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Effects of Technological Treatments and Storage on Probiotics Inoculated into Biscuits (Cookies) Made from Millet (*Pennisetum glaucum* L. R. Br.) and Tiger Nuts (*Cyperus esculentus* L.)

Drissa Siri[1]*, Sami Eric Kam[1,2], Benjamin Kouliga Koama[1,3], Windmi Kagambega[1], Alain Hien[1,2,4], Clarisse Ouedraogo[1], Franck Téounviel Somda[1], Baperman Abdel-Aziz Siri[5], Roland Nâg-Tiéro Meda[1]

[1]Laboratoire de Recherche et d'Enseignement en Santé et Biotechnologies Animales, Université Nazi Boni, Bobo-Dioulasso, Burkina Faso
[2]Laboratoire de Recherche en Bactériologie, INSP/Centre MURAZ, Bobo-Dioulasso, Burkina Faso
[3]Institut de Recherche en Sciences de la Santé, Bobo-Dioulasso, Burkina Faso
[4]Institut Supérieur des Sciences de la Santé, Université Nazi Boni, Bobo-Dioulasso, Burkina Faso
[5]Ministère de la Santé, Direction Générale de la Santé Publique, Ouagadougou, Burkina Faso
Email: *idris_sir@yahoo.com

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Abstract

Background: The introduction of probiotics into food processing could give food products additional nutritional and functional properties. The objective of this study was to analyze the effects of technological treatments and storage on the vitality and viability of probiotics inoculated into biscuits made from millet and tiger nuts. **Methods:** Four types of biscuits were produced, depending on the heat treatment (37˚C or 40˚C) and the kind of sourdough used: Lactic Bacteria (LB) and Lactic Bacteria + Yeast (LB + S). The effects of the manufacturing processes on the fermentation and lactofermentation capacities (vitality) of the inoculated probiotics were evaluated by measuring the rate of pH decline. The viability of the strains was characterized according to ISO 21527, 2008 standard. **Results:** Technological treatments differently impacted the properties of probiotics inoculated into biscuits. The highest fermentation capacities were recorded with LBS biscuits treated at 40˚C and 37˚C, followed by LB biscuits treated at 37˚C and 40˚C. LB and LBS biscuits treated at 37˚C presented the best vitality. For viability, lactic bacteria in co-culture with yeasts (LBS) in biscuits showed a higher survival rate (32.50%) than that of lactic bacteria in monoculture (1.83%). The best lactofermentation capacities and vitality of probiotics were observed on Day + 1. **Conclusion:** This study could contribute to the development of adapted diagrams to increase the tolerance of probiotics to various stresses associated with technological processing and storage environment.

Keywords

Technological Treatments, Storage, Effects, Probiotics, Vitality, Viability

1. Introduction

Since ancient times, fermentation has been one of the most common food preservation methods [1] [2]. In addition to its preservative function, fermentation also contributes to improving the nutritional quality and bioactive properties of foods [3] [4]. These properties give fermented foods better qualities, not only for the dietary needs of humanity but also for preventing and treating infectious, metabolic, or chronic diseases [5]-[7].

Fermentation requires both a substrate rich in organic materials [8] and an appropriate physicochemical environment, such as humidity, temperature, or pH [9], as well as the presence of specific microorganisms [10]. Using starters, which involves introducing exogenous microbial communities, allows for the artificial triggering of fermentation, through a faster lowering of pH at the expense of undesirable microorganisms [11] [12]. Moreover, synergistic interactions between beneficial strains limit the growth or metabolic activities (such as toxin production) of certain pathogens [13]. However, the interactions between microbial communities can evolve differently during fermentation [9]. According to Han *et al.* [11], the cooperation between *Acetobacter pasteurianus* and *Lactobacillus helveticus*, mutualists at the beginning, becomes amensalism over time. Indeed, the acetic acid produced by *A. pasteurianus* exerts an inhibitory and lethal effect on *L. helveticus*. Thus, certain combinations of microorganisms can be ineffective or even negative for the desired virtues using fermentation [14] [15]. In addition, technological treatments and storage conditions (temperature, humidity, etc.) are factors that consequently influence the physicochemical and microbiological characteristics of fermented products [16].

