PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.4169

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RESEARCH ARTICLE

The effect of thermal and non-thermal treatments on shelf-life of ready to drink whey-based functional beverages

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OPEN ACCESS

ARTICLE HISTORY

Received: 24 June 2024
Accepted: 18 September 2024

Available online
Version 1.0 : 12 November 2024

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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CITE THIS ARTICLE

Bhatia R, Chauhan K, Taneja N K, Kumar V, Singh G, Kaur K. The effect of thermal and non-thermal treatments on shelf-life of ready to drink whey-based functional beverages . Plant Science Today (Early Access). https://doi.org/10.14719/pst.4169

Abstract

Whey protein-based beverages are popular because of their many health benefits, high protein, pleasant flavor and vibrant hue. The hue of protein beverages is the most reliable barometer of how various quality characteristics will influence buyers' decisions. Protein beverages are typically processed using conventional thermal treatment, which diminishes their nutritional content and alters their appearance. Controlling the protein beverage quality and approximating the nutritional change can be achieved by examining microbial load during storage. This study analyzed the total microbial load such as coliform, yeast and molds of protein beverages, along with the impacts of various thermal (microwave, retort-pasteurization) and non-thermal (ultrasonication) treatments. The microbiological evaluation of the protein beverage was carried out at regular intervals (weekly) during the storage period under refrigeration and room temperature. In results, the protein beverage remained good in terms of overall acceptability and maintained the quality up to 90 days and 65 days after thermosonication treatment and was stored at refrigerated temperature (4 °C) followed by room temperature (28 °C) respectively. Hence, this study focuses on maximizing the shelf-life efficiency and maintaining protein beverage quality by using thermal and non-thermal procedures in tandem.

Keywords

beverage; protein; thermal; non-thermal sonication; microwave

Introduction

As the demand for functional beverages increases, researchers and manufacturers are continuously exploring ways to enhance the shelf-life and quality of these products. One critical aspect of ensuring the longevity of ready-to-drink whey-based functional beverages is the implementation of appropriate thermal and non-thermal treatments (1). These treatments play a vital role in preserving the nutritional value, sensory attributes and safety of the beverages, which are essential factors in meeting consumer expectations and regulatory standards (2). Thermal procedures, such as pasteurization and sterilization, involve subjecting beverages to specific temperatures for predetermined durations in order to eliminate or reduce spoilage-causing pathogens and enzymes (3). Thermal and non-thermal treatments eliminate microbes by disrupting their cellular structures or metabolic functions. Thermal treatments, such as pasteurization, use heat to denature

microbial proteins, leading to cell death. Non-thermal methods, like thermosonication, combine heat with ultrasonic waves to create cavitation, which physically damages microbial cells and enhances heat transfer. While thermosonication effectively reduces microbial load and preserves sensory qualities, its limitations include uneven microbial inactivation in complex food matrices and the potential for heat-sensitive nutrients to degrade under prolonged exposure or with the combination of thermal and non-thermal techniques (4).

In the food and beverage industry, the application of heat has been a time-honored and effective method of preservation, but it may also alter the nutritional composition and sensory qualities of the beverages. To achieve a balance between microbiological safety and product quality (5), it is essential to comprehend the optimal conditions and duration of thermal treatments. Non-thermal processes, such as high-pressure processing (HPP) (6), pulsed electric fields (PEF) (7) and ultraviolet (UV) radiation (8), have emerged as potential alternatives to thermal processes (9). These non-thermal technologies offer the benefit of preserving the nutritional value and organoleptic qualities of beverages while ensuring their microbial inactivation. Research into the efficacy of these treatments in extending shelf-life and preserving functional properties is crucial for realizing their maximum commercial potential (10). This exploration into the effect of thermal and non-thermal treatments on the shelf-life of ready-to-drink whey-based functional beverages is an endeavor to contribute to the advancement of the functional beverage industry (11).

Shelf-stable ready-to-drink (RTD) products can be broken down into 4 distinct groups. Beverage aseptically processed in a commercial setting; beverage processed in a retort setting; beverage processed by tunnel pasteurization; beverage filled either hot or cold and pasteurized (12). It's possible to thermosonication, retort, pasteurize or ultra-high-temperature-process a low-pH shelf-stable beverage (UHT). A retort is an extremely high-temperature treatment performed between 250 °F and 300 °F for 20 to 40 min (13). The UHT method only requires a brief period (5 sec) at temperatures exceeding 275 °F (14). Aseptic packaging ensures that UHT drinks won't go bad when sitting on a shelf. Hot fill is used in the production of acidic drinks. After being microwaved for 2 min at over 180 °F, the beverage is poured into a bottle while still hot and allowed to cool. In terms of infectious disease, high-acid beverages are preferable to low-acid ones. In this context, basic pasteurization is a frequent processing option, especially for smoothies (15).

