender set at a velocity of 10,000 revolutions per minute for a duration of 90 seconds. Upon completion of the preparation process, the cultured buttermilk samples were promptly placed in a refrigerated environment at a temperature range of 6–8 °C.



The cultured buttermilk samples were designated as follows: T_0 for the control buttermilk, T_1 for the buttermilk with 0.25% *Spirulina,* T_2 for the buttermilk with 0.45% *Spirulina*, T_3 for the buttermilk with 0.65% *Spirulina*, T_4 for the buttermilk with 0.85% *Spirulina* and T_5 for the buttermilk with 1.5% *Spirulina.*

The evaluation of the product was conducted through a comprehensive analysis encompassing chemical parameters such as acidity and pH, as well as the estimation of its antioxidant properties using the DPPH assay and total phenolic content (TPC) determination.

Additionally, sensory characteristics were also taken into consideration during the evaluation process. Upon careful examination of those tests, it was determined that the optimal level has been chosen for the subsequent phase of the investigation.

Determination of pH

The pH of the *Spirulina* fortified spiced buttermilk was determined using an electronic pH meter, specifically the Thermo Scientific model Sn B21899, which was sourced from Singapore.

Determination of acidity

The method outlined in IS: 1479, part I, 1960 was used to determine the titratable acidity of *Spirulina* fortified spicy buttermilk.

Determination of DPPH

The evaluation of the antioxidant capacity was conducted using the DPPH (2, 2 diphenyl - 1 -picryl hydrazyl) inhibition technique, following the protocol outlined by Kang and Saltveit (2002), with minor adjustments. A volume of 1 mL of the sample was transferred into a test tube and subsequently diluted by a factor of 10 using methanol. The resulting mixture was then completely dispersed by means of a vortex. In order to eliminate the suspended particle, the sample performed centrifugation at a speed of 3000 revolutions per minute for a duration of 10 minutes. To prepare blank samples, a 5 ml volume of methanol was placed in a test tube covered with an aluminum sheet. After that, 1 ml of a freshly prepared solution of DPPH (4 mg of DPPH dissolved in 10 ml of methanol) was added to the test tube. A volume of 5 ml of sample extracts was collected, followed by the addition of 1 ml of methanol to the collected volume. The solution was vigorously agitated and thereafter placed in a light-restricted environment for a duration of 30 minutes. The spectrophotometric monitoring of the resultant solution involved measuring the decrease in absorbance at a wavelength of 517nm.

The results were expressed as:

% DPPH scavenging activity =

$$\frac{\text{Absorbance of blank-Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

Determination of TPC

The Folin-Ciocalteau method (Singleton and Rossi, 1965) was employed to determine the total phenolic content, with a few variations. A volume of 0.5 mL of the sample extract was transferred into a test tube. Subsequently, 2.5 mL of Folin-Ciocalteu Reagent (FCR) diluted with distilled water at a ratio of 1:9 (1 mL of FCR in 9 millilitres of distilled water) was added, followed by the addition of 1 mL of a 7.5% sodium carbonate (Na_2CO_3) solution. The test tube was securely wrapped with aluminum foil and subjected to a period of incubation in darkness at ambient temperature for a duration of one hour. The measurement of absorbance was conducted at a wavelength of 760 nm using a UV-1800 spectrophotometer. A standard calibration curve was generated using varying concentrations of Gallic acid ranging from 0 to 800 mg/L. The quantification of the total phenolic content was reported in mg of Gallic acid equivalents (GAE) per 100 g. The standard curve was generated by plotting the absorbance values against the concentration of gallic acid (mg/100gm). The regression equation for this standard curve is $y = 0.0769x + 0.0041$, with an R-squared value of 0.998.

Sensory analysis

The sensory evaluation of both the control and *Spirulina*-fortified spiced buttermilk samples was conducted utilising a 9-point hedonic scale. A group of twenty expert panelists was chosen for their extensive background and expertise in sensory evaluation of dairy and milk-based products, with a specific focus on individuals who regularly drink cultured buttermilk. The sensory panelists rated many sensory aspects, including color and appearance, body and mouthfeel, flavor, and overall acceptability. The cultured buttermilk samples were removed from refrigeration, and the temperature at which they were served ranged from 10 to 12 °C. The cultured buttermilk samples were contained in 100 g plastic cups, each filled with 50 g of cultured buttermilk. These cups were then labeled with a unique 3-digit code. The acceptance level of the product was assessed by considering its score on a 9-point hedonic scale:

Table 1. 9-point Hedonic scale score card

Hedonic Scale	Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

Results and discussion

Effect on titratable acidity and pH of Spirulina fortified spiced buttermilk

The pH and acidity levels of the various treatments are depicted in Fig. 2. In the process of cultivating buttermilk, it has been observed that caseinate particles undergo complete precipitation within a pH range of 4.6 to 4.7 (Mehta *et al*., 2012). The observed phenomenon of increasing rates of titratable acidity and decreasing rates of pH can be attributed to the strong buffering properties of milk and the rapid proliferation of bacteria during the fermentation process (Beheshtipour *et al.,* 2012). The addition of *Spirulina platensis* to the buttermilk samples resulted in a significant reduction in the time required to reach a pH of 4.5 ($p < 0.05$). The pH of the *Spirulina*-fortified buttermilk exhibits a notable decrease within a relatively short period of 2 hours, in contrast to the control sample which necessitates a significantly extended duration of 3 hours for a similar pH drop to occur. The samples incorporating *Spirulina* powder at concentrations of 0.25%, 0.45%, 0.65%, 0.85%, and 1.5% did not exhibit any statistically significant variations in case of acidity and pH. Moreover, it was observed that the treatments fortified with *Spirulina* exhibited a significantly accelerated rise in acidity levels. The outcomes of the present investigation concurred with published reports (Varga *et al*., 2002; Gyenis *et al*., 2005; Barkallah *et al*., 2017; Patel *et al*., 2019). The incorporation of *Spirulina* into milk, along with the addition of S. *thermophilus* and *L. bulgaricus* cultures, exhibited an accelerated decline in pH levels compared to the control samples throughout the fermentation process. Consequently, this led to a reduced incubation period.

Effect of Spirulina concentration on DPPH activity and Total Phenolic Content (TPC)

The assessment of antioxidant activity in fermented milk products can be effectively conducted through the utilization of in-vitro methods. The in-vitro analyses performed in this study are based upon the assessment of reactive oxygen species (ROS) scavenging capacity and the evaluation of activity against stable non-biological radicals. Specifically, the 2-2-Diphenyl1-picrylhydrazyl (DPPH) method is utilized for this purpose. The present investigation aimed to assess the antioxidant activity through the quantification of free radical scavenging activity (RSA), with the subsequent expression of the obtained outcomes in terms of percentage activity.

According to the findings presented in Fig. 3, the experimental samples demonstrated significant antioxidant characteristics in relation to their ability to scavenge DPPH radicals. The control sample exhibits an antioxidant activity of 41.73%. In contrast, the spiced buttermilk fortified with *Spirulina* has antioxidative activities of 46.19%, 48.01%, 52.13%, 56.43%, and 58.77% at doses of 0.25%, 0.45%,

