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Optimization of co-fermentation of carrot and tomato juices by probiotic bacteria and yeast using a central composite design

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Abstract

In the present study, experiments were conducted on the suitability of tomato, carrot and mixed tomato+carrot juice for probiotication by *Lactobacillus plantarum*, *Lactobacillus fermentum, Lactobacillus casei* and *Lysinibacillus sphaericus* individually and in combination with a yeast (*Saccharomyces boulardii*). The combination of *Lys. sphaericus* and *S. boulardii* showed good results. Further optimization was carried out using a central composite design (CCD). The autoclaved juices were inoculated with probiotic cultures both alone or in combination with the yeast culture and incubated at 37°C for 72 h. After 24 h of fermentation, the pH levels had decreased from 6.1 to 4.0. Titratable acidity also increased from 0.12% to 0.36%, while the viable cell counts of probiotic bacteria and yeast gradually increased from 6.5 to 7.0 CFU/ml and from 5.4 to 7.9 CFU/ml, respectively. Subsequently, following further fermentation, viable cell counts decreased due to a decrease in pH and an increase in acidity as well as a lack of nutrients in the medium. The antimicrobial activity of mixed juice was found to have a maximum zone of inhibition of 11.8 mm and 9.8 mm against *Pseudomonas aeruginosa* MTCC 741 and *Bacillus subtilis* MTCC 2394, respectively. Probioticated tomato and carrot juices showed good sensory attributes.

Introduction

According to the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO), probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [1]. The dietary use of live microorganisms has a long history. Cultured dairy products are mentioned in the Bible and in sacred Hindu texts [2]. Soured milk and cultured dairy products such as kefir, koumiss, leben and dahi were often used therapeutically before microorganisms were discovered. In the early 1200s, the great armies of Genghis Khan consumed cultured horse milk [3]. The use of probiotic products has risen over the last two decades due to the increasing health awareness of consumers [4]. Probiotics as living microbial supplements have shown beneficial effects on the host by controlling intestinal infection and serum cholesterol levels, influencing the immune system, improving lactose utilization in lactose maldigesters, and inhibiting mutagenic activity [5]. According to Salminen *et al* [6], probiotics can be defined

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as 'viable microbial cultures that influence the health of the host'. This definition emphasizes that probiotics can be either non-viable cells or components of microbial cells that have an effect on the health and well-being of the host (it is proposed that probiotics be defined as 'microbial cell preparations').

Functional foods are defined as foods that in addition to supplying nutrients also offer potential health benefits that can enhance the well-being of individuals [7]. Lactic acid bacteria, predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, constitute a significant proportion of probiotic cultures in nutritional supplements, pharmaceuticals and functional foods [8]. Currently, the majority of products containing probiotics are dairy based and include yogurt and fermented milk beverages. However, due to some drawbacks related to dairy products, there is emerging interest in using non-dairy ingredients as substrates for delivering the physiological benefits of probiotics to a wider group of consumers [7].

Sheehan *et al* [9] showed that different probiotic cultures added to orange and pineapple juices varied in their ability to tolerate low pH (~3.5) and to survive during storage at low temperatures (~4°C). In addition, the majority of the probiotic bacteria were killed if the fermented juices were subjected to thermal or high pressure pasteurization. Among the cultures studied, *Lactobacillus casei*, *Lb. rhamnosus* and *Lb. paracasei* displayed good survival in orange and pineapple juices compared to cranberry juice. The cultures studied survived at levels of above 10^6 CFU/ml for at least 12 weeks when the bacteria were added to shelf-stable orange and pineapple juices and not subject to further pasteurization. Yoon *et al* [10] reported that *Lb. acidophilus* and *Lb. plantarum* grew well in non-supplemented beetroot and cabbage juices to nearly 10^8 CFU/ml at 30°C after 48 h of fermentation.

Carrot juice was chosen as a model for vegetable fermentation and as a new potential carrier for probiotics mainly because of its sweeter taste. Fermentation of carrot juice decreases the level of sugars and the acidification provides a fresh taste. Fermentation of carrot juice has been found to positively influence the availability of some minerals and vitamins such as calcium, phosphate, iron, carotene and vitamin C [11]. Tomatoes are widely used and versatile and are consumed fresh or as industrially processed products. Processed tomatoes include canned and sun-dried tomatoes, juices, ketchups, pastes, purees, salads, sauces and soups [12]. Tomatoes contain abundant health-promoting components such as lycopene, provitamin A, vitamin E and antioxidants [13]. Regular consumption of tomatoes has been associated with a reduced risk of various types of cancer [14].

The aim of this investigation was to study the introduction of probiotic lactic acid bacteria individually and in combination with a probiotic yeast into vegetable and fruit juice-based products for human consumption and to optimize the process in order to maximize product probiotic viability.

Materials and methods

Preparation of tomato and carrot juices

Tomatoes and carrots were purchased from a local vegetable market in Tirupati, and stored in a box at room temperature to ripen further. They were then washed with tap water to remove soil and other impurities, air-dried at room temperature, and blanched in water for 20 min at 60°C.

Juices were prepared from the tomatoes and carrots separately using a laboratory grinder and filtered through a muslin cloth in a sieve (0.8–1.1 mm pore diameter) to obtain a clear juice.

Probiotic cultures and inoculum preparation

Lactobacillus plantarum (Lp) (MTCC 1325), *Lactobacillus fermentum* (Lf) (MTCC 903), *Lactobacillus casei* (Lc) (MTCC 1428) and *Lysinibacillus sphaericus* (Ls) were maintained in MRS (de Man, Rogosa and Sharpe) agar stabs as pure cultures. Cultures were activated by two successive transfers in MRS broth at 37°C for 24 h. The activated cultures were again grown in MRS broth for 24 h at 37°C and used as inocula.

Saccharomyces boulardii was isolated from the dietary supplement Darolac obtained from a local chemist and maintained as a pure culture by growing it at 30°C for 48 h on PDA (potato dextrose agar) slants and stored at 4°C. The culture from the slant was transferred into PD broth and grown for 48 h at 30°C and then used as inoculum for

fermentation. For co-fermentation, actively growing individual lactic acid bacteria and *S. boulardii* were mixed in a ratio of 1:1 and used as inocula for probiotication.

Probiotication of tomato and carrot juices

Samples (100 ml) of tomato, carrot or tomato + carrot (50 ml of tomato juice and 50 ml of carrot juice) juice were placed individually into 250 ml Erlenmeyer flasks. All flasks were autoclaved for 15 min at 121°C and cooled and inoculated separately with 24-hour-old culture broth (~10^8 CFU/ml) of *Lb. fermentum*, *Lb. plantaram, Lb. casei* and *Lys. sphaericus* at a rate of 1–2 ml per 100 ml medium, either individually or in combination with *S. boulardii*, and incubated at 37°C for 72 h.

Physicochemical analysis

The pH of the probioticated juices was measured using a pH meter (CyberScan; Eutech Instruments, Singapore). Total soluble solids (TSS) were estimated in °Brix using a hand refractometer (Erma, Japan). Reducing sugars were determined spectrophotometrically using the DNS method. Titratable acidity (TA) was estimated by titration with 0.1N NaOH standard solution and expressed as percent lactic acid [15].

Viable cell count of probiotics

Viability was determined in duplicates by using the pour plate method [16] on MRS agar medium with 2.5 mg/l amphotericin B to inhibit yeast growth. The viable cell count of *S. boulardii* was determined by the pour plate method using PDA medium. A 10 ml aliquot of each sample of probioticated tomato juice, carrot juice or tomato+carrot juice was added individually to 90 ml of sterile 0.85% (w/v) saline and vortexed for 30 s. The resulting suspension was subjected to serial decimal dilution, while appropriate dilution was used for selective enumeration by the pour plate technique. The cell growth of each organism was assessed by counting the bacterial/yeast population after 12, 24, 48 and 72 h of probiotication of tomato, carrot and tomato+carrot juices on MRS agar or PDA. Plates containing 25–250 colonies were examined and colony forming units (CFU) counted and recorded as CFU per ml sample.

