

Research Article

Fermentation of roselle juice by lactic acid bacteria

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Abstract: This research was conducted to determine the suitability of roselle calyces as a raw material for the production of probiotic roselle juice by lactic acid bacteria (*Lactobacillus plantarum* and *Lactobacillus casei*). The juice was fermented for 72 h at 30°C and 37°C separately and then analyzed for pH, acidity, sugar content and viable cell counts. Both lactic cultures were found capable of rapidly utilizing roselle juice for cell synthesis and lactic acid production. They grew significantly better at 30°C for the first 24 h with an increase in cell numbers greater than 1.5 log cfu/ml. However, the viable cell count at 72 h of both temperatures was not much different (less than 0.6 log cfu/ml). Both strains produced a similar amount of titrable acidity expressed as lactic acid. However, the titrable acidity produced was about two-times higher at 30°C compared with that at 37°C (0.5% vs. 0.2-0.3% lactic acid). After 4 weeks of cold storage at 7°C, *Lb. plantarum* grown at 30°C survived significantly better than that grown at 37°C (1.8×10^6 cfu/ml vs. 2.9×10^4 cfu/ml). The addition of sucrose at the beginning of fermentation increased the amount of titrable acidity by at least two times (more than 1.1% lactic acid). However, both cultures did not survive well at the low pH and high acidity conditions in fermented sugar-added roselle juice, the viable cell count was up to 4×10^4 cfu/ml after 4 weeks of cold storage at 7°C.

Keywords: food, beverages, probiotics, *Lactobacillus plantarum*, *Lactobacillus casei*, *Hibiscus sabdariffa*

Introduction

Research has shown that the addition of probiotics to food provides several health benefits including improved gastrointestinal function, enhanced immune system and lower risk of colon cancer [1-3]. The majority of probiotics recommended are the species of *Lactobacillus* including *Lb. acidophilus*, *Lb. plantarum*, *Lb. casei* and *Streptococcus lactis*, etc. [4]. For health benefits, probiotic bacteria must be viable and available at a high concentration, typically above 10^6 cfu/ml or per gram of product [5].

Currently, probiotic products are usually marketed in the form of fermented milk and yogurt. However, lactose intolerance and the cholesterol content are two drawbacks related to their consumption, particularly in Asia. It has been suggested that fruit juice could serve as a good medium for cultivating probiotics [6]. Fruit and vegetables are healthy food, because they are rich in antioxidants, vitamins, dietary fibre and minerals. Furthermore, fruit and vegetables do not contain any dairy allergens that might prevent usage by certain segments of the population [7].

Roselle (*Hibiscus sabdariffa* L.) is widely cultivated in Thailand where it is known as *krachiap daeng*. Roselle calyces contain 9% moisture, 1.14% protein, 2.61% fat, 12% fibre and 6.9% ash. It is high in calcium, niacin, riboflavin and iron [8] and it is commonly used to make beverage and food colouring. The brilliant red colour and unique flavour make it a valuable food product [9]. It is claimed as a Thai traditional medicine for kidney stones. It is also used as an antibacterial, antifungal, hypocholesterolemic, diuretic, mild laxative and antihypertensive substance [10]. The objective of this study was to determine the suitability of roselle calyces as a raw material for production of probiotic roselle juice by *Lb. plantarum* and *Lb. casei*.

Methodology

Preparation of roselle juice

Dry roselle calyces (*Hibiscus sabdariffa* L.) were purchased from a local store and kept at 4°C prior to use. Roselle juice was prepared by boiling the dried calyces with water for 15 minutes using low heat. The boiled roselle juice was filtered through a thin white cloth to separate the calyces and the pH of the juice was adjusted with CaCO₃ before being sterilized for 15 min at 110 °C.

Strains and cultures

Probiotic lactic acid bacteria (*Lactobacillus plantarum* TISTR 863 and *Lactobacillus casei* TISTR 390) were obtained from the Thailand Institute of Scientific and Technological Research, Pathumthani. The cultures were grown at 37°C for 24 h in MRS broth and were used as an inoculum.

Fermentation of probiotic roselle juice

Fermentation experiments were conducted in 250-ml erylenmeyer flasks, each containing 100 ml of sterile roselle juice. All samples were inoculated with a 24-h culture ($>10^5$ cfu/ml) and incubated at 30 or 37°C for 72 h. Samples were taken every 24 h for chemical and microbiological analyses. The effect of sucrose concentration on the growth of lactic acid bacteria in roselle juice was also studied using 7% and 14% final sucrose concentration.

Effect of cold storage on cell viability in probiotic roselle juice

After 72-h of fermentation, the samples were stored at 7°C for 4 weeks. Samples were taken at weekly intervals and the viability of probiotic cultures in the juice was determined and expressed as colony forming units (cfu/ml).

