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Impact of *Citrus reticulata* Peel Extract and *Bifidobacterium longum* on *Staphylococcus aureus* during Cold Storage of Functional Yoghurt

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Abstract

THIS STUDY aimed to evaluates the effect of Citrus reticulate peel extract and Bifidobacterium longum on Staphylococcus aureus *inoculated in milk used* for manufacturing of yoghurt an attempt to improve the safety of yoghurt. Seven batches of yoghurt were prepared from cow's milk with Streptococcus thermophilus and Lactobacillus bulgaricus then inoculated with S. aureus, the first batch was a control (containing no probiotic bacteria, Citrus reticulata or S. aureus), the second batch contains S. aureus only, and the third batch contains B.longum. The following two batches (4,5) contain both citrus reticulata at concentrations of 0.5 and 1%. The remaining 6th, 7th batches contain B. longum and Citrus reticulata 0.5 and 1%, respectively. The yoghurt batches were examined physically and bacteriologically at zero time and after 3, 5, 10, and 12 days of refrigerated storage. The reduction percent of S. aureus count *once the storage term has expired* (12 days) was <56%, < 94,8% in yoghurt containing Citrus reticulata at concentrations of 0.5 and 1%, respectively. While the reduction percent in the case of yoghurt containing B. longum was < 95.4% But in the case of the combined addition of both citrus reticulata and B. longum, the reduction percent was < 96.2% and < 96.8% for 0.5 and 1% added citrus reticulata and B. longum, respectively. At the same time, in the control positive batch, the increase percent of S. aureus *after the storage time has ended* (*Twelve days*) was > 96.4%. This study offers important insights into the potentially beneficial uses of citrus peels, in particular, Citrus reticulata peel extract and B. longum as functional food additions in manufacture of yoghurt.

Keywords: Yoghurt, *S. aureus, Citrus reticulate*, *B. longum*.

Introduction

Food is referred to as "functional food" if it contains a dietary ingredient which enhances additional physiological processes within a human being. Naturally occurring foods can also be considered functional foods, which are typically ingested at an effective level as part of a regular, diverse diet [1]. One type of functional food is yoghurt, which is made through lactic acid fermentation, mostly by species of Lactobacillus and Streptococcus [2]. Because of its rich nutritional content, yoghurt is one of the most popular fermented milk products that is good for health [3]. It contains various health-fostering properties, like enhancing the defense mechanism, fat digestibility, stomach microbiota activity, and antibacterial activity. It also reduces sensitivity to lactose and its absorption through enhancing digestive functions. Including probiotic microorganisms, prebiotic substances, bioactive peptides, and other additives can enhance the health benefits of yoghurt [4]. Because of their high nutritional value, dairy products like yoghurt, which are staple foods in many nations, create an ideal habitat for the growth of numerous bacteria [5]. Along with other antimicrobial compounds, the acidity, which is created by fermenting microbes, is the main metabolic byproduct that prevents the growth of undesirable microbes [6]. *Staphylococcus aureus (S. aureus)* can be found in milk as a result of post-harvest contamination, contamination during the milking process [7]. *Staphylococcus aureus* is one of the most prevalent causes of clinical and subclinical bovine mastitis [8] and it represent a serious risk to