Optimization using a central composite design (CCD)

The probiotication process was optimized using a response surface methodology (RSM) protocol [17, 18]. The effect of pH (X_1), temperature (X_2), time (X_3) and sucrose (X_4) on the acidity (Y_1), cell viability (Y_2) and biomass (Y_3) of the combination of *Lys. sphaericus* and *S. boulardii* in the juices was studied using a central composite design (CCD) according to RSM using Design-Expert version 9 software (Stat-Ease Inc., Minneapolis, MN, USA). The range and levels of the variables investigated in this CCD study are given in Table 1. A 2^4-factorial CCD, with six replications at the centre points (n_0=6) for a total of 30 experiments was employed (Table 2) for the optimization of the probiotication conditions. The second degree polynomial equations were calculated with a statistical package to approximate the response of the dependent process variable. The variance determined for each factor was divided into linear, quadratic and interactive components which were represented using the second order polynomial function as follows:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{44}X_4^2+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+b_{23}X_2X_3+b_{24}X_2X_4+ b_{34}X_3X_4$$

where Y is the predicted response, X_1, X_2, X_3 and X_4 are independent variables, b_0 is the offset term, b_1, b_2, b_3 and b_4 are linear effects, b_{11}, b_{22}, b_{33} and b_{44} are squared effects, and b_{12}, b_{13}, b_{14}, b_{23}, b_{24} and b_{34} are interaction terms. The significance

Factor	Name	Low actual	Middle actual	High actual	Low coded	Middle coded	High coded
X_1	pH	3.1	5.2	6.8	–1	0	1
X_2	Temperature (°C)	20	37	42	–1	0	1
X_3	Time (h)	0	24	72	–1	0	1
X_4	Sucrose (%)	5	9	14	–1	0	1
Response	**Name**	**Units**	**Obs.[a]**	**Min.**	**Max.**	**Mean**	**SD**
Y_1	Cell viability	CFU	30	1.1	9.1	4.9	2.80
Y_2	Biomass	OD	30	0.04	0.86	0.42	0.28
Y_3	Acidity	%	30	0.27	0.49	0.37	0.07

[a]Observed run values

Table 1 - Actual and coded values of the variables studied

S. No	A	B	C	D	Cell viability (CFU/ml)	Biomass (OD)	Acidity (%)
1	-1	-1	-1	1	2.42 (3.06)	0.28 (0.43)	0.72 (0.52)
2	0	0	0	0	9.84 (9.95)	0.95 (0.73)	0.37 (0.32)
3	-1	1	-1	1	2.35 (2.47)	0.09 (0.23)	0.68 (0.37)
4	1	1	-1	-1	2.40 (2.83)	0.43 (0.75)	0.69 (0.77)
5	0	0	0	0	11.20 (10.16)	1.96 (1.73)	0.59 (0.47)
6	0	0	0	-2	2.46 (1.94)	0.49 (0.38)	0.82 (0.65)
7	1	1	1	-1	5.32 (6.73)	0.74 (0.52)	0.48 (0.36)
8	1	1	-1	1	2.97 (3.14)	0.27 (0.29)	0.73 (0.66)
9	0	1	0	1	10.63 (11.46)	1.86 (1.46)	0.73 (0.56)
10	0	0	0	0	9.50 (8.92)	1.92 (2.13)	1.09 (0.89)
11	1	-1	-1	-1	1.86 (1.46)	1.26 (1.05)	0.59 (0.55)
12	0	0	0	0	0.73 (0.56)	1.72 (1.53)	0.85 (0.64)
13	0	0	-2	0	4.15 (3.07)	1.06 (0.86)	0.53 (0.38)
14	-1	1	-1	-1	3.16 (3.06)	0.58 (0.47)	0.73 (0.56)
15	-1	-1	1	1	6.30 (7.17)	0.61 (0.54)	0.86 (0.45)
16	-1	-1	-1	-1	1.90 (2.17)	0.99 (0.64)	0.83 (0.63)
17	0	-2	0	0	10.23 (9.46)	1.79 (1.06)	1.71 (1.15)
18	0	0	0	0	9.30 (8.56)	1.62 (1.33)	1.79 (0.69)
19	-1	-1	1	-1	6.80 (5.14)	1.01 (0.55)	0.41 (0.76)
20	1	-1	1	1	6.17 (5.22)	0.82 (1.76)	0.82 (0.29)
21	-1	1	1	-1	3.98 (4.48)	1.54 (1.28)	1.23 (0.65)
22	0	2	0	0	9.50 (8.42)	0.95 (0.64)	0.82 (1.86)
23	2	0	0	0	5.12 (4.99)	1.82 (0.93)	1.29 (0.64)
24	1	-1	1	-1	7.40 (6.91)	1.71 (1.59)	1.35 (0.94)
25	-1	1	1	1	5.28 (5.19)	1.27 (1.35)	1.27 (0.76)
26	0	0	0	0	10.30 (9.27)	0.82 (0.83)	0.69 (0.31)
27	1	-1	-1	1	2.70 (2.30)	1.07 (0.47)	0.71 (0.32)
28	1	1	1	1	7.27 (6.10)	1.35 (1.35)	1.94 (0.72)
29	-2	0	0	0	1.40 (1.08)	0.15 (0.09)	0.45 (0.45)
30	0	0	0	2	2.70 (0.46)	0.61 (0.83)	0.73 (0.98)

S. No. serial number

Table 2 - Central composite design (CCD) experimental design matrix

of all terms in the polynomial functions was assessed statistically using the *F* value at a probability (*p*) of 0.001, 0.01 or 0.05. The three-dimensional (3D) plots were generated by keeping one variable constant at the centre point and varying the other variables within the experimental range. The optimized values of four independent variables for maximum activities were determined using the numerical optimization function of Design-Expert 9.0.1.

Antimicrobial activity

The agar well diffusion method was used to determine the antimicrobial property of the probioticated juices. A 24 h culture of the pathogenic strains *Pseudomonas aeruginosa* MTCC 741 and *Bacillus subtilis* MTCC 2394 was grown individually in Lysogeny Broth (LB) medium and the cell suspension was spread over the surface of Mueller Hilton agar plates using a sterile spreader. The plates were allowed to dry and a sterile well borer of 5 mm diameter was used to cut uniform wells in the agar. Each well was filled with 100 µl of probioticated tomato, carrot juice and tomato+carrot juice, and lactic acid or Ampicillin used as control. After incubation at 37°C for 24 h, the plates were observed for a zone of inhibition (ZOI) around the well. Results were considered positive if the diameter (mm) of the ZOI was greater than 1 mm [19].

Sensory analysis

The sensory characteristics of the juices were evaluated according to Dias *et al* [20] by a 20-member panel. Preferences for taste, acidity, mouth feel, aroma, flavour, colour and overall acceptability were determined using a 9-point hedonic scale. Randomized refrigerated (10°C) samples (50 ml) were served in clear tulip-shaped glasses coded with a random 3-digit code. The mean intensity scores of all attributes were calculated and plotted.

Statistical analysis

All experiments were carried out in triplicate and the mean value and standard deviation were presented. The data were analyzed by one-way analysis of variance (ANOVA) using SPSS v 16.0. Regression analysis and calculation of the correlation coefficient of the optimization experiments were performed with Design-Expert 9.0.1.

Results and discussion

Changes in pH

During the probiotication of individual or mixed juices with *Lb. plantarum* (Lp), *Lb. fermentum* (Lf), *Lb. casei* (Lc) and *Lys. sphaericus* (Ls) individually and in combination with probiotic *S. boular-*

dii (Sb), pH ranged from 5.9 to 6.1, which reduced to 5.3–5.2 after 24 h and to 4.4–4.2 after 48 h of probiotication (Fig. 1).

The pH of tomato juice fermented with Ls reduced from an initial pH of 5.9 to a maximum of 5.1, followed by Lc with a reduction to 5.2 and by Lf with a reduction to 5.1, while the pH of juice probioticated with Ls+Sb decreased to 4.5 at 24 h. After 24 h, pH further reduced to 4.6–4.1 with Ls (the maximum reduction) followed by Lc to 4.3, Lf to 4.6 and Lc to 4.4. The same pattern was observed in combination with yeast.