By understanding the intricate balance between preservation techniques and product characteristics, we aspire to facilitate the creation of beverages that not only cater to consumer preferences but also meet the stringent requirements of a dynamic and competitive market. This study aims to shed light on the effect of thermal and non-thermal treatments on the shelf-life of ready-to-drink whey-based functional beverages stored at refrigerated temperature (4 °C) and room temperature (28 °C) and provide valuable insights for the beverage industry and consumers alike.

Materials and Methods

Material collection and RTD whey-protein-based beverage preparation

Whey protein isolate (WPI) powder (raw whey protein 80 %, containing 80 % pure whey protein, 7 % carbohydrates, 4.21 % saturated fatty acids, BCAA 17.65 % and other micronutrients) was obtained from Muscle Blaze (Himachal Pradesh, India).

Thermal treatment

Retort pasteurization

RTD whey-protein-based beverages were pasteurized under the category of pasteurization "High-Temperature Short-Time (HTST) pasteurization". The HTST pasteurizer produces a constant flow of protein beverage that is heated to 72 °C for 15 sec and then rapidly chilled to 5 °C or below (10). After the HTST treatment, the sample was stored at refrigerated temperature (4 °C) as well as room temperature (28 °C). Total plate count, coliform count and yeast and mold count were estimated according to the method (16), with minor modifications and sensory evaluation on a 9-point Hedonic Scale of both treated and untreated (control) sample at a regular interval (weekly) to assess the overall quality and accessibility of the product.

Microwave

The microwave was used to treat 2 bottles of ready-to-drink (RTD) whey protein beverage, while another bottle was kept as the control. The microwave treatment was given at power level 60 (i.e., 600 w) for 3 different time duration i.e. 18 sec, 20 sec and 25 sec. The treated samples were kept under refrigerated conditions (4 °C) and ambient temperature or room temperature (28 °C). Total plate count, coliform count and yeast and mold count according to the method (16). Sensory evaluation on a 9-point Hedonic scale were done for each sample that was treated and untreated and stored at refrigerated temperature and room temperature at regular intervals (weekly) till they were acceptable based on consumer acceptance.

Non-thermal treatment

Ultrasonication (US)

Ultrasonication treatment was given to each sample of RTD whey-protein-based beverage except the control sample. The ultrasonication treatment was given for 3 different time durations: 1 min, 5 min and 10 min and after treatment, the treated and non-treated (control) samples were stored at refrigerated temperature (4 °C) and room temperature (28 °C). Total plate count, coliform count and yeast and mold count according to a method (16) and sensory evaluation on a 9-point Hedonic Scale were done for both treated and control samples at regular intervals (weekly) till the overall good acceptability of the product based on consumer acceptance.

Thermosonication (TS)

RTD whey-protein-based beverage samples were subjected to thermosonication. An untreated sample was used as a control for further analysis. The thermosonication treatment was given in 3 different combinations, as given below:

- Time 1 min + 72 °C for 15 sec.
- Time 5 min + 72 °C for 15 sec.
- Time 10 min + 72 °C for 15 sec.

Treated and non-treated samples (control) were stored and refrigerated (4 °C) and room temperature (28 °C) respectively. To check the quality and overall acceptability of the product, the total plate count, coliform count, yeast and mold count according to the method (16) with minor modifications and sensory evaluation on a 9-point Hedonic Scale were evaluated at regular time intervals (5 days).

Sensory analysis

Thirty volunteers (aged 22 to 45) at the NIFTEM campus in India evaluated the color, flavor, body texture and overall acceptability of the RTD whey-protein-based beverage for this study. On a 9-point hedonic scale, 9 = extremely like, 8 = very much like, 7 = moderately like, 6 = slightly like, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = extremely dislike, the ratings were conducted. The volunteers were instructed to consume a cream cracker and a glass of water between each taste to purge their palates. The results are presented as the mean ± SD score across all records.

The study was reviewed and approved by the NIFTEM Ethical Committee for Human Research (NECHR), vide protocol no. 5/7E/NECHR/23 and informed consent was obtained from each subject before they participated in the study.

Results and Discussion

Effects of retort pasteurization on shelf-life of RTD whey-protein-based beverage

Effect on total plate count

According to the U.S. FDA Pasteurized Milk Ordinance (http://www.thinkusadairy.org/2017) and the International Dairy Federation (IDF), pasteurization treatment is deemed adequate for the eradication of the most dangerous milk-borne pathogens, as *Coxiella burnetii* and *Mycobacterium tuberculosis/Mycobacterium bovis* (17).

The total plate count decreased due to the pasteurization treatment and increased in treated and non-treated samples during the storage period under refrigerated conditions (4 °C) and room temperature (28 °C). The total plate count in the fresh sample was 3.4×10^4 CFU mL^{-1}. Following the pasteurization, the plate counts were significantly reduced to 0.5×10^3 CFU mL^{-1}. During the storage period under refrigerated conditions (4 °C), the total plate count of the product remained < 10^4 for up to 19 days (Table 1a). Similarly, at room temperature, the product remained good for consumption for up to 14 days. Both the conditions were ideal for consumption as per FSSAI guidelines. Heat, through pasteurization or sterilization, denatures proteins and enzymes, leading to the disruption of microbial cellular processes. High temperatures cause proteins to unfold and lose their functional shape. This impairs vital enzymes involved in metabolism, cellular repair and replication. Heat also disrupts microbial cell membranes, affecting the integrity of the cell. As a result, the cell loses its ability to regulate the passage of molecules, causing cell death (18).

