European Journal of Nutrition & Food Safety

Volume 16, Issue 7, Page 304-312, 2024; Article no.EJNFS.118795
ISSN: 2347-5641

Effect of Storage on Probiotic Viability, Physicochemical and Sensory Properties of Probiotic-enriched Orange Juice

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Authors' contributions

This work was carried out in collaboration among all authors. Author BOO conceived and supervised the work, author ELO performed the methodology, author TEO performed the data curation and wrote the original manuscript, authors ELO and TEO performed the data analysis and initial report writing, authors ELO and BOO performed the Reviewing and author TOA performed the Editing while all Authors gave the Resources read and approved the final manuscript. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ejnfs/2024/v16i71480

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/118795

Original Research Article

Received: 21/04/2024
Accepted: 25/06/2024
Published: 03/07/2024

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Cite as: *Orike, Emmanuel Lucky, Temidayo Emmanuel Olajugbagbe, Bridget Okiemute Omafuvbe, and Titilola Oyenike Animasahun. 2024. "Effect of Storage on Probiotic Viability, Physicochemical and Sensory Properties of Probiotic-Enriched Orange Juice". European Journal of Nutrition & Food Safety 16 (7):304-12. https://doi.org/10.9734/ejnfs/2024/v16i71480.*

ABSTRACT

The effect of enrichment of orange juice with probiotic *Lactobacillus plantarum* and *Lactobacillus bulgaricus* as single and mixed cultures on the cell viability, physicochemical parameters and sensory properties of the juice during a 20day refrigerated (4ºC) and room temperature (30± 2ºC) conditions was studied. The viable cell count remained relatively stable at 4ºC over the storage period for both strains but was at its peak after 10 days of storage at 30±2ºC with *L. bulgaricus* accounting for the highest count (9.36±0.04 CFU/mL). The acidity of the enriched juice increased as the storage period progressed at 30ºC with the highest acidity observed with *L. bulgaricus* (pH 3.00±0.02, titratable acidity 16.27±0.05mg lactic acid/mL). Sensory evaluation indicated that the juice enriched with *L. bulgaricus* was more acceptable. The study concluded that orange juice enriched with *L. bulgaricus* is suitable in the development of non-dairy based functional food and could act as an ideal food matrix for probiotic beverage.

Keywords: Probiotic; fruit; storage; functional foods; Lactobacillus plantarum; Lactobacillus bulgaricus; human health.

1. INTRODUCTION

Fruits and vegetables are important in the maintenance of good health. They are good sources of carbohydrates, vitamins, minerals, fiber, and numerous bioactive compounds and their regular intake is reported to reduce the risk of chronic diseases in human [1,2]. The World Health Organization (WHO) recently promoted the inclusion of at least 400g of fruit and vegetables per day for the prevention of cancer, diabetes, obesity and heart disease [3,4]. Modern food technology has introduced the act of transferring the valuable fruit components into juices containing all the essential physical, chemical, organoleptic and nutritional characteristics which are found in the original healthy and ripe fruit [5]. It is believed that with the presence of these nutrients and absence of competing starter cultures, fruit juices would be an ideal medium for the delivery of probiotics [6].

Probiotics are regarded as live microorganisms that, when administered in minimum concentration of 10^6 CFU/ml or gram, confer a health benefit on the host [7,8]. Traditionally, dairy products including fermented and unfermented milk and cheese have been found to be an ideal carrier for delivering probiotics to the human gastrointestinal tract. However, to increase consumers' consciousness in probiotic functionalities and overcoming the challenges posed by lactose intolerance, milk allergies, vegetarianism as well as dyslipidemia, it is important to improve the functional product vehicles through other non-dairy products such as fruit juices [9].

The cultures of microorganisms mostly used for producing probiotic foods are lactic acid bacteria (LAB) such as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbrueckii* subsp. *bulgaricusi, Lactobacillus plantarum, Bifidobacterium bifidum, Bifidobacterium infantile and Streptococcus* spp [10]. Probiotic strains mainly of the species of *Lactobacillus plantarum* and *Lactobacillus bulgaricus* have been used extensively in the development of many probiotic fruit and vegetable juices and their suitability as carrier for probiotic bacteria as well as the sensory acceptability by the consumer has been reported [11,12]. It has been reported that *Lactobacillus plantarum* and *Lactobacillus bulgaricus* have potential to modify the phenolic composition fruit juice and enhance its overall antioxidant capacity [13,14]. This is done by incorporating the probiotic strain(s) directly into the acidic and other unfavorable processing conditions of the juice.