The pH of carrot juice probioticated with Ls was reduced to a maximum of 4.6 followed by Lp at 5.1, Lf at 4.9 and Lc at 5.0. Probiotication with Ls+Sb resulted in pH 4.8 at 24 h of probiotication. The same pattern was observed at 48 h.

The pH of tomato+carrot juice probioticated with Ls was reduced to 4.4, followed by Lc at 4.6, Lf at 4.7 and Lp at 4.9. Probiotication with Ls+Sb resulted in pH 4.5 at 24 h. After 24 h, a further reduction was observed in pH to 4.6–4.1 by Ls (the maximum reduction), followed by Lc to 4.1, Lf to 4.2 and Lp to 4.3. The same pattern was observed in combination with yeast (Fig. 1).

These results are in agreement with earlier reports [21]. Tomato juice was probioticated with four lactic acid bacteria and changes in pH, acidity and viable cell counts during fermentation analyzed. The lactic acid cultures reduced the pH to 4.1 or below and increased acidity to 0.65% or above, while viable cell counts reached nearly 1.0–9.0 CFU/ml after 72 h of fermentation.

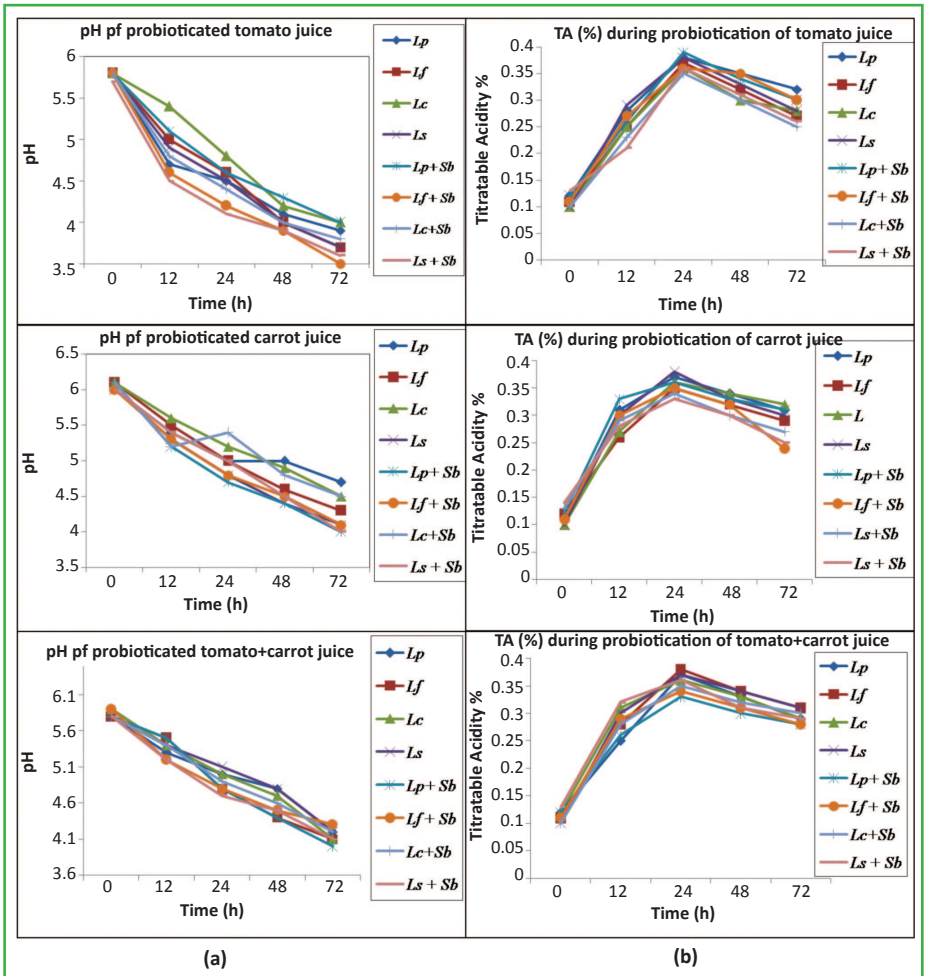

Figure 1 - Changes in (a) pH and (b) titratable acidity during probiotication of tomato, carrot and tomato+carrot juice. *Lc Lactobacillus casei, Lf Lactobacillus fermentum, Lp Lactobacillus plantarum, Ls Lysinibacillus sphaericus, Sb Saccharomyces boulardii*

Changes in TA

Changes in TA during the probiotication of the juices are presented in Fig. 1. During the probiotication of tomato, carrot and tomato+carrot juices with Lp, Lf, Lc and Ls individually and in combination with probiotic yeast (Sb), TA ranged from 0.09% to 0.12%, which increased to 0.32–0.36% at 24 h and to 0.36–0.24% at 48 h (Fig. 1).

In tomato juice, there was an increase in TA from 0.11–0.12% to 0.29–0.33% at 24 h. Probiotication with Ls showed the highest TA (0.33%), followed by Lp at 0.29% and Lf at 0.26%, with individual values dropping from 0.33–0.26% at 24 h to 0.30–0.23% at 48 h. Probiotication with Ls+Sb showed the highest TA at 0.32%, followed by Lc+Sb at 0.30% and Lp+Sb at 0.28%, with values dropping from 0.34–0.28% at 24 h to 0.32–0.21% at 48 h. In carrot juice, there was increase in TA from 0.10–0.13% to 0.33–0.37% at 24 h. Ls showed the highest TA at 0.37%, followed by Lp

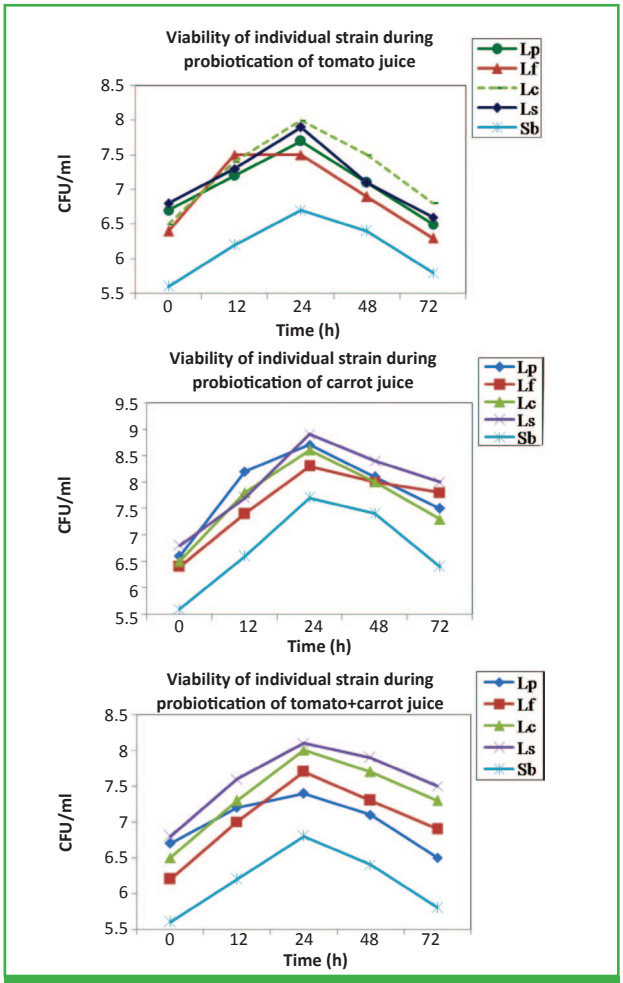

Figure 2 - Changes in the cell viability of individual probiotics during the probiotication of tomato, carrot and tomato+carrot juice. *Lc Lactobacillus casei, Lf Lactobacillus fermentum, Lp Lactobacillus plantarum, Ls Lysinibacillus sphaericus, Sb Saccharomyces boulardii*

at 0.35% and Lf at 0.32%, with individual values of 0.37–0.32% decreasing to 0.29–0.21% after 24 h. Probiotication with Lf+Sb showed the highest TA, followed by Ls+Sb at 0.35% and Lp+Sb at 0.30%, with values of 0.37–0.33% decreasing to 0.30–0.28% at 48 h.