Table 1a. Effect of retort pasteurization (temp. 72 °C; 15 sec) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at refrigerated temperature (4 °C).

No. of days	Total plate count (CFU/mL)				Coliform count (CFU/mL)				Yeast and mold count (CFU/mL)			
	Untreated sample (control CFU/mL)	Treated sample			Untreated sample (control CFU/mL)	Treated sample			Untreated sample (control CFU/mL)	Treated sample		
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	5	Nil	Nil	2.2×10^3	Nil	Nil	Nil	1.2×10^2	Nil	Nil	Nil
5	1.5×10^6	12	Nil	Nil	1.3×10^6	Nil	Nil	Nil	2.8×10^3	Nil	Nil	Nil
10	2.1×10^8	22	Nil	Nil	2.7×10^8	Nil	Nil	Nil	1.8×10^5	Nil	Nil	Nil
15	TNTC	61	Nil	Nil	TNTC	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil
20	TNTC	83	7	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
25	TNTC	94	12	2	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
30	TNTC	TNTC	38	4	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
35	TNTC	TNTC	TNTC	351	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

Statistical analysis

Experimental results were presented as mean with standard deviation. Statistical analyses were performed using Microsoft Office Excel 2007. Group differences were evaluated through one-way analysis of variance (ANOVA) using SPSS Base 19.0 statistical software. Each test was conducted in triplicate and statistical significance was determined at $p<0.05$.

Effect on coliform count

Microwave treatment affected the growth of coliform as well. The coliform count in the RTD whey-protein-based beverage was 2.2×10^3 CFU mL^{-1} before pasteurization. After pasteurization, it was completely reduced and no growth was observed during the storage period at room temperature (28 °C) for up to 22 days. Similarly, no growth was observed in the treated sample stored under refrigerated conditions (4 °C) for up to 34 days (Table 1a). Hence,

the study's findings revealed that refrigerated temperature (4 °C) is suitable compared to room temperature to prevent coliform growth. The findings of the present study corroborate with other study, which also highlighted the inhibition of *E coli* and *Staphylococcus aureus* at a refrigerated temperature of 4 °C (19).

Effect on yeast and mold count

Due to pasteurization, a significant change in the yeast and mold count relative to other microbial counts in RTD whey -protein-based beverages was observed. Before treatment, the yeast and mold count in protein beverage was observed as 1.2×10^2, after treatment, no growth of yeast and mold was observed up to 22 days under room temperature (28 °C) (Table 1b). Similarly, under refrigerated conditions (4 °C), the sample persisted in good conditions for up to 31 days (Table 1a). Hence, the refrigerated temperature is more suitable for preventing the growth of yeast and mold in the product. Similar outcomes were observed that the pasteurization treatment at 72 °C for 15 sec destroyed *Aspergillus, Penicillium, Rhizopus*, etc. in foods (15). Moreover, supported similar results with whey-based mango beverages when pasteurized at 72 °C for 15 sec and stored at refrigerated temperature (4 °C) for 28 days, which caused the prevention of *Rhizopus* and *Aspergillus* (20).

Effects of microwave on shelf-life of RTD whey-protein-based beverage

Effect on total plate count

The microwave treatment at a similar power level of 60 (i.e., 600 W) with different time combinations(18 sec, 20 sec and 25 sec) significantly reduced the microbial load, while storage of the samples at refrigerated temperature (4 °C) and room temperature (28 °C) lead to an enhancement in microbial load.

Notably, the total plate count in the fresh sample was recorded as 1.4×10^4 CFU mL^{-1}. Following the microwave treatment at 600W for 18 sec, 20 sec and 25 sec, the total plate count of the treated sample was recorded as 2.5×10^3, 1.9×10^2 and 1.1×10^2 CFU mL^{-1} respectively. During the storage period, the product was found to be stable for 25 sec under refrigerated conditions for up to 34 days (Table 3a). During the storage at room temperature (28 °C), the product had a shelf-life of 25 days only (Table 3b). Notably, as the plate count was < 10^4 (FSSAI guideline), the microbial safety of the product was attested as per FSSAI guidelines. The findings of the study were duly supported (10), who also observed a significant increase in shelf-life after microwave treatment of whey-based functional beverages containing orange juice and probiotics increased up to 24 days at refrigerated temperature (4 °C).

Table 1b. Effect of retort pasteurization (temp. 72 °C; 15 sec) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at room temperature (28 °C).