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the public's health [9]. *S. aureus* cannot withstand pasteurization temperatures, suggesting that raw milk can be pasteurized or boiled to efficiently destroy these germs [10]. The bacterium's enterotoxins have been connected to staphylococcal food contamination caused by inadequate food handling hygiene, inadequate packing, improper sterilization, and contamination of surfaces, utensils, and equipment used in handling food for consumption [11]. Staphylococcal poisoning incidents linked to dairy products are associated with modest quantities of staphylococcal enterotoxins, although malfunctioning starter cultures during fermentation and temperatures exceeding 10 °C are contributing elements [12]. Lactic acid inhibition lowers the danger of food-borne illnesses while extending the shelf life and nutritional value of yoghurt [13]. Despite yoghurt's nutritious content, its existence of pathogens and rotting bacteria lacking a minimal feasible LAB level renders it dangerous for consumption [14]. Food deterioration resulting from oxidative degradation and microbial action is a significant cause of food waste as it ultimately lowers the food's nutritional value, safety, and healthfulness. Artificial preservatives are frequently employed to impede additionally or postpone these procedures [15]. However, customer well-being worries additionally feelings opposition to artificial preservatives have rekindled consumers' aversion to and avoidance of meals that include these additives. Nitrates and nitrites, for example, are added to processed meals. have historically been linked to cancer [16]. The public's purchasing behavior is influenced by this information, favoring foods preserved with natural preservatives and fostering trust in the industry [16]. These discoveries have led scientists to investigate naturally occurring preservatives, which are mostly produced from plants and are thought to be safe, wholesome, nourishing, and supportive of sustainable food systems [15]. Whether Tropical or subtropical, citrus (*Citrus reticulate* Blanco) is a fruit that is found all over the world. Its nutritional qualities are significant in addition to its taste. 2020 saw 23.12 million tons of tangerines (*Citrus reticulate*) produced annually in China, making it the world's greatest producer [17]. Family Rutaceae includes about 140 genera and 1300 species, genus citrus includes mandarin (*Citrus reticulata*), and orange (*Citrus sinensis*), lemon (*Citrus lemon*) [18].Bioactive chemicals is located in large amounts inside the massive waste developed in conjunction with enterprises that process fruits and vegetables. In several sectors, these bioactive chemicals are ideal as natural coloring agents, antioxidants, preservatives, and antimicrobials [19]. The industry that processes citrus is among the many food processing sectors that produce a large number of leftovers, including rags, seeds, peels, and pomace. The overall citrus fruit production in 2019 was close to 158 million metric tons. This waste generation, which is roughly 10 million metric tons a year, contains 50–70% weight percentage of processed fruits. [20]. The two fractions that make up the majority from citrus juice industry leftovers are pulp additionally peel. Bioactive substances such as dietary fibre, vitamins, phenolic acids, flavonoids, terpenes, minerals, and organic acids, carotenoids. are thought to be abundant in both fractions [21]. Despite the fact that milk is said to contain significant levels of polyphenols, the quantity of polyphenolic compounds and natural antioxidants in milk is reduced by pasteurization and bacterial effects on milk protein [22]. Furthermore, probiotic bacteria can be effectively delivered to the human gut and thrive in the optimum habitat found in fermented dairy products [23]. " organisms that are live and, when given in appropriate amounts, beneficial to the host's health " is how probiotics are defined [24]. The most prevalent probiotic bacterial strains found in dairy products that have fermented are lactic acid bacteria, which can be present either as naturally occurring raw material components or as starter cultures of Lactobacillus, Streptococcus, Lactococcus, Bifidobacterium, and Leuconostoc. Microorganisms are preserved, and their viability and production are enhanced by fermentation, all while maintaining their probiotic qualities [25]. Gram-positive, strictly anaerobic, pleomorphic rods are known as bifidobacteria. The Latin word "bifidus," which means "cleft in two parts," refers to the branching morphology of the bacteria that goes by the name "Bifidobacterium." Apart from their primary phenotypic trait of generating lactic acid, bifidobacteria also generate acetic acid, which has a potent bactericidal effect and inhibits the growth of pathogenic bacteria [26]. Therefore, a combination of *Citrus reticulata* and *B. longum* might be expected to exert greater inhibitory action on pathogenic organisms than would either alone. The main purpose of this investigation was to determine the outcome of *Citrus reticulata* and *B. longum* on inhibition of *S. aureus* in yoghurt kept refrigerated.

Material and Methods

Sources of Cultures

Streptococcus thermophiles (EMCC Number: 1044, Designations: DSM 20479). *Lactobacillus delbrueckii ss. bulgaricus* (Orla-Jensen 1919) (EMCC Number: 1102, Designations: DSM 20080). *Bifidobacterium longum* Reuter 1963AL (EMCC Number: 1548, Designations: ATCC 15708, S3). These 3 strains are supplied as Actively growing cultures from the Microbiological Resources Centre. (Cairo Mircin). *Staphylococcus aureus* (ATCC 8095) was obtained from the Laboratory of Bacteriology, Animal Health Research Institute, Department of

Food Hygiene, Doki, Egypt. All cultures were stored at 2 to 5°C and sub cultured three times immediately before use in the experiments.