In tomato+carrot juice, TA increased from 0.08–0.11% to 0.36–0.31% at 24 h. Ls showed the highest TA at 0.36%, followed by Lp at 0.35% and Lc at 0.33%. Probiotication with Lf+Sb showed the highest TA, followed by Ls+Sb at 0.33% and Lp+Sb at 0.30%, with values of 0.31–0.33% decreasing to 0.20–0.28% at 48 h.

Changes in cell viability and growth of probiotics

Changes in the viable count of probiotics during the fermentation of juices (Fig. 2) attested to the probiotication of tomato, carrot and tomato+carrot juice with *Lb. plantaram*, *Lb. fermentum*, *Lb. casei* and *Lys. sphaericus*, individually and in combination with probiotic *S. boulardii*. The viable cell count increased from 6.5–7.0 CFU/ml to 7.8–8.2 CFU/ml at 24 h with a marginal decrease from 7.8–8.2 to 7.2–7.8 CFU/ml at 48 h, as shown in Fig. 2. This result is in agreement with an earlier report [21] that the viable cell counts for lactic acid bacteria in fermented tomato juice increased from 6.5 CFU/ml to 7.6 CFU/ml during 72 h of fermentation.

In tomato juice, viability increased with initial viable cell counts rising from 6.5–6.8 CFU/ml to 7.9–8.2 CFU/ml at 24 h (Fig. 3). Ls showed a 16.25% increase in viability, while Ls in combination with Sb showed a 17.07% increase in viability. This indicates that yeast supported Ls growth in combined fermentation. The viability of the other isolates was increased by 12–13% at 24 h, with a marginal decrease in viability of 2.56% at 48 h.

In carrot juice, viability

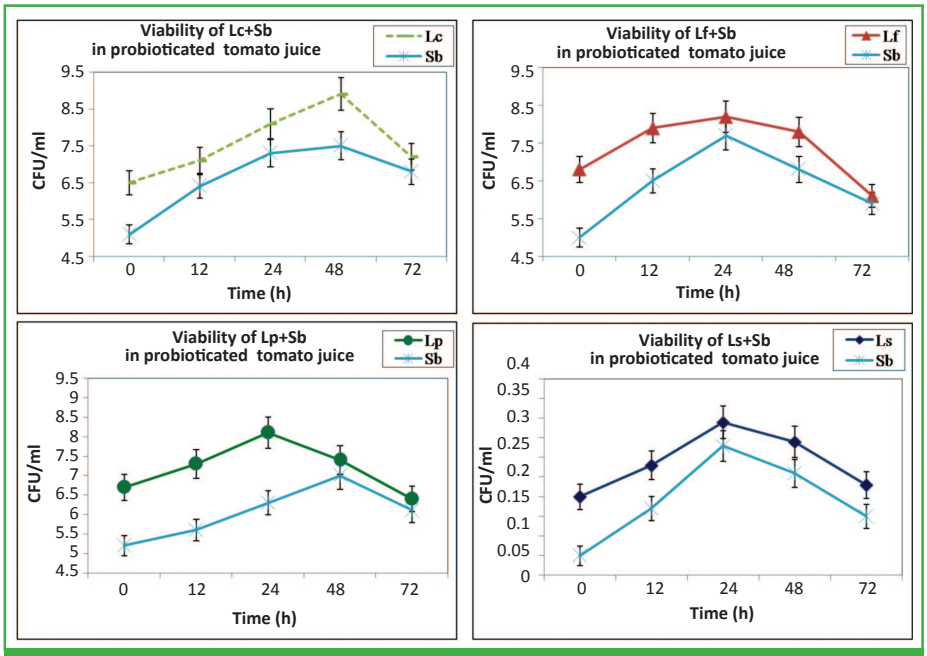

Figure 3 - Viability of probiotic bacteria with *Saccharomyces boulardii* during probiotication of tomato juice. *Lc Lactobacillus casei, Lf Lactobacillus fermentum, Lp Lactobacillus plantarum, Ls Lysinibacillus sphaericus, Sb Saccharomyces boulardii*

increased from an initial viable cell count of 6.5–6.9 CFU/ml to 7.8–8.1 CFU/ml at 24 h (Fig. 4). Ls alone showed a 15.21% increase in viability, while Ls in combination with Sb showed a 15.50% increase in viability. The viability of other isolates was increased by 12.34–13.8% by 24 h, with a marginal decrease in viability of 3.12% at 48 h.

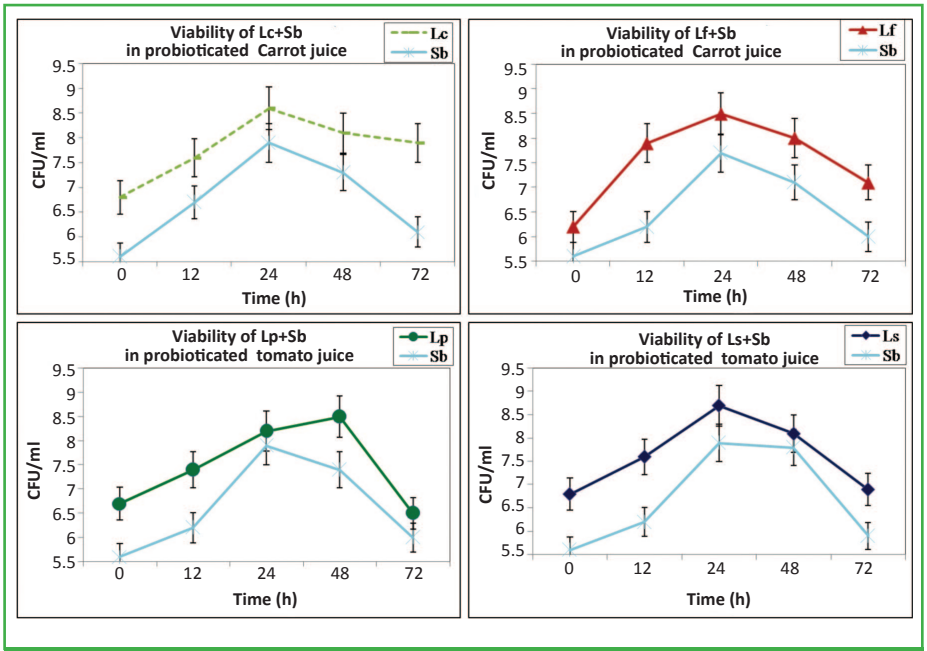

Figure 4 - Viability of probiotic bacteria with *Saccharomyces boulardii* during probiotication of carrot juice. *Lc Lactobacillus casei, Lf Lactobacillus fermentum, Lp Lactobacillus plantarum, Ls Lysinibacillus sphaericus, Sb Saccharomyces boulardii*

In tomato+carrot juice, viability increased from an initial viable cell count of 6.3–8.8 CFU/ml to 7.8–8.2 CFU/ml at 24 h (Fig. 5). Ls showed a 16.45% increase in viability, while Ls in combination with Sb showed a 17.28% increase in viability. This indicates that the yeast supported the growth of Ls in combined fermentation. The viability of other isolates was increased by 13.64–13.97% at 24 h, with a marginal decrease in viability of 2.96% at 48 h.