Millet is a cereal of high nutritional value [17], mainly grown in Burkina Faso [18] [19]. The average annual production of millet was estimated at 926,900 tons over the period 2015-2024, representing 1/3 of cereal consumption per year in Burkina Faso [20] [21]. Nutsedge is a cyperaceous plant with a triangular stem 10 to 50 cm high, whose tubers (tiger nuts) are the consumed parts. 2080.82 tons of tiger nuts were produced in 2017 in Burkina Faso [22]. Millet and tiger nuts, due to their proximal composition and technological suitability, have a certain food and nutritional interest [19] [23]. Introducing lactic bacteria and yeasts in the millet and tiger nuts transformation process could confer additional nutritional and functional properties to foods based on these commodities. As far as we know, few studies conducted in our setting have assessed the feasibility of such a product as well as its properties after different processing technological treatments.

This study aims to investigate the effect of technological processing and storage conditions on probiotics inoculated in biscuits formulated from millet and tiger nuts.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

Millet and tiger nuts were purchased at the local market in the city of Bobo-Dioulasso, Burkina Faso.

2.1.2. Probiotics

Freeze-dried capsules containing lactic bacteria strains from the Trunature and Spring Valley brands were used. These strains consisted of:

- ✓ Bifidobacterium (*B. bifidum*, *B. breve*, *B. infantis*, *B. lactis*, *B. longum*);
- ✓ Lactobacillus (*L. acidophilus*, *L. casei*, *L. paracasei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius*).

The yeast powder (*Saccharomyces cerevisiae Boulardii*), in the form of ultra-pharmaceutical yeast, was also used.

2.1.3. Reagents and Consumables

The cow's milk was sterilized by Ultra High Temperature (UHT) treatment for the evaluation of bacterial vitality. Culture medium was purchased from Liofilchem for the isolation of microbial strains: Salted tryptone (0.009%, pH = 7.0 ± 0.2), Sabouraud agar (0.005% chloramphenicol and 4% glucose), and MRS agar.

2.1.4. Equipment

- ➢ RoHS dehydrator, model FDS-018, with an adjustable heating element from 0˚C to 100˚C and a fan that propels a laminar air flow. This design makes this model a potential source of thermal, osmotic, and oxidative stress.
- ➢ Fisher pH meter, model Scientific AE 150, is an automatic pH reader (resolution 0.01 - 0.1) and temperature reader (accuracy ±0.3˚C).
- ➢ SZYTF brand hygrometer, model FY-10, is equipped with an extendable cord probe that allows continuous reading (reading range = 10% - 99%, resolution = 0.1%, accuracy ±1%).

2.2. Methods

2.2.1. Preparation of Sourdoughs

The tiger nut milk produced according to the process described by Oyedele *et al.* [24] was used as the culture medium for the preparation of two kinds of sourdough. Sourdough 1 was prepared with Bifidobacteria and Lactobacillus (1.2×10^{11} LB CFU/g). Sourdough 2 was made with Bifidobacterium, Lactobacillus, and yeast (1.2×10^{9} LB CFU/g + 4.6×10^{5} S CFU/g). They were then stored at 4˚C until use.

2.2.2. Production of Biscuits

The millet and tiger nut flours obtained by adapting the processes described by Oyedele *et al.* [24] were rolled; then the resulting granules were steamed. Cookies enriched with sourdoughs or without (negative control) were produced according to five main steps recorded in Figure 1.