Activation and Enumeration of Bacterial strains

B. longum was activated and counted by pour plate technique on previously prepared (MRS) agar plates. The plates were then incubated at 37°C for 72 hours under anaerobic condition according to [27].

A sterile inoculating loop was used to pick up isolated colonies of the *S. thermophilus*, and *L. bulgaricus*, which were then inoculated in sterile nutritional broth and allowed to incubate overnight at 37°C. The colony-forming unit per milliliter for each of the two organisms was calculated after dilutions up to 10^{-10} and were plated on previously prepared (MRS) agar plates. The plates were then incubated at 41°C for 24 hours [28].

S. aureus strain was activated on nutrient broth then serially diluted and counted on Baird- Parker agar plates that incubated at 37°C for 48 -hour period [29].

Preparation of Citrus reticulata extract

The majority of Egyptian governorates have *citrus reticulate* gardens, particularly in Upper Egypt (Assiut) and the delta (Mansoura). *Citrus reticulate* fruits were gathered in Mansoura, Dakahlia, Egypt, during December 20, 2022 – February 20, 2023. A plant taxonomist verified its authenticity, and the National Research Center Herbarium has a specimen (C-16) on file. The following procedures were used to produce these extracts according to [30] : pulverizing one kilogram of fresh fruit peel in a blender, After soaking the blender's output in sterile distilled water, heating it to 40°C, filtering it through Whatman No. 1 filter paper, concentrating it on rotavapor (Buchi, USA), drying it in a lyophilizer (Labconco, USA) under low pressure, and repeating the extraction process with the fine sediment particles resoaking in sterile distilled water, and repeat multiple times until a clear supernatant is obtained. To condense the water extract, a rotary evaporator was employed, which was later dried using lyophilization to generate a dried residue. Following that, this extract will be kept in a refrigerator at or below 10°C in an airtight container.

Inoculation of cultures and preparation of yoghurt [31]

In this investigation, seven batches of 200 cc each of raw cow's milk were used. After 30 minutes of laboratory pasteurization at 64°C, each batch was chilled to 37°C. Following two consecutive transfers, the live cultures of *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* were further inoculated in dairy medium for 6-7 hours at 37 °C, resulting in an initial population of 10^7 CFU mL−1. Every batch of pasteurized milk received a culture of *Staphylococcal aureus* to provide a concentration of, 5 × 10^7 cells /ml, except the first batch, which was control -ve (No *S. aureus*). The sole bacteria in the second batch (control +ve) were *S. aureus.* While *B. longum* (ATCC 15708) was added in the third batch solely to yield a concentration of 63 × 10^7 cells per milliliter. *Citrus reticulata* was added at concentrations of 0.5% and 1% to the fourth and fifth batches, respectively, while *B. longum* (63 × 10^7 cells /ml) and citrus reticulata at concentrations of 0.5% and 1% were inoculated into the sixth and seventh batches. *Streptococcus thermophiles* strain and *Lactobacillus delbrueckii ss. Bulgaricus* were added to all batches and incubated in their sterile containers at 41°C until curds formed. Every yoghurt curd was maintained at 4°C and monitored for acidity percentage, and bacteriologically investigated for *S. aureus* and *B. longum* counts at zero time and again after 3, 5, 10, and 12 days of refrigeration.

Titratable acidity*:* determined by Thörner method titration [32].

Staphyloccus aureus **count***:* performed according to [29].

Bifidobacterium longum **count***:* by using technique described by [33].

Statistical Analysis

The information was gathered, tallied, and presented as means ± SE. Using the SPSS 20 Evaluation Version computer program (SPSS Inc., Chicago, IL), post-hoc tests (least significant difference, or "LSD") were performed after one-way analysis of variance (ANOVA) was used to compare statistical data between the groups. P<0.05 was chosen as the threshold for statistical significance.