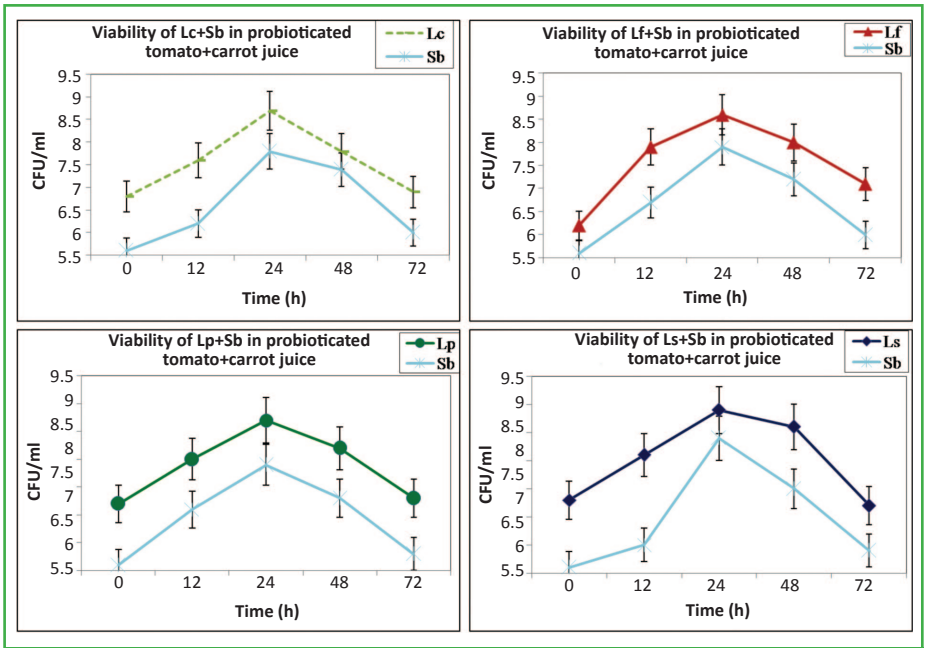

Figure 5 - Viability of probiotic bacteria with *Saccharomyces boulardii* during probiotication of mixed tomato and carrot juice. *Lc Lactobacillus casei, Lf Lactobacillus fermentum, Lp Lactobacillus plantarum, Ls Lysinibacillus sphaericus, Sb Saccharomyces boulardii*

Babu *et al* [22] reported that the addition of tomato juice to skimmed milk stimulated the growth of *Lb. acidophilus* and resulted in higher viable counts. More sugar utilization resulted in more acid production and lower pH. It was also reported that probiotic fermentation of indigenous food mixtures containing tomato pulp using *Lb. casei* and *Lb. plantarum* showed a decrease in pH, increase in acidity and improved digestibility of starch and protein [23].

For maximum health benefits, it is important that a significant number of viable lactic acid bacteria are present in probiotic products [24]. Several factors could affect the cell viability of lactic acid cultures in probiotic food products. Probiotic cultures are commonly used in the dairy industry and some products produced during lactic acid fermentation, such as lactic acid, diacetyl and acetaldehyde, could be associated with the loss of

viability of added probiotic bacteria [25]. Lactic acid starters are reported to produce bacteriocins against probiotic bacteria and vice versa [26].

In general, cell viability depends on the strains used, interaction between species present, culture condition, oxygen content, final acidity of the product, and the concentration of lactic acid and acetic acid. The main factors affecting the loss of viability of probiotic organisms have been suggested to be a decrease in the pH of the medium and the accumulation of organic acids as a result of growth and fermentation [27]. In the present study, the probiotic bacteria and yeast survived better in the fermented tomato and carrot juices with high acidity and low pH. These results suggest that fermented tomato and carrot juices might serve as probiotic beverages for vegetarians or consumers allergic to dairy products.

It has been suggested that co-fermentation of probiotic bacteria and yeast in tomato and carrot juices establishes mutualism, in which the growth of *S. boulardii* is stimulated and in turn promotes the growth of lactic acid bacteria by utilizing organic acids formed during the fermentation of vegetable juices [28]. The *S. boulardii* is also able to survive at low pH and creates a special environment for its enhanced survival, with no inhibition effect on lactic acid bacteria found in this study.

The tomato, carrot and tomato+carrot juices had a normal physical appearance with good colour, aroma, flavour and texture. pH decreased and TA simultaneously increased in all samples with probiotic bacteria in combination with probiotic yeast during first 24 h of fermentation, with marginal variations during the remaining period of fermentation. pH and TA influenced physical stability, flavour and the aroma of the probioticated juices. This study has shown that the optimum pH for the probiotication of these juices by bacteria in combination with yeast was 5.5–5.3, and that the increase in TA was due to acid production during fermentation. However, probiotic bacteria present in fermented vegetable juices are unstable. Their poor survival is attributed to low pH and their low acid tolerance. Yeast has the ability to utilize organic acids, thereby increasing pH. Thus the growth of probiotic yeast in association with probiotic bacteria has been suggested for enhancing the viability of lactic acid bacteria [28].

In tomato, carrot and tomato+carrot juices fermented with *Lb. plantaram, Lb. fermentum, Lb. casei* and *Lys. sphaericus* along with the probiotic yeast *S. boulardii*, the highest increase in viability (17.11%) occurred by 24 h, with a marginal (3.24%) decrease until 48 h. The maximum growth increase in probiotic bacteria in the juices was found at 24 h of fermentation. The growth of yeast *S. boulardii* in all fermented juice samples showed its maximum increase (7.8%) after 48 h of fermentation. The maximum increase in growth among all juice cultures was found in cultures with lactic acid bacteria combined with yeast. *Lys. sphaericus* gave maximum growth both in probiotic bacteria isolates and also in combination with the yeast *S. boulardii*. Of the three juice samples, the tomato+carrot juice was the best medium for increased growth and probioticated properties.

Optimization of process conditions

Optimization of process conditions is critical during the development of an efficient and economic bioprocess [29]. Hence the influence of pH, temperature, time and sucrose on acidity, cell viability and biomass was investigated using RSM. The results are presented in Table 2. The effect of each factor and their interactions were analyzed using ANOVA and the χ^2 test as appropriate. A regression equation for the optimization of probiotic conditions showed that cell viability (Y_1, %), biomass (Y_2, %) and acidity (Y_3, %) are a function of pH (X_1), temperature (X_2, °C), time (X_3, min) and sucrose (X_4, %). By applying multiple regression analysis to the experimental model data, the following second order polynomial equation is found to effectively represent cell viability, biomass and acidity:

Cell viability (CFU) $Y_1 = 35.4 + 5.3X_1 + 2.1X_2 + 0.16X_3 + 2.9X_4 – 0.91X_1^2 – 0.11X_2^2 – 6.26X_3^2 – 0.79X_4^2 + 8.53X_1X^2 + 2.73X_1X_3 + 1.02X_1X_4 – 9.51X_2X_3 + 4.1X_2X_4 + 7.63X_3X_4$

Biomass (%) $Y_2 = 4.28 + 0.56X_1 + 0.20X_2 + 8.52X_3 + 1.28.X_4 – 1.35X_1^2 – 0.43X_2^2 – 4.72X_3^2 – 6.63X_4^2 + 4.63X_1X_2 + 4.43X_1X_3 + 3.46X_1X_4 – 7.44X_2X_3 + 2.55X_2X_4 + 5.82X_3X_4$

Acidity (%) $Y_3 = 9.16 + 1.26X_1 + 1.27X_2 + 6.02X_3 + 1.54.X_4 - 1.47X_1^2 - 1.35X_2^2 - 7.56X_3^2 - 7.36X_4^2 + 4.75X_1X_2 + 7.81X_1X^3 + 4.62X_1X_4 - 4.50X_2X_3 + 2.58X_2X_4 + 3.94X_3X_4$

The predicted levels of cell viability, biomass and acidity in probioticated juices using the above equations are given in Table 2. The R^2 values for all response variables were above 0.90, so the regression model was reliable (Tables 3–5).