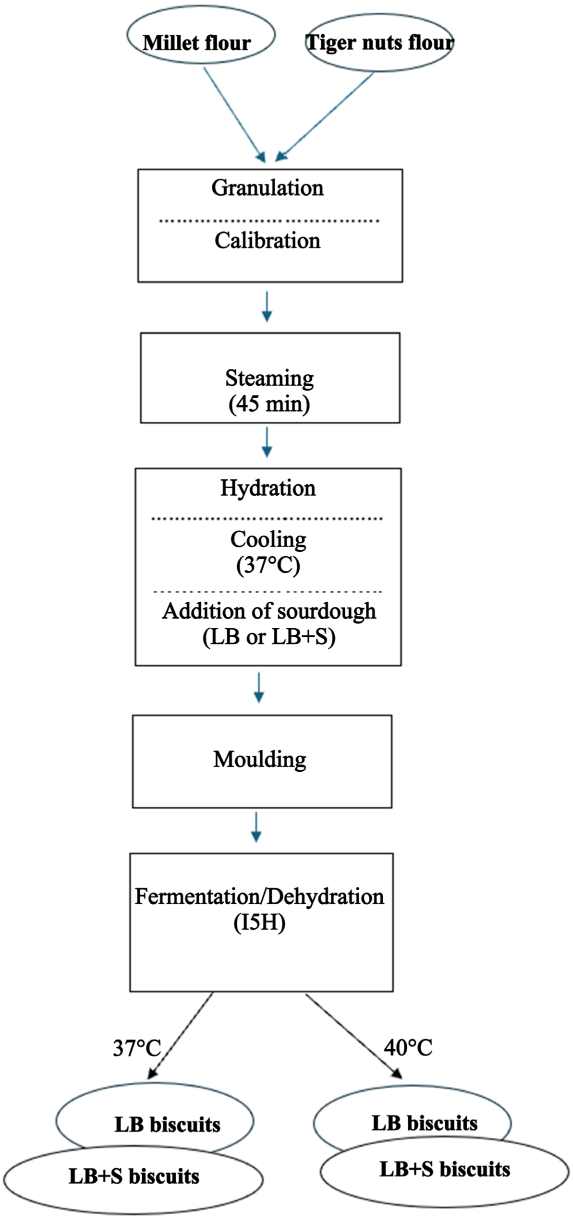


Figure 1. Flow diagram for biscuit production. LB = Sourdough prepared with lactic bacteria; LB + S = Sourdough prepared with lactic bacteria + *S. cerevisiae*.

2.2.3. Evaluation of the Effects of Technological Treatments on the Fermentation Capacity of Probiotics Inoculated into Biscuits during Processing

Fermentation allows a rapid decrease in pH. The effects of technological treatments on the fermentation capacity of probiotics during the process were evaluated through the measurement of pH during fermentation-dehydration (37˚C and 40˚C) according to the AOAC (Volume 1, 15th Edition, 1990) method. The pH was measured every three hours for 15 hours by randomly sampling 10 g from each batch of biscuits. The relative rates of pH decline (ΔpH) and relative humidity decline (ΔRH) were calculated using Equation (1) and Equation (2) below:

$$\Delta \mathrm{pH} = \frac{(\mathrm{pHtn} - \mathrm{pHtn} - 1)}{\mathrm{pHtn} - 1} \times 100 \tag{1}$$

$$\Delta \mathrm{RH} = \frac{(\mathrm{RHtn} - \mathrm{RHtn} - 1)}{\mathrm{RHtn} - 1} \times 100 \tag{2}$$

2.2.4. Evaluation of the Impacts of Technological Treatments and Storage on the Vitality of Probiotics in Dehydrated Biscuits

The post-process vitality of probiotics was evaluated by measuring their lactofermentative capacity within the produced biscuits. A sample of UHT milk (20 mL) was inoculated with biscuit powder (1 g). Another sterile sample of UHT milk (20 mL) without biscuit powder was used as a negative control. The inoculated milk samples were incubated at 37˚C; then the relative rates of pH reduction were monitored for 12 hours on D + 1 and at D + 90 (storage at 30˚C). ΔpH was calculated using Equation (1).

2.2.5. Evaluation of the Impacts of Technological Treatments and Storage on the Viability of Probiotics in Dehydrated Biscuits

The effects of technological treatments and storage on the survival rate of probiotics were evaluated through microbiological analyses on D + 1 and D + 90 (storage at 30˚C). A random sample of biscuits (10 g) was crushed in sterile physiological water using sterile gloves. A suspension (10 mL) of the crushed biscuits was then taken under sterile conditions, and a series of successive decimal dilutions was performed. The isolation was performed according to ISO 21527-1:2008 [24]. For bacteria, samples were carried out after inoculation of specific selective MRS agar medium, and incubation at 37˚C under CO_2 for 72 hours (bacteria). For yeasts, samples were carried out after inoculation of specific selective Sabouraud chloramphenicol agar, and incubation at 25˚C for 5 to 7 days (yeasts). The number of Colony-Forming Units (CFUs) per gram of product was determined using culture plates from two successive dilutions, at least one of which had a minimum of 15 colonies, using the formula below:

$$N = \frac{\sum C}{1.1 \times d \times V} \tag{3}$$

N: Number of CFU/g of the sample.