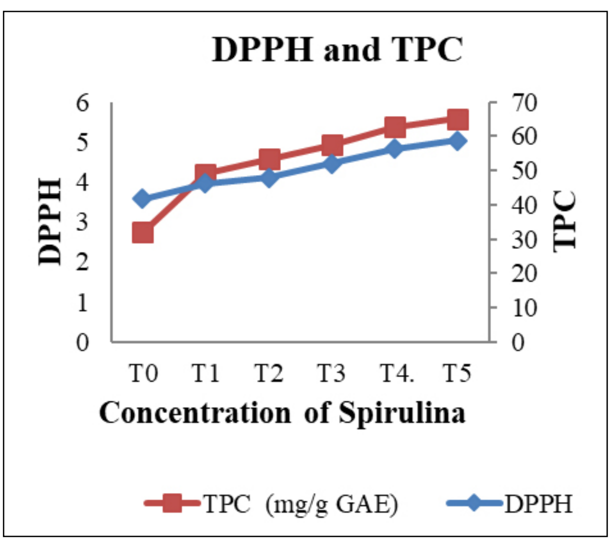


Fig.2. Effect on titratable acidity and pH

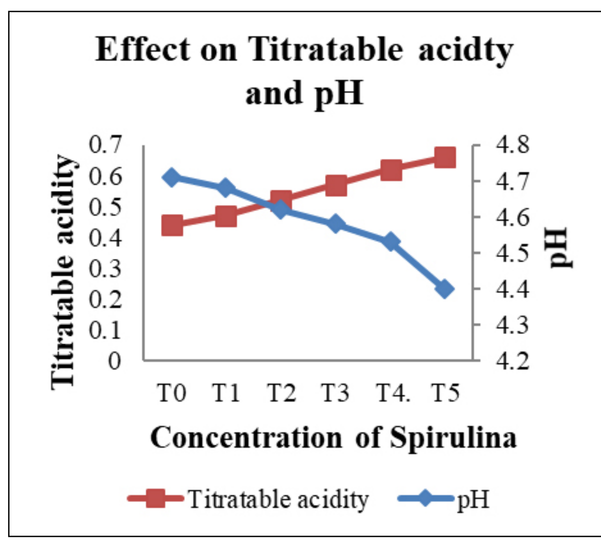


Fig.3. Effect on DPPH and TPC

Table 2. Effect of *Spirulina* on pH, Titratable acidity, DPPH inhibition and TPC

	T_0	T_1	T_2	T_3	T_4	T_5	p value
pH	4.71±0.02	4.68±0.13	4.62±0.03	4.58±0.07	4.53±0.02	4.4±0.10	*0.39
Titratable acidity	0.44±0.03	0.47±0.03	0.52±0.02	0.57±0.04	0.62±0.01	0.66±0.02	0.01
DPPH	41.73±0.03	46.19±0.02	48.01±0.12	52.13±0.10	56.43±0.03	58.77±0.07	0.01
TPC(mg/g GAE)	2.74±0.01	4.21±0.02	4.58±0.04	4.92±0.01	5.37±0.03	5.58±0.02	0.00

Results are expressed as mean ± standard deviation (n=3),
values are significantly different at p<0.05; *p<0. 5. *(no significant difference)

Table 3. Results of sensory evaluation

	T_0	T_1	T_2	T_3	T_4	T_5
Appearance	8.82±0.01^{a}	8.58±0.12^{b}	7.84±0.11^{c}	6.12±0.01^{d}	4.01±0.02^{e}	3.28±0.02^{f}
Odour	8.66±0.01^{a}	8.42±0.01^{b}	8.1±0.05^{c}	6.87±0.03^{d}	4.96±0.01^{e}	3.7±0.01^{f}
Colour	8.8±0.04^{a}	8.62±0.01^{b}	7.32±0.04^{c}	6.34±0.01^{d}	3.98±0.01^{e}	3.04±0.01^{f}f
Flavour	8.69±0.09^{a}	8.48±0.04^{b}	7.02±0.07^{c}	6.28±0.01^{d}	3.77±0.01^{e}	3.01±0.03^{f}
Mouthfeel	8.56±0.01^{a}	8.23±0.01^{b}	7.99±0.02^{c}	5.72±0.02^{d}	3.21±0.03^{e}	2.98±0.01^{f}
Overall acceptability	8.706±0.02^{a}	8.466±0.01^{b}	7.654±0.01^{c}	6.266±0.04^{d}	3.986±0.01^{e}	3.20±0.01^{f}

Results are expressed as means±SD (n=3). a-f Means within columns with different superscript are significantly ($p<0.05$) different from each other

0.65%, 0.85%, and 1.5% respectively. According to a report, a greater proportion of microalgae lead to increased antioxidant activity (Fradinho *et al.*, 2020). Research conducted on yoghurts supplemented with *Chlorella vulgaris* and *Dunaliella* sp. has demonstrated the improved antioxidant properties resulting from the incorporation of these microalgae into the yoghurt formulation. The use of *Spirulina* powders in one's dietary routine has the potential to elevate the concentrations of chlorophylls (Ismaiel *et al*., 2016), carotenoids (Goiris *et al*., 2012), and phycocyanin (Anbarasan *et al*., 2011), thereby leading to an enhancement in the capacity to scavenge free radicals.

Statistically significant difference ($p < 0.001$) was observed between T_0 and T_5 in terms of total phenolic content (TPC). The inclusion of *Spirulina platensis* in the cultured buttermilk led to a significant increase in the overall phenolic content observed in T5. A comparable reaction was noted upon the introduction of microalgae into trials of gluten-free pasta (Goiris *et al*., 2012). Furthermore, Niccolai *et al*. (2019) observed an augmentation in phenol concentration due to the fermentation process of Spirulina biomass. In a previous study, Liu *et al.* (2011) documented that *Arthrospira platensis* exhibits a notable abundance of phenolic compounds, with a recorded level of 19.47 mg/g GAE. Furthermore, the researchers observed that the process of fermentation amplifies the beneficial properties of *Spirulina platensis* (Liu *et al*., 2011).