Analysis of response variables

Cell viability

Probiotic juice fermentation is the result of many interactions and depends both on the strains and on the physico-chemical factors of the medium including sugar content, acidity and temperature [30]. Yeast strains differ in their responses to temperature as seen in wine making [31]. In this study, from the cell viability regression model (Y_1), the value of the coefficient of determination (R^2=0.9414) indicates that only 2.86% of the total variations were not explained by the model. The value of the adjusted coefficient of determination (adj. R^2=0.8957) was also high, supporting the strength of the model. Cell viability response surface plots demonstrate that the maximum yield was achieved at different sucrose concentrations and temperatures with time being a limiting factor (supplementary Fig. S1). Among the model terms, X_1, X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant with a probability of 99% (Table 3). However, the interaction between X_1, X_2 and X_1, X_3 had no significant influence on cell vi-

Source	Sum of squares	df	Mean square	*F* value	*p* Value probably >*F*
Model	208.73	14	14.91	11.13	<0.0001
X_1	0.40	1	0.40	0.30	0.5926
X_2	0.84	1	0.84	0.63	0.4397
X_3	27.43	1	27.43	20.48	0.0004
X_4	3.750	1	3.750	2.800	0.9585
X_1X_2	6.250	1	6.250	4.667	0.9830
X_1X_3	0.18	1	0.18	0.13	0.7186
X_1X_4	1.38	1	1.38	1.03	0.3260
X_2X_3	0.53	1	0.53	0.39	0.5404
X_2X_4	0.11	1	0.11	0.079	0.7827
X_3X_4	0.18	1	0.18	0.13	0.7186
X_1^2	85.58	1	85.58	63.91	<0.0001
X_2^2	7.45	1	7.45	5.56	0.0323
X_3^2	4.77	1	4.77	3.56	0.0787
X_4^2	110.30	1	110.3	82.37	<0.0001
Residual	20.09	15	1.34		
Lack of fit	19.95	9	2.22	96.98	<0.0001
Pure error	0.14	6	0.023		
Correlated total	228.82	29			

Table 3 - ANOVA for cell viability quadratic model

Source	Sum of squares	df	Mean square	*F* value	*p* Value probably >*F*
Model	2.22	14	0.16	12.42	<0.0001
X_1	2.400	1	2.400	0.19	0.6708
X_2	0.011	1	0.011	0.88	0.3624
X_3	0.29	1	0.29	22.63	0.0003
X_4	6.667	1	6.667	5.221	0.9434
X_1X_2	2.250	1	2.250	0.018	0.8962
X_1X_3	0.018	1	0.018	1.43	0.2507
X_1X_4	5.625	1	5.625	0.44	0.5169
X_2X_3	3.600	1	3.600	0.28	0.6032
X_2X_4	4.000	1	4.000	0.031	0.8619
X_3X_4	9.000	1	9.000	0.070	0.7942
X_1^2	0.88	1	0.88	68.79	<0.0001
X_2^2	0.074	1	0.074	5.79	0.0295
X_3^2	0.097	1	0.097	7.58	0.0148
X_4^2	1.10	1	1.10	86.21	<0.0001
Residual	0.19	15	0.013		
Lack of fit	0.19	9	0.021	92.43	<0.0001
Pure error	1.371	6	2.286		
Correlated total	2.41	29			

Table 4 - ANOVA for biomass response surface quadratic model

Source	Sum of squares	df	Mean square	*F* value	*p* Value probably >*F*
Model	0.14	14	9.928	9.86	<0.0001
X_1	1.500	1	1.500	0.15	0.7050
X_2	1.500	1	1.500	0.15	0.7050
X_3	0.043	1	0.043	42.27	<0.0001
X_4	6.667	1	6.667	0.066	0.8005
X_1X_2	1.000	1	1.000	0.099	0.7570
X_1X_3	3.025	1	3.025	3.00	0.1036
X_1X_4	9.000	1	9.000	0.89	0.3595
X_2X_3	1.000	1	1.000	0.099	0.7570
X_2X_4	6.250	1	6.250	0.62	0.4431
X_3X_4	4.000	1	4.000	0.40	0.5381
X_1^2	0.044	1	0.044	43.30	<0.0001
X_2^2	7.003	1	7.003	6.95	0.0187
X_3^2	3.072	1	3.072	0.031	0.8637
X_4^2	0.068	1	0.068	67.91	<0.0001
Residual	0.015	15	1.007		
Lack of fit	0.015	9	1.641	28.71	0.0003
Pure error	3.429	6	5.714		
Correlated total	22.41	29			

Table 5 - ANOVA for acidity response surface quadratic model

ability during the fermentation of probiotic juice. Maximum cell viability was observed in nine of 30 runs for predicted values (Table 2).

Biomass

Biomass production by bacteria and yeast is influenced by many growth and environmental factors [32]. Several studies have shown that an increase in temperature results in higher biomass production [33]. It is reported that the optimum temperature for maximum biomass production by the commercial probiotic strains *Lb. casei, Lb. plantarum, Lb. fermentum, Lys. sphaericus* and *S. boulardii* varies between 22°C and 32°C [34]. The response surface plot shows the actual (1.86) and coded (1.46) values of biomass at different sucrose concentra-

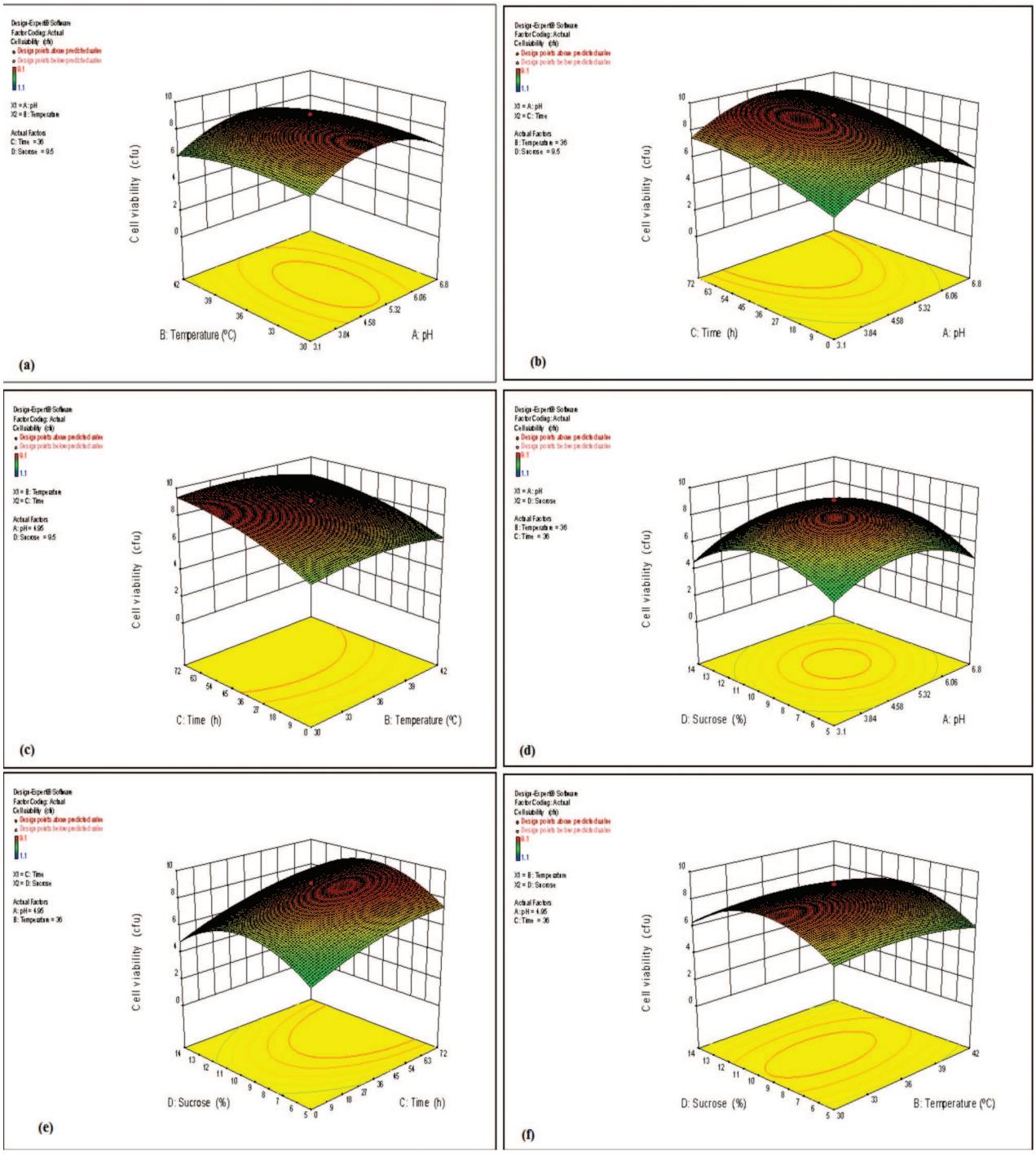

Figure S1 - Response surface plots of cell viability showing the interactive effect of (a) temperature and pH on cell viability, (b) time and pH on cell viability, (c) time and temperature on cell viability, (d) sucrose and pH on cell viability, (e) sucrose and time on cell viability, and (f) sucrose and temperature on cell viability

tions and temperatures with pH being a limiting factor (supplementary Fig. S2). From the experiments, the coefficient of determination of biomass is R^2=0.9238, with only 6.53% of the total variation not explained, while the adjusted R^2=0.8631 of the model is highly significant. The model terms X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant with a probability of 99% and X_1, X_2X_3 were significant with a probability of 95% (Table 4). Biomass production was not significantly influenced by the interactions between X_1, X_2 and X_1, X_3.