$\sum C$: Sum of colonies from the two successive plates selected.

d: First dilution selected.

V: Volume of inoculum (1 mL).

The survival rate of microorganisms was evaluated using the following formula:

$$\text{Survival rate} = \frac{\text{Number of CFU by g of biscuits}}{\text{Number of CFU by g of sourdough}} \times 100 \tag{4}$$

2.3. Statistical Analysis

Three shots were used for each measurement. Excel Office 365 and R Studio 3.14 software were used for data processing and analysis, as well as for designing graphs. Data are reported either as mean ± Standard Deviation (SD) or, when the distribution deviated from normality, as median with the corresponding Interquartile Range (IQR). The Shapiro-Wilk test was applied to verify distribution normality.

Comparisons of proportions between independent groups with small expected frequencies were performed using Fisher's exact test. For paired categorical data, McNemar's exact test was applied.

Median differences between two independent groups were assessed using the Mann-Whitney U test, while comparisons involving more than two groups relied on the Kruskal-Wallis test with suitable post hoc procedures when needed. The Wilcoxon signed-rank test was used to compare values in paired measurements. Statistical significance was established at a p-value threshold of <0.05.

3. Results

3.1. Effects of Technological Treatments on the Fermentative Capacity and Vitality of Probiotics

The study evaluated the impact of technological treatments on fermentation. Capacity and the vitality of probiotics were determined by measuring the relative rates of pH decrease (ΔpH), as a function of the variation in the relative rates of humidity lowering (ΔHR). Figure 2 shows the effects of technological treatments on the evolution of the pH of biscuits. Globally, three phases of pH evolution were observed during fermentation-dehydration. A first phase (T0 - T6) of rapid deceleration of the pH-lowering rates occurred concomitantly with an acceleration of

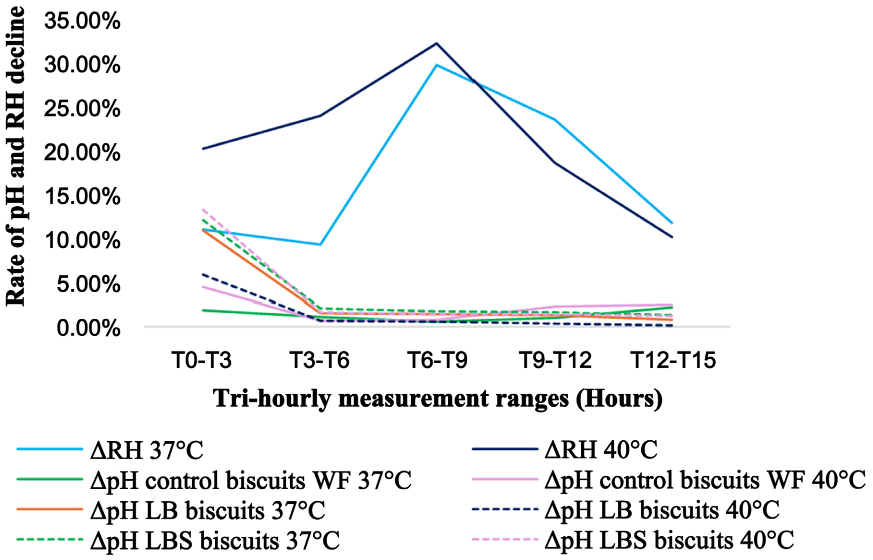
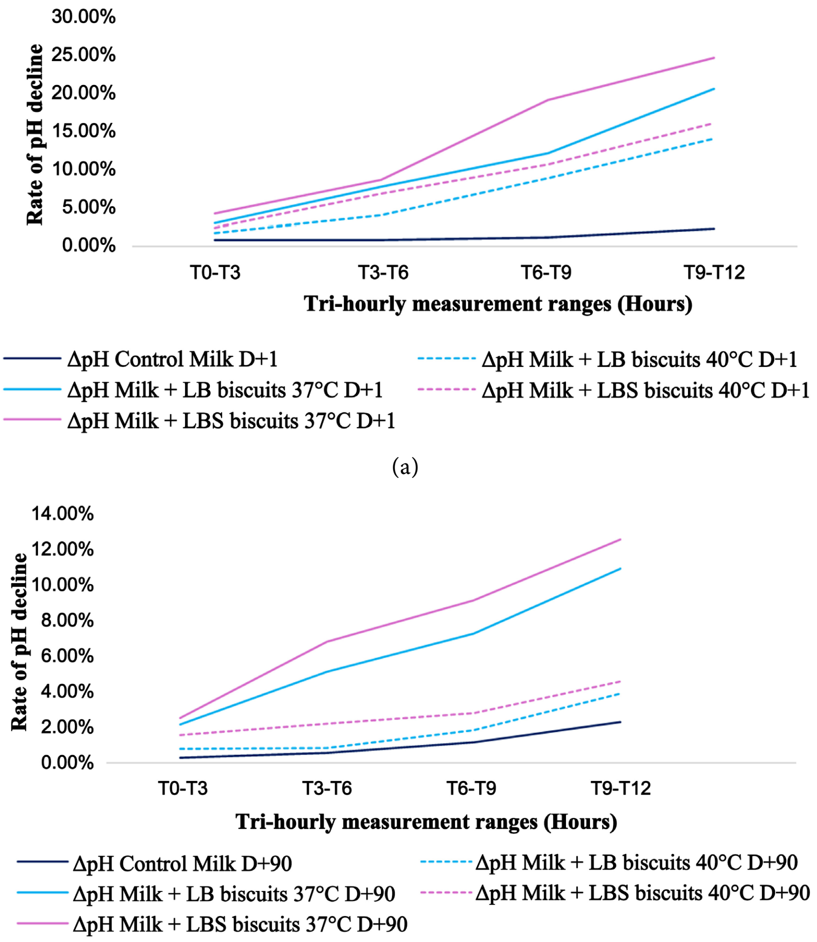