No. of days	Untreated sample (control CFU/mL)	Total plate count (CFU/mL)			Untreated sample (control CFU/mL)	Coliform count			Untreated sample (control CFU/mL)	Yeast and mold count (CFU/mL)		
		Treated sample										
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	5	Nil	Nil	2.2×10^3	Nil	Nil	Nil	1.2×10^2	Nil	Nil	Nil
5	1.9×10^6	26	Nil	Nil	2.3×10^6	Nil	Nil	Nil	2.8×10^3	Nil	Nil	Nil
10	2.1×10^9	72	Nil	Nil	2.9×10^9	Nil	Nil	Nil	1.8×10^5	Nil	Nil	Nil
15	TNTC	105	Nil	Nil	TNTC	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil
20	TNTC	211	16	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
25	TNTC	TNTC	113	38	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
30	TNTC	TNTC	TNTC	205	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
35	TNTC	TNTC	TNTC	TNTC	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

Effect on Sensory Evaluation

The flavor, color, consistency and visual appearance of pasteurized RTD whey-protein-based beverages were identical to those of untreated samples. The treated sample stored under refrigerated conditions (4 °C) displayed sensory stability for up to 12 days as compared to the untreated (control) sample that displayed good sensory evaluation for up to 6 days due to the overgrowth of microorganisms such as bacteria and fungi (Table 2). The sensory evaluation results are supported by another study (21).

Effect on coliform count

Microwave treatment at power level 600 W with different time combinations, i.e., 600W:18sec, 600W:20 sec and 600W:25 sec, significantly reduced the coliform count from the microwave sample as compared to the control. After being heated in the microwave at 600 watts for 18 sec, 20 sec and 25 sec, the coliform count in the fresh RTD whey-protein-based beverage was 3.4×10^3. The values were reduced by 0.1×10^2, 0.02×10^2 and 0 respectively, because the coliform growth was below the FSSAI-required threshold of 10^4; the sample kept in the refrigerator (4 °C) had a

Table 2. Sensory data table.

Sample	Flavor	Color	Appreance	consistency	Mouth feel	Overall acceptability
Untreated sample (control)	5.12 ± 1.42	5.6 ± 1.61	6.3 ± 1.31	6.4 ± 1.29	5.8 ± 1.23	5.71 ± 1.13
Treated sample	6.5 ± 1.35	6.0 ± 1.46	6.4 ± 2.01	6.6 ± 1.21	6.2 ± 1.27	6.34 ± 0.82

shelf life of up to 34 days (Table 3a). In contrast, a coliform count of 1.8×10^5 was found in the product held at room temperature (28 °C) for 16 days (Table 3b). Hence, the microwave treatment at 600 W for 25 sec was found to be the most optimal condition for inhibiting coliform growth. Our findings were reinforced by a previous study, who found that the shelf-life of inexpensive beverages made with soy milk was extended by up to 28 days after microwave treatment (22).

perature (4 °C), the treated sample showed product safety for up to 18 days, while the control sample was rejected after 12 days due to the overgrowth of yeast and mold. Our findings align with the study conducted, who developed innovative whey mango-based beverages (23). In their research, they observed mold growth during the storage period under refrigerated conditions (4 ± 1 °C). Specifically, after 10 days of storage, the mold count reached 1.26 ± 0.05. This indicates that despite refrigeration, mold

Table 3a. Effect of microwave (600w; 25 sec) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at refrigerated temperature (4 °C).

No. of days	Untreated sample (control CFU/mL)	Total plate count (CFU/mL)			Untreated sample (control CFU/mL)	Coliform count (CFU/mL)			Untreated sample (control CFU/mL)	Yeast and mold count (CFU/mL)		
		Treated sample										
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	2	Nil	Nil	2.2×10^3	Nil	Nil	Nil	1.2×10^2	Nil	Nil	Nil
5	1.5×10^6	8	Nil	Nil	1.3×10^6	Nil	Nil	Nil	2.8×10^3	Nil	Nil	Nil
10	2.1×10^8	17	Nil	Nil	2.7×10^8	Nil	Nil	Nil	1.8×10^5	Nil	Nil	Nil
15	TNTC	24	Nil	Nil	TNTC	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil
20	TNTC	32	7	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
25	TNTC	38	12	2	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
30	TNTC	140	38	4	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
35	TNTC	252	TNTC	24	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
40	TNTC	TNTC	TNTC	58	TNTC	12	3	Nil	TNTC	8	2	Nil

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

Table 3b. Effect of microwave (600w; 25 sec) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at room temperature (28 °C).