Results and Discussion

Table 1 presents data on the percentage rise in acidity during the first (0) and last (12) days in a refrigerated store for yoghurt. The percentage increase was found to be 13.04% and 30.00% for yoghurt treated with *B. longum* and *Citrus reticulata* 1 and 0.5%, respectively. The yoghurt treated with *B. longum* showed a 26.09 % increase in acidity, but the yoghurt treated with 1 and 0.5% C*itrus reticulata* alone showed an increase in acidity of 40.00 percent and 29.17 percent, respectively. In reference to the control batches, the percentage rise in acidity at the 12-day storage period was 31.82% for the control positive batch and 31.58% for the control negative batch.

The outcomes were almost identical to those published by [34]. In previous study, strains of *Bifidobacterium bifidum* ATCC 11863 and *Lactobacillus acidophilus* ATCC 4356 were employed. to create both acid-adapted and non-adapted yoghurts were kept for 21 days at +4°C. The yoghurts' pH and titratable acidity levels were assessed, while it's being stored, also the probiotics' viability levels. Overall yoghurt collectives during storage, a drop in pH values and a rise in titratable acidity were noted. Yoghurt interventions prepared with regular starter were more acidic than equivalent interventions made with strains of Bifidobacteria. The reason for the decrease in titratable acidity observed with the increase in Bifidobacteria strains ATCC 15696, ATCC 29521, and *B. longum* BL-04 could be attributed to their combative impact on other bacteria or to their reduced generation of acid [35]. The titratable acidity of *Citrus reticulata* 1% fortified yoghurt was much greater than the control for the identical 12-day storage period. The outcomes concurred with an earlier study that found adding various amounts of orange marmalade to yoghurt raised its acidic flavor and lowered its pH [36]. Furthermore, for the same duration of storage (14 and 28 days), the titratable acidities of the Acidophilus- bifidus-thermophilus (ABT) symbiotic yoghurts fortified with citrus peels were considerably higher than those of the control [37].

Table 2 displays the percentages of *B. longum* count reduction after 12 days of cold storage for yoghurt. The reduction percentages were less than 97.2% and less than 99.6% for yoghurt treated with 1% and 0.5% of *Citrus reticulate* respectively with *B. longum*. Conversely, the yoghurt treated with *B. longum* showed a drop in *B. longum* count of less than 94.3%.

According to a related study [38], the essential oil reduced the quantity of the microorganisms under study. These findings demonstrated that, Gram-positive bacteria were more vulnerable to the antibacterial qualities of lemon essential oils than Gram negative bacteria. Two strains of *B. breve* were among the Gram-positive bacteria that were examined, and the citric essential oil inhibited these bacteria.

Citrus peels added to synbiotic yoghurt when keeping chilled also improved the probiotic starter's survivability, according to a different paper by [37]. As a result, this study offers important insights into the potentially beneficial uses of citrus peels, especially sour and sweat orange peels, as multipurpose meals additions in ABT-style symbiotic yoghurt. The *S. aureus* counts reduction percentages after 12 days of cold storage for treated yoghurt are displayed in Fig1. The reduction percentages were less than 96.8% and less than 96.2% for 1 and 0.5% C*itrus reticulata* with *B. longum* treated yoghurt respectively. While the percentage of *S. aureus* reduced in yoghurt handled with *B. longum* was less than 95.4%, the percentages of *S. aureus* count reduction in yoghurt handled with 0.5% and 1% *Citrus reticulata* were less than 56.4% and less than 94.8%, respectively. After the 12-day storage period, the control +ve batch's *S. aureus* increase percentage was greater than 96.4%. Our results showed that after 12 days of cold storage, there was a drop in the number of *S. aureus* in treated yoghurt batches except the control +ve batch. In yoghurt batches number five (containing 1% *Citrus reticulata*), number six (containing *B. longum* + 0.5% *Citrus reticulata*), and number seven (containing *B. longum* + 1% *Citrus reticulata*), there was a noticeable decrease in *S. aureus* populations. These outcomes could be attributed to the antibacterial compounds that bifidobacteria produced, which suppressed a significant percentage of Gram-positive and Gram-negative bacteria [35].

Many probiotic strains' antibacterial properties are crucial as natural preservation agents in a variety of milk products [39].