Orange is the fruit of the citrus species *Citrus sinensis*. It contains high concentration of vitamin C, flavonoids and a good source of hesperidin. Orange juice is highly acidic due to its citric acid content [15]. Probiotic strains involved in the fruit juice probiotication process therefore must remain viable through the harsh conditions via a stable and favourable storage conditions over a reasonable period of time without jeopardizing the organoleptic properties of the juice itself.

The aim of this study was to investigate the suitability of orange juice as a possible non-dairy based probiotic carrier through the monitoring of the viability of probiotic strains of *L. plantarum* and *L. bulgaricus* (isolated from fermenting cassava), the pH, titratable acidity and the organoleptic evaluation of the probiotic orange juice stored at room and refrigerated temperatures.

2. MATERIALS AND METHODS

Probiotic strains (*Lactobacillus plantarum* (A_2) and *Lactobacillus bulgaricus* (C_2) used in this study were previously isolated from fermenting cassava, characterized and screened for probiotic potentials [16].

Preparation of pasteurized orange juice: The ripeness index that was considered during the selection of the fruits are: skin colour, aroma and texture or firmness.

Fresh, sizable, mature, ripened and yellow-oranges were purchased from the fruit market in Ile-Ife, Nigeria, washed and squeezed by pressing the pulp through a juice extractor to extract the juice. The extracted orange juice was filtered through a sieve and the filtered orange juice was dispensed in 40 mL portion into several sterile 100 mL capacity conical flasks, covered with foil paper and then pasteurized for 30 seconds in a water bath set at 95 ^oC [17].

Inoculum preparation: The cell suspension of each of the two probiotic organisms (*L. plantarum* and *L. bulgaricus*) was prepared by adding 10 mL of sterile normal saline to 18-24 h old De Man Rogosa and Sharpe (MRS) agar slant cultures of the test strain in McCartney bottle and shaken to wash the cells. The suspension was centrifuged at 8000 rpm for 10 min and pellets were washed with sterile normal saline. The cells were re-suspended in sterile normal saline and standardized to contain 1x 10^9 CFU /mL using a spectrophotometer [18].

Inoculation of pasteurized orange juice: The pasteurized orange juice (40 mL) in conical flask was aseptically inoculated with 1% (v/v) of the standardized cell suspension of the selected probiotic lactic acid bacteria as single and mixed culture (1:1). The freshly produced probiotic orange juice was divided into two batches. One batch was stored at 4 ^oC in the refrigerator and the other half at room temperature (30^oC ± 2^oC) for a period of 20 days. The cold storage (4 ^oC) temperature was stabilized and maintained by placing a thermometer in the refrigerator powered by two different power sources; government and power generating plants in order to avoid any power fluctuation. During the storage period, samples were withdrawn from the triplicate flasks for analysis at 5 days intervals starting from time zero.

Evaluation of changes in the viable counts of lactic acid bacteria in probiotic orange juice: The viable count of lactic acid bacteria (LAB) in the probiotic orange juice was determined following standard plate count method with De Man Rogosa and Sharpe (MRS) agar. An aliquot (1.0 mL) of the probiotic orange juice sample was serially diluted in sterile maximum recovery diluent (MRD) up to 10^{-5} and 0.1 mL of the appropriately diluted sample was spread-plated on De Man Rogosa and Sharpe (MRS) agar and incubated at 35 ^oC for 24-48 h anaerobically. After incubation, the plates were observed and colonies were enumerated and expressed as log CFU / mL probiotic orange juice.

Determination of changes in pH and titratable acidity of probiotic orange juice: The pH of the probiotic orange juice sample was measured using an electronic digital pH meter (HANNA INSTRUMENT 8021). The juice sample (10 mL) was poured into a clean 100 mL capacity beaker and the calibrated pH electrode was dipped into it and read electronically [19].