No. of days	Untreated sample (control CFU/mL)	Total plate count (CFU/mL)			Untreated sample (control CFU/mL)	Coliform count (CFU/mL)			Untreated sample (control CFU/mL)	Yeast and mold count (CFU/mL)		
		Treated sample										
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	2	Nil	Nil	2.2×10^3	Nil	Nil	Nil	1.2×10^2	Nil	Nil	Nil
5	1.9×10^6	15	Nil	Nil	2.3×10^6	Nil	Nil	Nil	2.8×10^3	Nil	Nil	Nil
10	2.1×10^9	28	Nil	Nil	2.9×10^9	Nil	Nil	Nil	1.8×10^5	Nil	Nil	Nil
15	TNTC	52	Nil	Nil	TNTC	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil
20	TNTC	95	16	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
25	TNTC	TNTC	113	26	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
30	TNTC	TNTC	TNTC	112	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
35	TNTC	TNTC	TNTC	TNTC	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
40	TNTC	TNTC	TNTC	TNTC	TNTC	7	4	Nil	TNTC	3	1	Nil

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

Effect on yeast and mold count

After microwave treatment of RTD whey-protein-based beverages, there was no discernible difference in yeast and mold count compared to other microbiological counts. After microwave treatment for 18 sec, 20 sec and 25 sec, the yeast and mold count in the sample did not significantly drop. Hence, the microwave treatment is no longer used for the increased shelf-life of products. The microwave-treated sample and untreated sample (control) showed similar growth when stored at room temperature (28 °C). However, when the sample was stored at refrigerated tem-

growth still occurred, which is consistent with the microbial activity typically observed in perishable beverages (23).

Effect on sensory evaluation

The sensory score for flavor, color, consistency and appearance of microwave-treated RTD whey-protein-based beverage was found to be the same as that of untreated samples for up to 10 days (Table 4). Hence, it can be presumed that the sensory evaluation of the product was appropriate up to 10 days after that the overgrowth of yeast and mold led to alteration in the sensory properties. As previously reported, the growth of molds significantly

Table 4. Sensory data table.

Sample	Flavor	Color	Appearance	consistency	Mouth feel	Overall acceptability
Untreated sample (control)	5.36 ± 1.28	5.42 ± 1.05	5.3 ± 1.21	6.2 ± 1.72	6.1 ± 1.10	5.67 ± 1.27
Treated sample	5.17 ± 2.16	6.32 ± 1.32	6.20 ± 1.41	6.37 ± 1.39	6.4 ± 0.83	6.09 ± 1.13

altered the sensory properties of whey mango-based beverages during storage, particularly after 10 days of refrigeration at 4 ± 1 °C (23). The presence of molds led to the production of various enzymes, such as proteases and amylases, which initiated biochemical reactions within the whey protein beverages. These enzymes are known to degrade proteins and carbohydrates, resulting in undesirable changes in texture, flavor and overall quality. As a consequence, the sensory attributes of the product, including taste, smell and mouth feel were negatively impacted, rendering the beverage unpalatable. Due to these significant sensory and quality degradations, the product was deemed unacceptable and was ultimately rejected. This finding underscores the importance of stringent microbial control and the potential need for enhanced preservation strategies to maintain the stability and acceptability of whey-based beverages during extended storage (23).

Effects of ultrasonication on shelf-life of RTD whey-protein-based beverage

Effect on total plate count

The ultrasonication treatment was given to RTD whey-protein-based beverages at 30 kHz/min for 1 min, 5 min and 10 min respectively. The effect of ultrasonication treatment on RTD whey-protein-based beverages is presented in Table 5a. The total number of plates in the fresh sample was recorded as 3.40×10^4. Only the 30 kHz/10 min ultrasonication treatment reduced the microbial load to (1.2×10^3) in the treated sample, while the other two combinations (30 kHz/1 min and 30 kHz/5 min) showed no significant ($p>0.05$) difference in reducing the microbial load. In the control sample, the microbial load remained unchanged at 2.3×10^4. Both refrigerated storage (4 °C) and room temperature (28 °C) were used to keep the treated and untreated samples respectively.

At refrigerated temperature (4 °C), the product displayed acceptability for up to 5 days only (Table 3a), while at ambient temperature (28 °C), both treated and untreated samples were rejected due to overgrowth of the total count before 5 days (Table 5b). The results demonstrated that after 5 days of storage at room temperature (28 °C), the microbial load in the treated sample was 2.4×10^6 CFU mL^{-1}, while in the untreated sample, it was 5.4×10^7 CFU mL^{-1}. Overgrowth of 1.5×10^4 also renders the treated sample unusable after 5 days in the refrigerator. Therefore, protein drinks no longer have their shelf lives extended with ultrasonication.

It was found similar results after the ultrasonication treatment at 30 kHz for 10 min and reported that the ultrasonication for 10 min is not suitable for reducing the maximum microbial load from the product, hence it is no longer used to increase the shelf-life of the final product (24). It was also obtained comparable results following the ultrasonication treatment at 30 kHz for 10 min (24). They claimed that the ultrasonication for 10 min is not suitable for reducing the maximum microbiological load from the product and as a result, it is no longer employed to enhance the shelf-life of the final product (24).

Effect on coliform count

Given the ultrasonication treatment at different conditions, it was observed that the coliform count reduced only

Table 5a. Effect of ultrasonication (30 wHz; 10 min) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at refrigerated temperature (4 °C).