Figure 2. Evolution of the pH of biscuits during fermentation-dehydration. WF: Without ferment; ΔpH: pH variation; ΔHR: Humidity variation; LB: Lactic Bacteria; LBS: Lactic Bacteria + *S. cerevisiae*; T: Time in hours.

the relative humidity lowering rates. The highest pH reduction rates were observed successively with LBS biscuits at 40˚C and 37˚C; followed by LB biscuits at 37˚C and 40˚C. The lowest rates were recorded with the control biscuits. The second phase (T6 - T9) of the evolution of ΔpH was characterized by a stabilization of pH and a peak in the reduction of humidity. The third phase (T9 - T15) of evolution was marked by a slight increase in pH reduction rates and a progressive regression in the lowering of hygrometry.

3.2. Impact of Technological Treatments and Storage on the Vitality of Probiotics

The determination of lactofermentative (UHT milk) capacities of bacteria allowed the evaluation of the vitality of probiotics. Three-hourly pH monitoring showed a continuous increase in pH reduction rates on Day + 1 and Day + 90 (**Figure 3(a)** and **Figure 3(b)**). The highest reduction rates were observed successively with biscuits treated at 37˚C (with LB and LBS) and at 40˚C (with LB and LBS). The best pH reduction rates were noted at Day + 1.



(a)

(b)

Figure 3. Lactofermentative capacities of biscuits: (a) Day + 1; (b) Day + 90. ΔpH: pH variation; ΔHR: Humidity variation; LB: Lactic Bacteria; LBS: Lactic Bacteria + *S. cerevisiae*.

3.3. Impact of Technological Treatments and Storage on the Viability of Probiotics

Table 1 describes the survival rates of probiotics inoculated into biscuits after technological processing and storage. The best survival rates were obtained with processing at 37˚C on Day + 1 and during storage (Day + 90). The highest survival rates were observed at Day + 1 with yeast multiplication (313.04%). Lactic bacteria in co-culture with yeast had a higher survival rate (32.50%) than lactic bacteria in monoculture (1.83%). A significant reduction in survival rates was recorded during storage.

Table 1. Survival rates of probiotics inoculated into biscuits dehydrated at 37˚C and at 40˚C

	Survival rate at D + 1 (%)		Survival rate at D + 90 (%)		p-value
	37˚C	40˚C	37˚C	40˚C	
Lactic acid bacteria in monoculture	1.83	0.20	-	-	
Lactic acid bacteria (in co-culture with yeast)	32.50	20.00	0.01	0.001	0.03
Yeast	313.04	2.61	89.13	0.92	<0.001

D + 1 = 1 day after biscuit production; D + 90 = 90 days after biscuit production.