Total titratable acidity (TTA) of the probiotic orange juice was determined using titration method with phenolphthalein as end point indicator [19]. Exactly 10 mL of the sample (probiotic orange juice) was diluted with equal volume of distilled water and titrated against 0.1N NaOH solution with two drops of phenolphthalein (1% w/v) indicator to give a faint pink colour end point of pH 8.3 (monitored with a pH meter). Each mL of 0.1 NaOH is equivalent to 90.08 mg.

Titratable Acidity = Volume (mL) of NaOH x Normality (N) of NaOH x Lactic acid Equivalent / volume of sample used.

Organoleptic analysis of the probiotic orange juice: The organoleptic property of the probiotic orange juice stored at refrigeration temperature for 20 days was assessed by a trained panel of 10 regular consumers of orange juice. The probiotic orange juice samples were evaluated for colour, taste, aroma, appearance and general acceptability. Uninoculated orange juice served as control. The parameters were scored using a 5-point Hedonic scale of dislike extremely (1), dislike (2), neither dislike nor like (3), like (4) and like extremely (5) [20].

Statistical analysis: The data obtained in this study were subjected to one-way analysis of variance followed by Student -Newman - Keuls post hoc test (Primer for Biostatistics software package version 3.01) for difference between means [21]. Statistical significance was accepted at *P* value equal to or less than 0.05.

3. RESULTS AND DISCUSSION

3.1 Changes in the Viable Counts of LAB in Probiotic Orange Juice During Storage

There was no significant viable cell count in the control set-up as shown in the Table 1, since there was no inoculation.

The viable counts (Log CFU / mL) of LAB in probiotic orange juice inoculated with single and mixed cultures of *L. plantarum* (A2) and *L. bulgaricus* (C2) during storage at 4°C and room temperature (30°C ± 2°C) for 20 days is presented in Table 1. The probiotication involving *L. plantarum* (A2) and *L. bulgaricus* (C2) applied as single culture showed no significant difference ($P \leq 0.05$) in the viable cell counts of LAB during storage at 4°C. There was a slight decrease in the viable count of probiotic LAB strains from 8.16 ± 0.03 to 7.51 ± 0.04 and from 8.23 ± 0.02 to 7.81 ± 0.03 for *L. plantarum* and *L. bulgaricus* respectively after 15 days of storage. Also, *L. plantarum* and *L. bulgaricus* as mixed cultures maintained a continuous viability ranging between 8.05 ± 0.01 and 8.50 ± 0.03 till the 15th day followed by a slight drop in viable count to 7.54 ± 0.03 at the end of the storage period. This viability could be attributed to the inactive state and the behaviour of the probiotic strains added to the juice. This corresponds with the report of Boudjou et al. (2014). Similarly, a stable pH of the juice at this temperature condition could contribute to the abundance of probiotic strains exceeding the minimum bacteria populations required for probiotic foods to possess health claims as fermentation was limited by keeping the samples in refrigerator. This conforms with the report that refrigeration could promise a more prolonged survival of probiotics while thermal abuse could be detrimental to the viability of probiotics in orange and vegetable juices [9]. It is imperative from the health point of view that probiotic strains selected for probiotication retain their viability and functional activity throughout the shelf-life of the delivery product; an attribute which is strain dependent [22]. Probiotics need to maintain a minimum therapeutic level of 10^6 - 10^7 CFU/mL in food product in order to confer health benefit [23]. On the other hand, at room temperature (30°C ± 2°C), both *L. plantarum* and *L. bulgaricus* maintained a steady growth till the 5th day. The strains gained a significant increase in viable counts at the 10th day of storage after which a significant decrease was observed till the end of the storage period. The viability of *L. plantarum* and *L. bulgaricus* as mixed cultures showed a steady growth till the 10th day followed by a sharp decrease from 9.09 ± 0.03 to 7.53 ± 0.02 at the 15th day and further decreased to 6.91±0.03 at the end of the storage period. The steady cell count of probiotic strains suggests that orange juice favors a synergistic relationship and enables beneficial bacteria to thrive as mixed probiotic cultures during an extended storage period. This has been effective in the treatment of several gastrointestinal disorders (Ouwehand et al., 2013). However, the drop in viable cell count of strains from 10^9 to 10^6 CFU / mL (although still within the healthy limit) after the storage period signals the inability of the probiotic strains to tolerate high acidic condition over an extended storage period (above 15 days). This agrees with the report of Ghafari and Ansari [6].