Acidity

Acidity (the degree of sourness of the probiotic juice) should be as low as possible. Lactic acid accounts for 90% of the acidity. By law, the acidity in

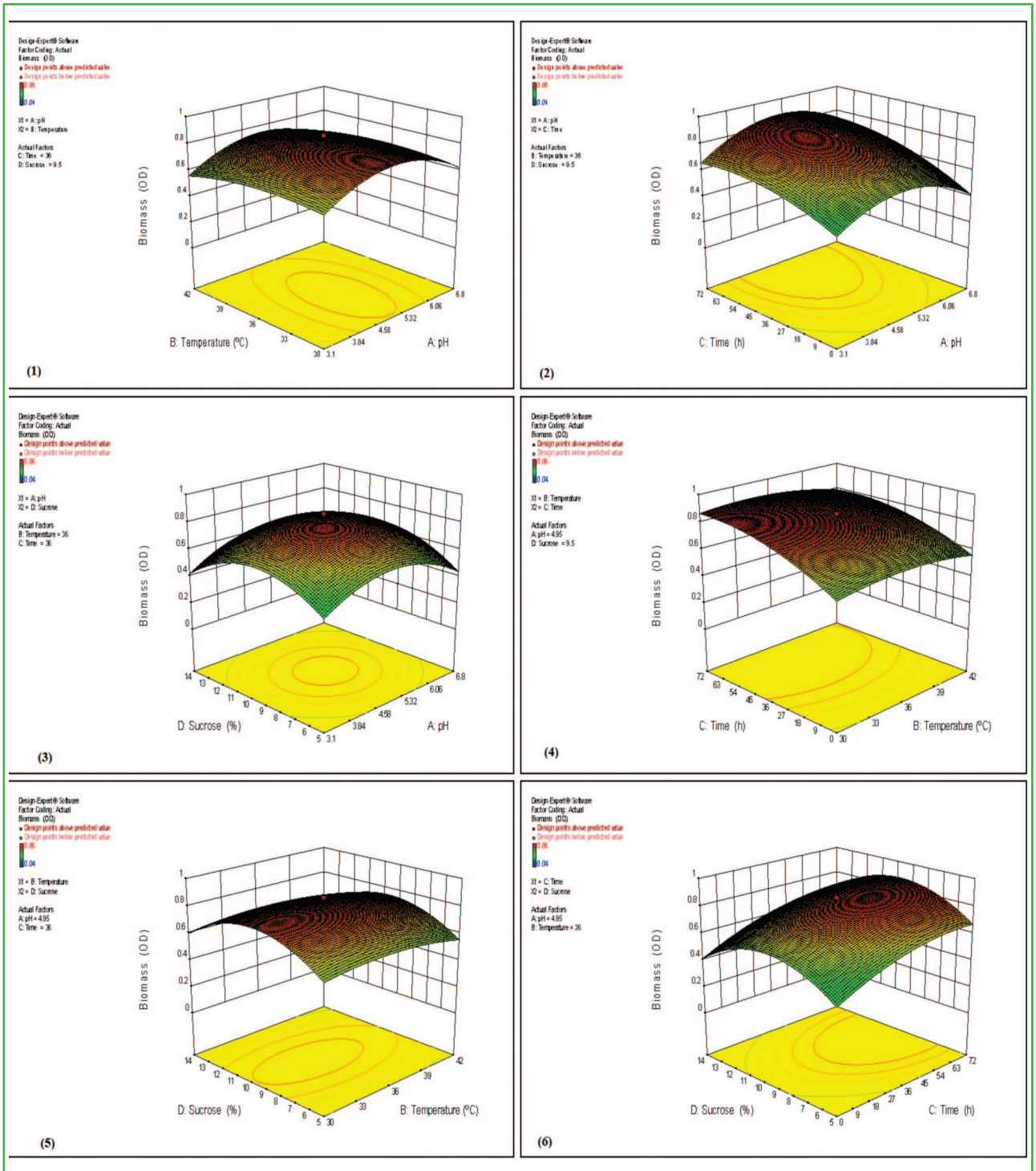

Figure S2 - Response surface plots of biomass showing the interactive effect of (1) temperature and pH on cell biomass, (2) time and pH on biomass, (3) sucrose and pH on biomass, (4) time and temperature on biomass, (5) sucrose and temperature on biomass, and (6) sucrose and time on biomass

probiotic juice cannot be higher than 1.0–1.5 g/l, depending on the country [35]. There is very little variation in the amount of lactic acid produced at fermentation temperatures of 10°C, 21°C and 33°C. Consequently, the values given in Table 5 for the acidity of the experimental probiotic juice are excellent, except for X_1X_3 variables, which showed an acid concentration of 1.24%.

The coefficient of determination R^2=0.9531 indicated that the model could explain 95.31% of variability with the remaining 4.69% unexplained. The predicted R^2 value of 0.7625 is in reasonable agreement with the adjusted R^2 value of 0.8924. The predicted value (0.56) shown in the acidity response plots is acceptable in light of the actual value (0.73) at the same levels of sucrose and temperature with the pH being a limiting factor (supplementary Fig. S3).