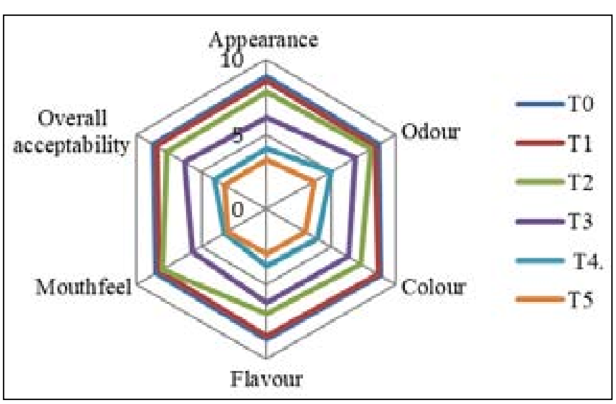


Fig. 4. Evaluation of sensory parameters

Effect on sensory profile of spiced buttermilk with different concentration of Spirulina

In accordance with the findings presented in Fig. 4, an in-depth evaluation was conducted on each individual product with regard to its appearance, odour, color, flavor, mouthfeel and overall level of acceptability. Based on the findings derived from the implementation of the hedonic scale, it is observed that a significant majority of 82% among the sensory panelists who underwent training exhibited a favorable and affirmative reaction towards both T_0 and T_1 samples. The findings of this study indicate that a significant proportion of the panelists involved in the evaluation process exhibited a tendency to undervalue T_4, as evidenced by their ratings falling below the threshold of 4 on the hedonic scale. The generation of compounds through the oxidation of minerals and lipids by Supplemental *Spirulina* has been observed to exhibit dual functionality as pro-oxidant molecules. Additionally, these compounds have the potential to induce metallic off-flavors, thereby leading to an undesirable taste experience (Rose *et al*., 2023). The observed results indicate that there was no statistically significant difference between the sample T_1 and T_0, as depicted in Fig 4. Upon subjecting the panelists to a sample of buttermilk containing a microalgae concentration of 1.5% (referred to as T_4), it was observed that the sensory attribute of said sample was deemed unsatisfactory, as indicated by a statistically significant $p<0.05$. Furthermore, it was observed that the presence of insoluble Spirulina particles resulted in a perceptible graininess, predominantly in treatment groups T_2 and T_3. Additionally, a statistically significant alteration in the oral texture was noted ($p < 0.05$).

The results indicated that the solubility of *Spirulina* decreased with increasing concentration, rendering higher concentrations unsuitable for acceptance. The samples characterized as T_1 exhibited the most favorable ratings in terms of appearance, aroma, color, flavor, mouthfeel, and overall acceptability. Conversely, treatments incorporating T_2, T_3, and T_4 received the lowest scores in these aforementioned attributes ($p < 0.05$). The sensory score and overall acceptance were found to be highest for T_1, which contained 0.25% *Spirulina*-fortified buttermilk.

Hence, the buttermilk enriched with a concentration of 0.25% *Spirulina* (T_1) was chosen as the optimal end product and was subsequently subjected to additional evaluation for further study.

The study conducted by Debbabi *et al*. (2018) aimed to explore the effects of incorporating *Spirulina* into yoghurt at varying concentrations of 0.12% and 0.24%. Based on the findings of their study, it was determined that the incorporation of *Spirulina* at a concentration of 0.12% into yoghurt yielded the most favorable outcomes in relation to sensory attributes. In a recent study conducted by Ustun-Aytekin *et al*. (2022), a novel approach was taken to enhance the nutritional value of traditional kefir. The researchers incorporated *Spirulina platensis* biomass into the kefir at varying concentrations, namely 0.05%, 0.1%, 0.5%, 1%, and 2%. The results indicated that these concentrations yielded the highest levels of homogeneity and texture, as well as the most favourable sensory scores. The incorporation of *Spirulina* at a concentration of 0.25% expedited the termination of the fermentation process, while simultaneously maintaining the desired textural attributes and sensory palatability of the resultant milk-based product.

Conclusion

The utilisation of *Spirulina* powder as a novel and captivating constituent presents a promising avenue for incorporating it into spiced buttermilk, thereby enhancing its nutritional and physico-chemical attributes while preserving its sensory appeal to a considerable extent. The findings of the study revealed that the fortification of buttermilk with a concentration of 0.25% *Spirulina,* denoted as T_1, emerged as the optimal choice for the ultimate product due to its commendable nutritional composition and favorable sensory attributes. Hence, it was subjected to a comprehensive evaluation in order to facilitate subsequent analysis.

Conflict of interest

The authors declare that they have no conflict of interest

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