3.2 Changes in pH and Titratable Acidity of Probiotic Orange Juice During Storage

The pH changes in orange juice inoculated with single and mixed cultures of probiotic *Lactobacillus* species are shown in Table 2. Probiotication of orange juice involving *L. plantarum* and *L. bulgaricus* as single culture and in combination showed no significant change in pH throughout the course of storage at 4°C due to low rate of fermentation and production of organic acids. However, at room temperature storage, a sharp increase in acidity was observed for the single and mixed culture probiotic orange juices. The probiotic orange juice inoculated with *L. plantarum* showed changes in pH from an initial value of 3.80 to 3.10 at the end of the room temperature storage. The probiotication involving *L. bulgaricus* and a mixture of both lactic acid bacteria also showed the same pattern in pH changes from an initial pH of 3.8 to 3.0 at the 20th day of storage. It is believed that fermentation was rapid at room temperature compared to the refrigeration condition thus increasing the acidity of the orange juice with time due to the accumulation of organic acid from the fermentation of fermentable polysaccharides by the probiotic strains. Similar result was reported by Gallina et al. [24] in the development and characterization of probiotic fermented smoothie beverage. The viability of probiotics and increase in acidity is of great importance to the quality of the juice as it minimizes the influence of spoilage bacteria stimulate protein digestion and enhance the sensory properties of the juice [25].

Table 1. Changes in viable counts of LAB in probiotic orange juice during storage

Storage Days	4 °C Storage			Room Temperature (30 ± 2 °c) Storage		
	L. plantarum	*L. bulgaricus*	*L. plantarum+ L.bulgaricus*	*L. plantarum*	*L. bulgaricus*	*L. plantarum+ L. bulgaricus*
0	8.00 ± 0.01[a]	8.00 ± 0.01[a]	8.05 ± 0.01[a] 8.00	8.04 ± 0.01[ab]	8.00 ± 0.01[ac]	8.07 ± 0.01[a]
5	8.03 ± 0.02[a]	8.19 ± 0.01[a]	8.25 ± 0.01[a] 8.01	8.43 ± 0.02[b]	8.68 ± 0.03[ab]	8.59 ± 0.01[ab]
10	8.16 ± 0.03[a]	8.23 ± 0.02[a]	8.50 ± 0.03[a] 8.00	8.74 ± 0.03[b]	9.36 ± 0.04[b]	9.09 ± 0.03[b]
15	7.51 ± 0.04[a]	7.81 ± 0.03[a]	8.02 ± 0.01[a] 8.01	7.03 ± 0.01[ac]	8.05 ± 0.02[ac]	7.53 ± 0.02[ac]
20	7.30 ± 0.02[a]	7.22 ± 0.03[a]	7.54 ± 0.03[a] 8.01	6.53 ± 0.03[c]	7.61 ± 0.03[c]	6.91 ± 0.03[c]

Viable counts expressed in Log CFU / mL
Each value is the mean ± standard deviation.
Mean values within columns with different superscripts are significantly different (p<0.05)

Table 2. Changes in the pH of orange juice inoculated with probiotic *Lactobacillus* species during storage

Storage days	4° C Storage			Room Temperature Storage		
	L. plantarum	*L. bulgaricus*	*l. Plantarum + L. bulgaricus*	*L.plantarum*	*L. bulgaricus*	*L. plantarum + L. bulgaricus*
0	3.80 ± 0.01[a]	3.80 ± 0.01[a]	3.80 ± 0.01[a]	3.80 ± 0.01[a]	3.80 ± 0.01[a]	3.80 ± 0.01[a]
5	4.00 ± 0.02[a]	3.90 ± 0.01[a]	4.00 ± 0.01[a]	3.30 ± 0.01[b]	3.35 ± 0.03[b]	3.30 ± 0.01[b]
10	4.00 ± 0.01[a]	4.00 ± 0.03[a]	4.10 ± 0.01[a]	3.25 ± 0.02[b]	3.15 ± 0.03[c]	3.20 ± 0.02[bc]
15	4.10 ± 0.03[a]	4.10 ± 0.01[a]	4.00 ± 0.01[a]	3.20 ± 0.01[bc]	3.10 ± 0.01[c]	3.10 ± 0.01[c]
20	3.90 ± 0.01[a]	4.00 ± 0.00[a]	4.00 ± 0.02[a]	3.10 ± 0.01[c]	3.00 ± 0.02[c]	3.00 ± 0.02[c]