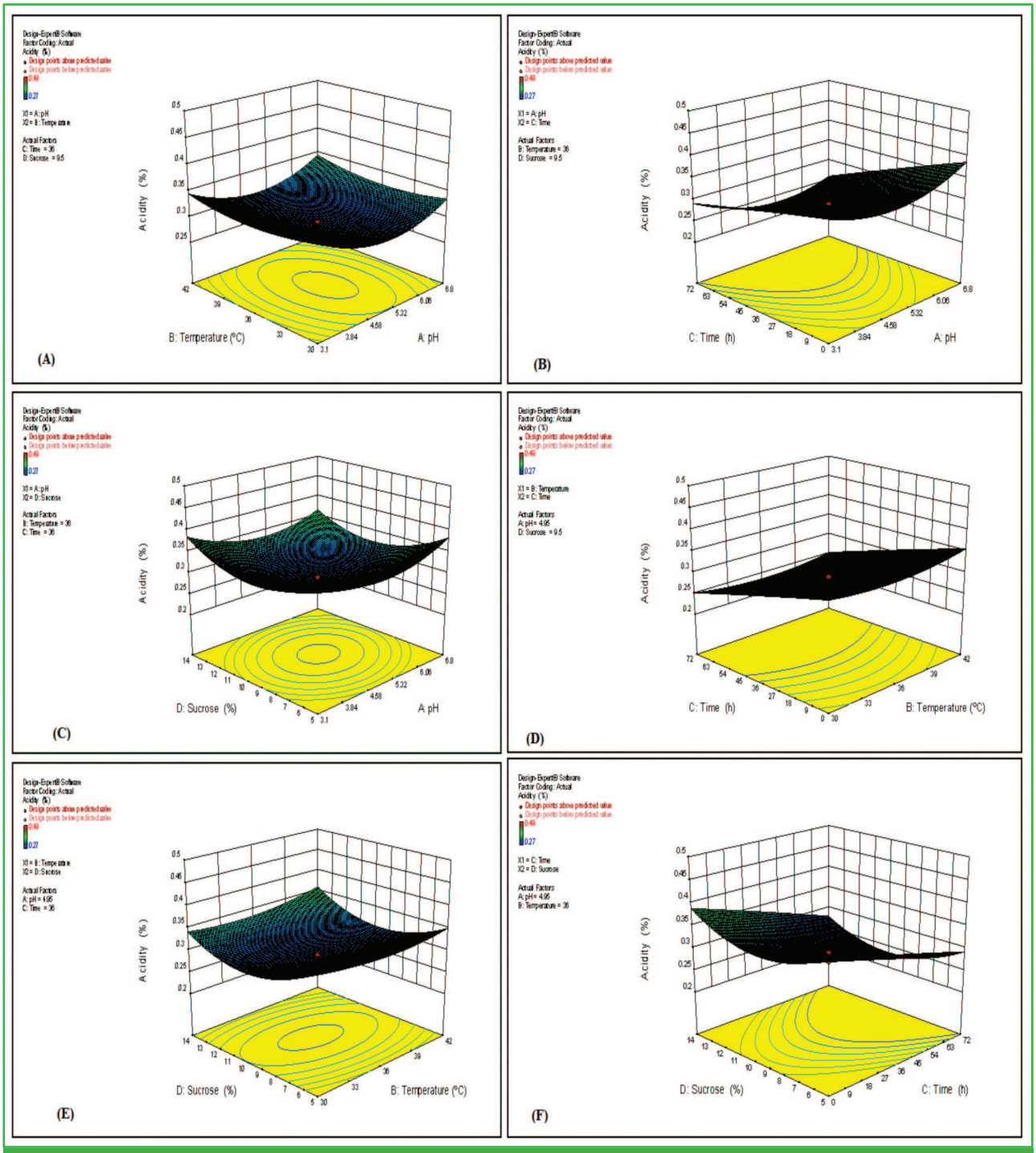

Figure S3 - Response surface plots of acidity showing the interactive effect of (A) temperature and pH on acidity, (B) time and pH on acidity, (C) sucrose and pH on acidity, (D) time and temperature on acidity, (E) sucrose and temperature on acidity, and (F) sucrose and time on acidity

The significant probability of 99% is with the model terms X_1, X_3, X_1X_2, X_1^2, X_2^2 and X_3^2. The model terms X_1X_3 and X_2X_3 have 95% significant probability. The pH (X_2) model term is not significantly involved in acid production during probiotic juice fermentation. Similarly, in this model the predicted values (<4 g/l) of biomass concentration were low, and observed in four runs (Table 5).

Change in TSS during probiotication of the juices

The levels of TSS and reducing sugars in tomato juice decreased during fermentation from 24 to 72 h, from 6.5±0.8 to 3.9±0.81 and from 29±0.01 to 15±0.02, respectively. The same values in carrot juice decreased from 8.2±0.47 to 5.5±0.47 and from 26±0.01 to 13±0.04, respectively, as shown in Table 6. The levels of TSS and reducing sugars in the tomato and carrot juices were similar to those reported by Kumar *et al* [36].

Juices	Incubation time (h)	TSS (°Brix)	Reducing sugars (mg/100 ml)
Tomato	24	6.5±0.8	29±0.01
	48	5.1±1.0	20±0.03
	72	3.9±0.81	15±0.02
Carrot	24	8.2±0.47	26±0.01
	48	6.6±0.81	18±0.02
	72	5.5±0.47	13±0.04

Table 6 - Change in total soluble solids (TSS) and reducing sugars during the probiotication of tomato and carrot juices

Sample	Zone of inhibition in (mm)	
	***Pseudomonas aeruginosa* MTCC 741**	***Bacillus subtilis* MTCC 2394**
PTJ	10.1±0.1	10.4±0.2
PCJ	9.42±0.2	8.3±0.1
PT+CJ	11.0±0.2	9.8±0.3
Lactic acid	11.8±0.1	8.8±0.2
Ampicillin	13.77+1.74	16.23+0.86

Values are the mean of three replicates (±SD)

Ls Lysinibacillus sphaericus, *PCJ* probioticated carrot juice, *PTJ* probiotic tomato juice, *PT+CJ* probioticated tomato+carrot juice, *Sb Saccharomyces boulardii*

Table 7 - Antimicrobial activity of tomato and carrot juice probioticated with Ls+Sb

Antimicrobial activity

The antimicrobial activity of the probioticated tomato, carrot and tomato+carrot juices was evaluated against *P. aeruginosa* and *B. subtilis* as compared to the Ampicillin control; the data are presented in Table 7. Probioticated tomato+carrot juice showed maximum inhibition against *P. aeruginosa* and *B. subtilis* with a zone of inhibition of 11.0 and 9.8 mm, respectively. In contrast, probioticated tomato and carrot juices showed smaller inhibition zones of 10.1 and 9.72 and of 9.42 and 8.3 mm against *P. aeruginosa* and *B. subtilis*, respectively, than those of the probioticated mixed juice.

Sensory analysis

Sensory evaluation produced good sensory scores for probioticated tomato, carrot and tomato+carrot juices (Fig. 6).

The panel members slightly preferred the probioticated juices to the control. The taste, acidity, mouth feel, aroma, flavour, colour and overall acceptance of probioticated juices differed from those of control juices. The results were in agreement with a previous report on the sensory evaluation of mango and sapota juices probioticated using *Lactobacillus* [37].

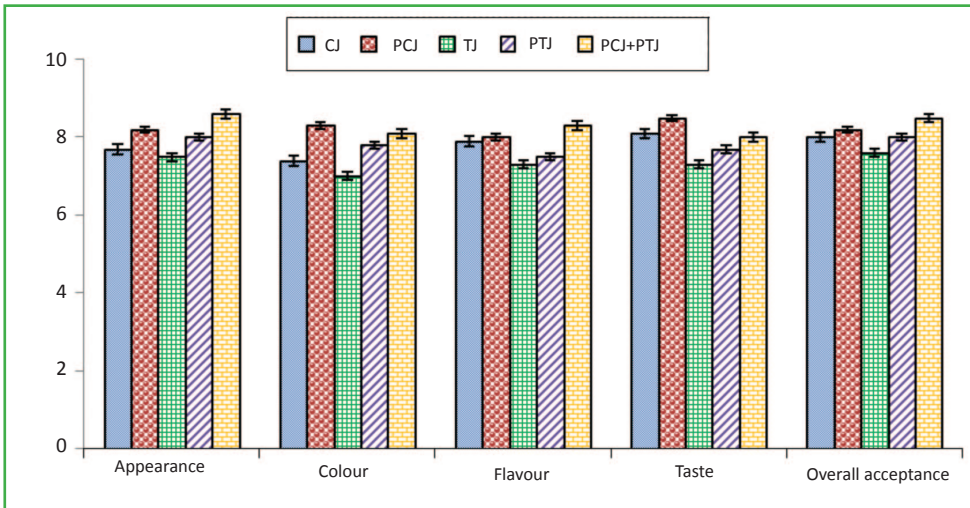

Figure 6 - Sensory evaluation of tomato, carrot and tomato+carrot juice probioticated with *Lysinibacillus sphaericus* and *Saccharomyces boulardii*. *CJ* carrot juice, *PCT* probioticated carrot juice, *PTJ* probiotic tomato juice, *TJ* tomato juice

Conclusions

The tomato and carrot juices and their mixture were probioticated using *Lb. fermentum*, *Lb. plantarum*, *Lb. casei* and *Lys. sphaericus*, individually and in combination with the yeast *S. boulardii*. The mixed juice probioticated with *Lys. sphaericus* and yeast supported good growth of probiotic strains and was superior to other cultures regarding tolerance to acidity and stable viable count. It also showed better antimicrobial properties and sensory characteristics and could be used as a probioticated drink. Studies are in progress to determine the type and quantity of bacteriocins produced by the above cultures, and investigations on how to scale up the process are ongoing.

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Conflict of Interest

All the authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the article.

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