[37] observed that the restriction zone grew from 6.50 millimeters to 7.40 millimeters on day 0 to 7.00 to 8.20 mm on day 14, and 7.80 to 9.30 mm on 28th day using the ABT milk with sour orang (SO) peel addition as an example. Therefore, in contrast to the control group for the identical storage duration, fortifying the ABT synbiotic yoghurt with 0.5 percent of various peel powders greatly increased the antimicrobial efficacy versus *S. aureus*. The outcomes were in line with a prior study that demonstrated the potent antibacterial activity of citrus peel extract with high polyphenol levels [40]. The results showed that citrus peels' high phenolic component content contributed to their antioxidant action. Also, the findings of [41] demonstrated the antibacterial action of mandarin (*Citrus reticulata*)—against all pathogenic bacteria and fungi.

The essential oils of lemon, mandarin, and orange have been demonstrated to suppress the expansion of certain bacteria in the food industry, *Lactobacillus curvatus*, *L. sakei*, *Staphylococcus carnosus*, and *S. xylosus* [42]

Conclusion

Adding *Citrus reticulata* peels extract at 1% concentration to the milk used for manufacture of yoghurt lead to increase in acidity during 12-day cold storage period. The increase % of acidity in 1% *Citrus reticulata* peel extract supplemented yoghurt was (40.0%) higher than that of control –ve (31.58 %) and the batch that contain 1% *Citrus reticulate* with *B. longum* (13.04%). Additionally, antibacterial effect of 1% *Citrus reticulata* peel extract against *S.aureus* in treated yoghurt was significantly higher than the effect of 0.5 % *Citrus reticulata* peel extract. Moreover combination of *B.longum* with *Citrus reticulata* enhanced the antibacterial effect against *S.aureus*.

As a result, this study offers important insights into the potentially beneficial uses of citrus peels, in particular, *Citrus reticulata* peel extract *and B. longum* as functional food additions in manufacture of yoghurt, but further studies may be needed to assess the effect of different concentrations on organoleptic characters and on other milk borne pathogens.

Conflict of interest statement

The writers have disclosed that They don't hold any conflicts of interest related to the publication of this article.

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Impact *of Citrus reticulata and B. longum on acidity% throughout the experiment.*

TABLE 1. Acidity percentage changes of the treated yoghurt groups during refrigerated storage

Yoghurt batches	Acidity Percentage		
	Day zero	Day 12	Increase %
B. longum + *Citrus reticulata* 1%	1.15 %	1.30 %	13.04 %
B. longum + *Citrus reticulate* 0.5%	1.00 %	1.30 %	30.00 %
Citrus reticulate 1%	1.00 %	1.40 %	40.00 %
Citrus reticulate 0.5%	1.20 %	1.55 %	29.17 %
B. longum	1.15 %	1.45 %	26.09 %
Control +ve	1.10 %	1.45 %	31.82 %
Control -ve	0.95 %	1.25 %	31.58 %

Changes of *Citrus reticulata on B. longum* count throughout the experiment.

TABLE 2. Comparison of B. longum count between groups

	Zero to 5 days	10 to 12 days	Reduction %
Bifid + Cit. 1%	$57.7 \times 10^7 \pm 36.9 \times 10^7$ a	$1.6 \times 10^7 \pm 1.4 \times 10^7$ a	↓ 97.2%
Bifid + Cit. 0.5%	$44 \times 10^7 \pm 24.8 \times 10^7$ b	$0.17 \times 10^7 \pm 0.035 \times 10^7$ b	↓ 99.6%
Cit. 1%	0 c	0 c	-
Cit. 0.5%	0 c	0 c	-
Bifido.+staph	$28.33 \times 10^7 \pm 25.6 \times 10^7$d	$1.6 \times 10^7 \pm 0.2 \times 10^7$ d	↓ 94.3%
Control +	0 c	0 c	-
Control -	0 c	0 c	-

Values are expressed as means ± standard deviations
Means in the same column without a common letter (a, b, c, d) differ significantly ($P < 0.05$).

Impact of *Citrus reticulata* and *B. longum* on *S. aureus* count throughout the experiment.