Values are means ± standard deviation.
Mean values with different superscript within columns are significantly different (P ≤ 0.05).

Table 3. Changes in total titratable acidity of orange juice inoculated with probiotic *Lactobacillus* species during storage

	4º C Storage			Room Temperature Storage		
Storage Days	***L. plantarum***	***L.bulgaricus***	***L. plantarum + L. bulgaricus***	***L.plantarum***	***L.bulgaricus***	***L. plantarum + L. bulgaricus***
0	6.52 ± 0.02[a]	6.46 ± 0.01[a]	6.51 ± 0.01[a]	6.52 ± 0.02[a]	6.46 ± 0.01[a]	6.52 ± 0.01[a]
5	6.56 ± 0.01[a]	6.50 ± 0.01[a]	6.44 ± 0.14[a]	13.15 ± 0.00[b]	13.11 ± 0.05[b]	13.11 ± 0.05[b]
10	6.47 ± 0.23[a]	6.53 ± 0.13[a]	6.42 ± 0.19[a]	15.31 ± 0.01[bc]	15.22 ± 0.09[c]	15.09 ± 0.14[c]
15	6.41 ± 0.31[a]	6.42 ± 0.00[a]	6.42 ± 0.00[a]	16.38 ± 0.15[c]	15.99 ± 0.05[cd]	15.94 ± 0.09[cd]
20	6.46 ± 0.05[a]	6.49 ± 0.00[a]	6.49 ± 0.00[a]	16.21 ± 0.00[c]	16.27 ± 0.05[d]	16.24 ± 0.05[d]

Titratable acidity expressed as mg lactic acid / mL. Values are means ± standard deviation.
Mean values within columns with different superscripts are significantly different (P ≤ 0.05)

Table 4. Sensory attributes of probiotic orange juice

		Organoleptic	Attributes		
Sample Code	**Colour**	**Taste**	**Aroma**	**Appearance**	**General Acceptability**
A	4.40 ± 0.52[a]	3.70 ± 0.48[b]	3.70 ± 0.82[a]	4.00 ± 0.47[a]	4.00 ± 0.67[b]
B	4.20 ± 0.42[a]	3.90 ± 0.74[a]	3.60 ± 0.70[a]	4.00 ± 0.00[a]	4.20 ± 0.63[a]
C	4.40 ± 0.52[a]	3.60 ± 0.70[b]	4.10 ± 0.74[a]	4.20 ± 0.42[a]	4.20 ± 0.42[a]
D	4.50 ± 0.53[a]	4.10 ± 0.57[a]	3.90 ± 0.74[a]	4.00 ± 0.47[a]	4.20 ± 0.63[a]

Values are mean scores ± standard deviation (n= 10).
Mean values in the same column with different superscripts are significantly different (P ≤ 0.05)
Key to sample code: Sample A- Orange juice inoculated with Lactobacillus plantarum (A_2)
Sample B - Orange juice inoculated with Lactobacillus bulgaricus (C_2)
Sample C - Orange juice inoculated with mixed cultures of L. plantarum (A_2) and L. bulgaricus (C_2)
Sample D - Uninoculated orange juice (control)

The total titratable acidity (expressed as mg lactic acid/mL) of probiotic orange juice involving *L. plantarum* and *L. bulgaricus* as single and mixed culture showed a slight reduction initially from 6.52 at day zero to 6.41, 6.46 to 6.42, and 6.51 to 6.42 respectively at the 15th day of cold storage period (Table 3). The TTA later increased to 6.46, 6.49 and 6.49 respectively at the end of storage at 4 ^oC. On the other hand, a sharp increase in TTA was observed throughout the room temperature storage of the probiotic orange juice. The *L. plantarum* and *L. bulgaricus* as single and mixed culture produced significantly more TTA (from between 6.46 and 6.52 mg lactic acid / mL at the onset to between 16.21 and 16.27 mg lactic acid/mL at the 20th day).