4. Discussion

The decrease in pH is an expression of the enzymatic fermentation activity of microorganisms, which contributes to the formation of metabolites such as organic acids [8] [25]. Thus, changes in pH could express vitality through the fermentation capacity of probiotics, subjected to the combined effects of heat (37˚C and 40˚C), dehydration, and storage.

The fermentative capacity and the vitality of probiotics subjected to the effects of technological treatments were evaluated by measuring the pH during the fermentation-dehydration (**Figure 2**). In the first six hours of fermentation-dehydration, LBS biscuits dehydrated at 40˚C showed the fastest rate of pH decrease. This could be explained by the stimulating effects of temperature (40˚C) on enzyme activity and the synergistic action between bacteria and yeasts. The second phase (T6 - T12) of fermentation-dehydration saw an acceleration in the decrease in relative humidity, which caused a stabilization of pH variation. Similar results have also been reported in previous studies [26]. A rapid dehydration is a hyperosmotic stress factor that can lead to the disorganization of microorganisms' cell membranes [27] [28]. The dehydrator is a source of a stream of air heated by an electric resistance. This air stream could also be a cause of oxidative stress for probiotics [29] [30]. Hyperosmotic and oxidative stress are inhibiting factors of the vitality and viability of probiotics in finished products [9]. The slight increase in pH reduction rates recorded in the third phase (T9 - T15) of fermentation-dehydration could be linked to the adaptability of microbial communities to prolonged periods of stress [31] [32].

The pH reduction rates described in **Figure 3** indicate that the best lactofermentative capacities of probiotics inoculated in cookies were noted at D + 1 and 37˚C with LB biscuits, followed by LBS biscuits. Low vitality of microorganisms was noticed with heat treatment at 40˚C and storage.

For the viability monitoring of probiotics, their survival rates after technological treatments were calculated. **Table 1** indicates that the best survival rates were observed at 37˚C, on Day + 1 with lactic bacteria in co-culture with yeasts. In co-culture with yeast, lactic bacteria tolerate thermal stress, and the effects of storage are better than those of lactic bacteria in monoculture. This tolerance could be linked to mechanisms such as the sporulation capacity or metabolic reprogramming of microorganisms [33]. However, these mechanisms may become exhausted or ineffective if heat cycles are prolonged or repeated several times [34].

Our results agree with the results reported by [34]-[37], but also by [38] [39]. Our results are not in agreement with the results reported by [40] [41].

The environmental conditions of a fermentation and storage process are of paramount importance for the growth and maintenance of probiotics [23] [42]-[44]. Yeast-mediated processes are associated with biological (safe) and sustainable food security low-cost strategies to improve productivity, prevent and control plant attacks, and grain spoilage [45] [46]. Yeast-mediated processes are also used for modifying food's physicochemical characteristics and enhancing sensorial and functional properties [47]-[49]. Other fields of yeast-mediated applications concern pharmacology, medicine, bioengineering, and environmental protection [50]-[53].

The use of substrates enriched with osmoprotectants, successive pre-treatments for acclimatization, and selection could increase the tolerance of probiotics to stress [54]-[57].

5. Conclusions and Future Challenges

This study aimed to determine the effects of technological treatments and storage on the vitality and viability of probiotics inoculated into biscuits made from millet and tiger nuts. Four types of biscuits were produced according to the heat treatment (37˚C and 40˚C) and the type of sourdough used (LB and LBS). The technological treatments had different impacts on the vitality and viability of the probiotics inoculated into the biscuits. The best fermentation capacity and vitality of the probiotics after treatment were obtained, respectively, with LBS biscuits dehydrated at 40˚C and LB biscuits treated at 37˚C after storage on Day + 1. The LBS-enriched biscuits dehydrated at 37˚C showed the highest survival rate on Day + 1.

One of the limitations of our study is that it did not test the gastrointestinal survivability of the inoculated probiotics.

Optimizing the process by using successive inoculated probiotics as starters could create adaptive conditions and help increase probiotics' tolerance to various stresses associated with technological treatments used in this formulation.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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