No. of days	Untreated sample (control CFU/mL)	Total plate count (CFU/mL)			Untreated sample (control CFU/mL)	Coliform count (CFU/mL)			Untreated sample (control CFU/mL)	Yeast and mold count (CFU/mL)		
		Treated sample										
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	42	19	6	2.2×10^3	34	11	4	1.2×10^2	Nil	Nil	Nil
5	1.5×10^6	114	13	26	1.3×10^6	91	18	28	2.8×10^3	11	4	Nil
10	2.1×10^9	TNTC	149	51	2.9×10^9	116	67	49	1.8×10^5	26	16	9

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

Table 5b. Effect of ultrasonication (30 wHz; 10 min) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at room temperature (28 °C).

No. of days	Untreated sample (control CFU/mL)	Total plate count (CFU/mL)			Untreated sample (control CFU/mL)	Coliform count (CFU/mL)			Untreated sample (control CFU/mL)	Yeast and mold count (CFU/mL)		
		Treated sample										
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	48	5	3	2.2×10^3	91	14	6	1.2×10^2	14	8	Nil
5	1.9×10^6	TNTC	TNTC	50	2.3×10^6	219	108	41	2.8×10^3	35	21	5
10	2.1×10^9	TNTC	TNTC	TNTC	2.9×10^9				1.8×10^5	73	49	16

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

in samples treated at 30 wHz for 10 min. The coliform count was noted at 3.4×10^4 in fresh RTD whey-protein-based beverages, but after treatment, it was reduced and remained at 1.2×10^2. Under room temperature conditions, an overgrowth of coliform count in the treated sample (2.5×10^5) as well as the untreated sample (4.2×10^6) was noted after 5 days. Hence, both the samples were rejected. Notably, under refrigerated conditions, an overgrowth of coliform in treated sample 1.8×10^5 and untreated sample 2.8×10^6 was also duly observed. The findings of the study indicated that ultrasonication is not an appropriate technique to enhance the shelf life of the product.

Effect on yeast and mold

The ultrasonication treatment at 30 wHz for 1 min, 30 wHz for 5 min and 30 wHz for 10 min was used to treat RTD whey-protein-base beverages. The results showed that the ultrasonication treatment was non-significant ($p>0.05$) in reducing the concentrations of yeast and mold from the treated samples. The yeast and mold counted 1.2×10^2 in the fresh samples after treatment at different combinations (30 wHz for 1 min, 30 wHz for 5 min and 30 wHz for 10 min). A major reduction was not observed in the treated sample. Hence, it is not an appropriate technique to increase the shelf-life of protein beverages.

Effect on sensory evaluation

The sensory score for flavor, color, consistency and appearance of ultrasonication-treated RTD whey-protein-based beverage was found to be the same as the untreated sample for up to 5 days (Table 6). Hence, it can be concluded that the sensory evaluation of the product was good for only up to 5 days. After 5 days, the overgrowth of microorganisms such as coliform and yeast appeared, which caused the unacceptability of the product.

Effects of thermosonication on shelf-life of RTD whey-protein-based beverage

Effect on total plate count

The thermosonication treatment was used in different combinations: 1 min, 72 °C and 15 sec, 5 min, 72 °C and 15 sec and 10 min, 72 °C and 15 sec to reduce the total microbial load from the sample. The effect of thermosonication treatment on RTD whey-protein-based beverages is shown in Table 7a. A total microbial load of 3.4×10^4 was noted in the fresh sample after treatment at 1 min, 72 °C and 15 sec, a non-significant ($p>0.05$) microbial reduction was observed. However, after treatment with 5 min, 72 °C and 15 sec and 10 min, 72 °C and 15 sec, significant ($p<0.05$) microbial reduction was observed at 1.4×10^3 and 1.1×10^2 respectively. The thermosonication treatment at 10 min, 72 °C and 15 sec is more effective than others. After treatment, the treated sample and untreated sample (control) were stored at refrigerated temperature (4 °C) and room temperature (28 °C) and found that the treated samples at 5 min, 72 °C and 15 sec and 10 min, 72 °C and 15 sec (at 4 °C) showed minimum microbial growth up to 55 days i.e. 5.4×10^3 and 4.7×10^3 respectively (Table 7a). Similarly, stored samples under room temperature (28 °C) showed minimum microbial load up to 35 days when treated at 5 min, 72 °C and 15 sec and 10 min, 72 °C and 15 sec at 4.4×10^3 and 3.8×10^3 respectively (Table 7b). Hence, it can be concluded that thermosonication at 10 min, 72 °C and

Table 6. Sensory data table.

Sample	Flavor	Color	Appearance	consistency	Mouth feel	Overall acceptability
Untreated sample (control)	5.24 ± 1.39	6.12 ± 2.16	5.2 ± 1.44	5.7 ± 1.06	5.8 ± 1.13	5.61 ± 1.41
Treated sample	6.02 ± 1.10	5.93 ± 1.41	6.4 ± 0.73	6.28 ± 1.15	6.24 ± 1.20	6.17 ± 1.46

Table 7a. Effect of thermosonication (30 wHz; 10 min) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at refrigerated temperature (4 °C).