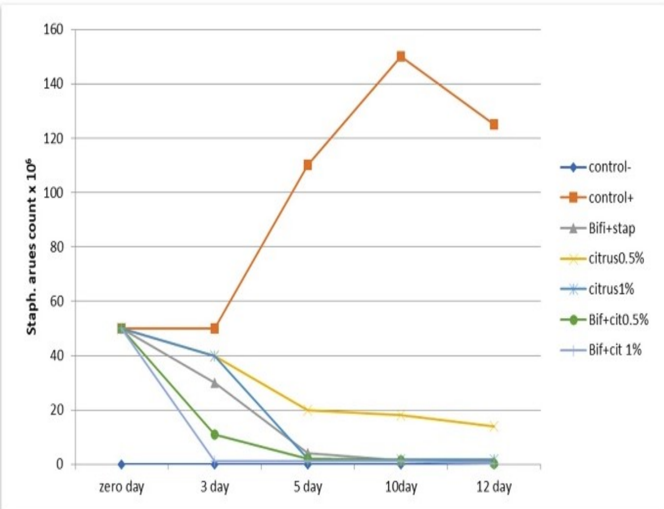

Fig. 1. Effect of *Citrus reticulata* and *B. longum* on *S. aureus* count throughout the experiment.

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تأثير مستخلص قشر اليوسفى و بكتيريا البيفيدو على المكورات العنقودية الذهبية أثناء التخزين البارد للزبادي الوظيفي

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المستخلص

تهدف هذه الدراسة إلى تقييم تأثيرمستخلص قشر اليوسفى بشكل منفرد او مع البيفيدوباكتيريوم كبروبيوتك على المكورات العنقودية الذهبية اثناء تصنيع الزبادي وحفظه باردًا في محاولة لتحسين سلامة الزبادي.حيث تم تحضير سبع دفعات من الزبادي من حليب البقر مع اضافة المكورات العقدية الحرارية واللاكتوباكيللوس البلغارية (1:1) بتركيز 10^7x1 خلية لكل مللى وتطعيمها بالمكورات العنقودية الذهبية تركيز5×10^7 خلية لكل مللى .الدفعة الأولى كانت عبارة عن مجموعة تحكم لا تحتوى على مستخلص قشر اليوسفى او المكورات العنقودية الذهبية او على البيفيدوباكتيريوم الدفعة الثانية تحتوي على المكورات العنقودية فقط بينما الدفعة الثالثة تحتوى على البيفيدوباكتيريوم فقط .تركيز 63×10^7 وحدة تشكيل المستوطنة الدفعتان التاليتان (الرابعة، الخامسة) على كل من قشراليوسفى بتركيزات 0.5 و 1٪.على التوالي وتحتوي الدفعات السادسة والسابعة المتبقية على.البيفيدوباكتيريوم وقشر اليوسفى بنسبة 0.5 و 1٪ على التوالي.

تم فحص دفعات الزبادي لنسبة الحموضة وبكتريولوجيا (لعدد المكورات العنقودية الذهبية والبيفيدوباكتيريوم) عنداليوم الاول وبعد 3، 5، 10 و12 يوم و بمجرد انتهاء مدة التخزين (12 يومًا) . كانت نسبة انخفاض عدد المكورات العنقودية الذهبية في الدفعتين السادسة والسابعة اللتان تحتويان على البيفيدوباكتيريوم ومستخلص قشر اليوسفى بنسبة 0.5% و 1% أقل من 96.2% و96.8% على التوالي ونسبة انخفاض ف الدفعتين الرابعة والخامسة على التوالي اللتان تحتويان على مستخلص قشر اليوسفى بتركيز0.5 و1% على التوالى أقل من 56% و 94.8%. ونسبة الانخفاض للدفعة الثالثة التي تحتوى على البيفيدوبكتيريوم اقل من 95.4% ونسبة الزيادة ف الدفعة الثانية التي تحتوى على المكورات العنقودية الذهبية فقط اعلى من 96.4%. لقد ألقت الدراسة الضوء على أهمية استخدام مستخلص قشر اليوسفى والبيفيدوباكتيريوم في تصنيع الأغذية الوظيفية كالزبادى . حيث أظهرت النتائج تأثير مثبط لمستخلص قشر اليوسفى بتركيز 1% سواء بشكل منفرد أو مع البيفيدواباكتيريوم على المكورات العنقودية الذهبية في الزبادى المصنع.