The titratable acidity of probiotic orange juice produced by *L. plantarum* and *L. bulgaricus* at the cold storage (4^oC) period can be linked to the inability of the probiotic strains to metabolize or produce organic acids because fermentation was not possible at refrigeration storage. On the contrary, probiotication involving *L. plantarum* and *L. bulgaricus* as single and mixed cultures at room temperature can be linked to the decrease in pH of the probiotic orange juice at room temperature. As the inoculated LAB strains ferment the carbohydrate content of the orange juice, lactic acid is produced which reduces the pH making the juice more acidic and a corresponding increase in the TTA. This result corresponds with the report of Shukla and Kushwaha [15].

3.3 Organoleptic Properties of the Probiotic Orange Juice

The probiotic orange juice produced by *L. bulgaricus* as a single culture (sample B) and in combination with *L. plantarum* (sample C) showed no significant difference ($P \le 0.05$) in colour, taste, aroma, appearance and general acceptability and were most preferred to the other probiotic orange juice involving *L. plantarum* as a single culture (sample A) (Table 4). This is an indication that *L. bulgaricus* plays a vital role in contributing to the development of these sensory attributes. Sample A (orange juice inoculated with *L. plantarum*) was rated low for taste and general acceptability and was significantly different from the other probiotic orange drinks. It is worthy of note that the probiotic orange drink inoculated with *L. bulgaricus* and the mixed culture of the two lactic acid bacteria were not significantly different from the uninoculated pasteurized orange juice in all the organoleptic attributes scored by the taste panel. The type of microorganism, the juice, storage conditions and the addition of other compounds may influence the sensory properties of the finished product [26]. The results showed that Sample B (inoculated with *L. bulgaricus*) and sample C (inoculated with mixture of *L. plantarum* and *L. bulgaricus*) were more preferred and acceptable by consumer than the sample involving *L. plantarum* as a single culture (sample A). This is an indication that *L. bulgaricus* may have played a vital role in the development of these sensory attributes. Similar result was reported by Maldonado et al. [27] in the potential application of four types of tropical fruits in lactic fermentation.

4. CONCLUSION

The combination of *L. plantarum* and *L. bulgaricus* as probiotics in orange juice is suitable in the development of functional foods. The combination of the LAB strains presented a favourable synergy from their metabolism without jeopardizing the integrity of the juice. Orange juice enriched with *L. plantarum* and *L. bulgaricus* singly and in combination may provide a new asset in the production of healthy functional drink which may solve the problem associated with probiotic dairy products especially for lactose intolerant individuals.

5. RECOMMENDATION

Results from the study has shown that orange juice enriched with *L. plantarum* and *L. bulgaricus* singly and in combination may provide a new asset in the production of healthy functional drink which may solve the problem associated with probiotic dairy products especially for lactose intolerant individuals. However, further research may be carried out to explore other non-dairy food such as legume-base milk as possible carrier for probiotic delivery to human.

6. LIMITATION

A challenge in probiotic fortification of juices is product acceptance by consumers, getting consent from participants could be tasking too. Refrigerated temperature storage was another limitation especially in areas where electric power supply is not constant.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc have been used during writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology

CONSENT

This study was conducted in line with the required guideline involving informed consent of all participants. All the participants were pre-informed of the nature of the study and they willingly volunteered to participate without any cohesion.

AVAILABILITY OF DATA MATERIALS

All data generated or analyzed during this study are included in this article.

FUNDING

This Research did not receive any specific grant from funding agencies in the public, commercial or non-for-profit sectors

ACKNOWLEDGEMENTS

The authors wish to appreciate the staff of the General Laboratory, Department of Microbiology, Obafemi Awolowo University, Ile-Ife for the assistance rendered during the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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