No. of days	Untreated sample (control CFU/mL)	Total plate count (CFU/mL)			Untreated sample (control CFU/mL)	Coliform count (CFU/mL)			Untreated sample (control CFU/mL)	Yeast and mold count (CFU/mL)		
		Treated sample										
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	Nil	Nil	Nil	2.2×10^3	Nil	Nil	Nil	1.2×10^2	Nil	Nil	Nil
5	1.5×10^6	Nil	Nil	Nil	1.3×10^6	Nil	Nil	Nil	2.8×10^3	Nil	Nil	Nil
10	2.1×10^8	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil	1.8×10^5	Nil	Nil	Nil
15	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil
20	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
25	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
30	TNTC	9	Nil	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
35	TNTC	15	6	Nil	TNTC	Nil	Nil	Nil	TNTC	Nil	Nil	Nil
40	TNTC	32	13	Nil	TNTC	12	4	Nil	TNTC	Nil	Nil	Nil
45	TNTC	58	29	2	TNTC	41	19	Nil	TNTC	2	Nil	Nil
50	TNTC	81	33	9	TNTC	91	48	16	TNTC	7	1	Nil
55	TNTC	113	46	21	TNTC	103	89	27	TNTC	11	4	Nil
60	TNTC	218	91	46	TNTC	154	114	39	TNTC	26	9	2

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

Table 7b. Effect of thermosonication (30 wHz; 10 min) in prevention of microbial growth (CFU/mL) in RTD whey-protein-based beverage and increase shelf-life during the storage period at room temperature (28 °C).

No. of days	Total plate count (CFU/mL)				Coliform count (CFU/mL)				Yeast and mold count (CFU/mL)			
	Untreated sample (control CFU/mL)	Treated sample			Untreated sample (control CFU/mL)				Untreated sample (control CFU/mL)			
		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}		10^{-2}	10^{-3}	10^{-4}
0	3.4×10^4	Nil	Nil	Nil	2.2×10^3	Nil	Nil	Nil	1.2×10^2	Nil	Nil	Nil
5	1.9×10^6	Nil	Nil	Nil	2.3×10^6	Nil	Nil	Nil	2.8×10^3	Nil	Nil	Nil
10	2.1×10^9	Nil	Nil	Nil	2.9×10^9	Nil	Nil	Nil	1.8×10^5	Nil	Nil	Nil
15	TNTC	16	Nil	Nil	TNTC	Nil	Nil	Nil	2.7×10^8	Nil	Nil	Nil
20	TNTC	37	18	Nil	TNTC	21	8	Nil	TNTC	Nil	Nil	Nil
25	TNTC	94	52	4	TNTC	64	28	2	TNTC	Nil	Nil	Nil
30	TNTC	164	43	16	TNTC	114	83	7	TNTC	Nil	Nil	Nil
35	TNTC	197	67	24	TNTC	249	121	18	TNTC	Nil	Nil	Nil
40	TNTC	TNTC	116	49	TNTC	TNTC	259	37	TNTC	3	1	Nil
45	TNTC	TNTC	276		TNTC	TNTC	TNTC	118	TNTC	11	4	2
50	TNTC	TNTC	TNTC		TNTC	TNTC	TNTC	236	TNTC	43	37	7
55	TNTC	TNTC	TNTC		TNTC	TNTC	TNTC	TNTC	TNTC	91	53	26
60	TNTC	TNTC	TNTC		TNTC	TNTC	TNTC	TNTC	TNTC	118	92	44

TNTC: Too Numerous to Count; **CFU**: Colony Forming Unit.

15 sec is more effective than others and may be used as an appropriate technique to increase the shelf life of RTD whey-protein-based beverages.

Effect on coliform count

The thermosonication treatment assisted in reducing the coliform count, as discussed above. In the fresh sample of RTD whey-protein-based beverage, a coliform count of 3.4×10^4 CFUmL^{-1} was noted. After the thermosonication treatment only at combinations 5 min, 72 °C and 15 sec and 10 min, 72 °C and 15 sec the coliform count was reduced to 1.8×10^2 and 1.1×10^2 respectively. At the refrigerated temperature, the coliform safety was measured up to 55 days and 35 days at refrigerated temperature (4 °C) and room temperature (28 °C) respectively, when treated at 5 min, 72 °C and 15 sec. The results obtained in this study are consistent with another study, who reported that thermosonication treatment extended the shelf life of pulque to 24 days when stored at 4 °C (25). Similarly, the coliform count ($<10^4$) of the RTD beverage was noted up to 55 days and 35 days at refrigerated temperature and room temperature (Table 4 a, b) when treated with 10 min, 72 °C and 15 sec. It was found that thermosonication at different conditions (5 min, 72 °C and 15 sec and 10 min, 72 °C and 15 sec) showed a similar effect in reducing coliform growth from the RTD samples.

Effect on yeast and mold

The thermosonication with 5 min; 72 °C; 15 sec and 10 min; 72 °C; 15 sec also reduces the yeast and mold count from the RTD whey-protein-based beverage and prevents the growth up to 60 to 65 days at refrigerated temperature (4 °C) and up to 50 to 55 days at room temperature (28 °C) respectively. The reduction of yeast and mold count is indicated in Tables 7 a, b. The thermosonication treatment can be used to extend the shelf-life of the RTD whey-protein-based beverage and can be used as an appropriate technique. A study highlighted the effectiveness of thermosonication (72 °C) as a treatment method for inhibiting microbial growth in fruit juices (26). The researchers demonstrated that sonication, a non-thermal preservation technique, proved to be highly efficient in eliminating or significantly reducing the growth of microbes, including yeasts and molds, in beverages. According to their findings, the application of sonication effectively suppressed the growth of yeast and mold, which are common spoilage organisms in fruit juices, for an extended period. They observed that while untreated juices experienced microbial growth, particularly yeast proliferation, within a much shorter timeframe, the sonicated juices remained free from significant microbial contamination for up to 28 days. This indicates that sonication can be a promising preservation method, offering a way to extend the shelf life of fruit juices without compromising their sensory and nutritional qualities, as it avoids the use of heat, which can degrade certain heat-sensitive compounds. The study emphasized the potential of thermosonication as an efficient, cost-effective approach to enhancing the microbial stability of beverages, making it a valuable alternative to traditional preservation methods (26).

Effect on sensory evaluation

The sensory score for flavor, color, consistency and appearance of thermosonication-treated RTD whey-protein-based beverage was found to be the same as that of untreated samples up to 65 days at refrigerated temperature (4 °C) and 55 days at room temperature when treated with 10 min; 72 °C; 15 sec (Table 8). The thermosonication for 10 min; 72 °C; 15 sec is more effective in maintaining the sensory attributes of RTD whey-protein-based beverage. It was explored the impact of sonication, specifically thermosonication, on the sensory properties of fruit juices (26).

Table 8. Sensory data table.

Sample	Flavor	Color	Appearance	consistency	Mouth feel	Overall acceptability
Untreated sample (control)	5.15 ± 1.11	6.21 ± 1.13	5.9 ± 1.37	5.36 ± 1.14	6.30 ± 1.21	5.78 ± 1.73
Treated sample	6.21 ± 1.43	6.63 ± 1.03	6.81 ± 1.25	6.40 ± 1.31	6.66 ± 1.05	6.54 ± 1.42

They found that thermosonication-treated juices demonstrated significantly improved stability compared to juices processed through traditional methods, during storage at refrigerated temperatures (4 ± 1 °C). The treatment, which combines a sonicator with mild heat (72 °C), not only effectively controlled microbial growth but also helped maintain key sensory attributes such as color, flavor and aroma over an extended period (26).

The study highlighted that the treated juices experienced less degradation in taste and appearance, remaining fresher and more palatable throughout the storage period. In contrast, untreated or conventionally treated juices often showed signs of spoilage, including changes in color, off-flavors and the growth of spoilage microorganisms like yeasts and molds. The enhanced sensory stability observed in thermosonicated juices is likely due to the ability of the process to inhibit enzymatic activities that contribute to nutrient loss and quality deterioration, such as browning or flavor degradation (26).

Thermosonication, while effective in microbial inactivation, has potential limitations, including high initial equipment costs and specialized maintenance requirements. The process also demands precise temperature and ultrasonic control, increasing operational complexity. Additionally, scalability for large industrial applications can be challenging, making it less feasible for small-scale producers.

Conclusion

Thermosonication-treated RTD whey-protein-based beverage samples were evaluated for 70 days at refrigerated temperature (4 °C), whereas it was evaluated for 60 days at room temperature. A control sample of untreated RTD whey-protein-based beverages was evaluated for 70 days at refrigeration temperature and 60 days at room temperature respectively. During storage, total plate count, coliform count, yeast and mold count and sensory evaluation or total overall acceptability and quality of the product remained up to 60-65 days and 55-60 days at refrigerated temperature (4 °C) and room temperature (28 °C) respectively. Here we conclude that thermosonication is more effective in increasing the product shelf-life compared to other techniques as discussed above. Therefore, it can serve as a suitable technique for extending the shelf-life of RTD whey-protein-based beverages at the industrial level.

Acknowledgements

The authors are sincerely grateful to NIFTEM for infrastructural and logistics support to prepare this manuscript; manuscript number NIFTEM-P-2024-125.

Authors' contributions

RB, KC and NKT contributed to conceiving, data acquisition and writing the manuscript. RB and VK carried out analysis, data interpretation and critical revision. VK, GS and KK contributed to refining the manuscript. RB, KC, analyzed, interpreted, refined and critically revised the manuscript. All authors have read and approved the manuscript.

Compliance with ethical standards

Conflict of interest: There is no declared conflict of interest.

Ethical issues: The study was approved by NECHR, NIFTEM, vide protocol no. 5/7E/NECHR/23 for sensory evaluation.

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