Chemical and microbiological analyses

The pH of probiotic roselle juice was measured with a pH meter. Total acidity, expressed as percent lactic acid, was determined by titrating roselle juice samples with 0.1 N NaOH using phenolphthalein as indicator. Sugar content was analyzed as glucose by the phenol sulphuric acid method of Dubois *et al.* [11]. Viable cell counts (cfu/ml) were determined by the standard plate method with MRS agar after 48 h of incubation at 37°C.

Statistical analysis

All experiments were carried out in triplicate and the results expressed as mean \pm SD (standard deviation). Data generated from the experiments were analyzed for significance by the Student's t-test. The values within rows that have no common superscript are significantly different ($p < 0.05$) according to this testing method. Any two means not marked by the same superscript (for example, a and b or b and c within rows) are significantly different ($p < 0.05$). Any two means marked by the same superscript (for example, a and a or b and b within rows) are not significantly different ($p < 0.05$).

Results and Discussion

Viable cell counts, pH and titrable acidity of sucrose-free roselle juice during 72-h fermentation period

Both *Lb. plantarum* and *Lb. casei* were found capable of growing well in sterilized roselle juice without nutrient supplementation. The time courses of lactic acid fermentation of roselle juices by these species are presented in Tables 1-4, respectively, and show that *Lb. plantarum* and *Lb. casei* grew significantly better at 30°C than at 37°C for the first 24 h. The increase in cell numbers was more than 1.5 log cfu/ml at 30°C, compared with less than 0.7 log cfu/ml at 37°C. This observation was different from that reported by Ma and Marquis [12] who found that the optimum temperature for the growth of *Lb. casei* was 37°C, not 32 or 45°C. On the other hand, Shamala *et al.* [13] reported that the optimum temperature for the growth of *Lb. plantarum* was 30°C. Extending the fermentation beyond 24 h resulted in a significant decrease in the viable cell counts of lactic acid bacteria at 30°C, but not at 37°C. However, the viable cell number of both strains at 72-h fermentation at both temperatures was not much different (less than 0.6 log cfu/ml).

Table 1. Time courses of lactic acid fermentation of roselle by *Lb. plantarum* at 30°C.

Time (h)	pH	Acidity (% lactic acid)	Sugar (mg/ml)	Cell number (cfu/ml)
0	4.79±0.01 ^a	0.25±0.00 ^d	9.66±0.9 ^a	3.3±0.4X10 ^{7 a}
24	3.90±0.00 ^b	0.50±0.00 ^c	2.75±0.28 ^b	2.7±0.3X10 ^{9 b}
48	3.89±0.00 ^b	0.46±0.01 ^b	1.82±0.35 ^c	5.1±1.0X10 ^{8 c}
72	3.91±0.00 ^b	0.44±0.01 ^b	1.82±0.21 ^c	4.6±0.5X10 ^{8 c}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Table 2. Time courses of lactic acid fermentation of roselle by *Lb. casei* at 30°C.

Time (h)	pH	Acidity (% lactic acid)	Sugar (mg/ml)	Cell number (cfu/ml)
0	4.74±0.04 ^a	0.25±0.00 ^a	9.36±0.76 ^a	8.2±3.7X10 ^{7 a}
24	3.92±0.02 ^b	0.51±0.01 ^b	4.71±0.42 ^b	4.2±0.7X10 ^{9 b}
48	3.88±0.00 ^b	0.50±0.04 ^b	4.76±1.45 ^b	3.0±0.2X10 ^{8 c}
72	3.90±0.00 ^b	0.52±0.00 ^b	4.07±0.48 ^b	2.7±0.2X10 ^{8 c}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

According to Tables 1-4, the roselle juice fermented at 37°C showed a significantly faster reduction in pH for the first 24 h, compared with the juices fermented at 30°C (1.2 units vs. 0.8 units). However, after 48-h fermentation the pH of the juices incubated at 30 and 37°C showed nearly the same final pH, especially that of the roselle juice fermented by *Lb. plantarum*. This observation agrees with Narvhus *et al.* [14], who studied the production of fermented milk at 22, 30 and 37°C by *Lactococci* and observed that products incubated at 37°C showed a faster reduction in pH early in the fermentation period, but that after 18 h the products incubated at 30 and 37°C showed the same final pH. Both *Lb. plantarum* and *Lb. casei* caused a stable pH in roselle juice during prolonged incubation between 24 and 72 h at both 30 and 37°C. This observation was different from Ostlie *et al* [15], who studied the effect of temperature on metabolism of probiotic bacteria in milk and observed that after 24-h incubation *Lactobacillus reuteri* SD2112 and *Lb. rhamnosus* GG caused a further decrease in pH on prolonged incubation between 24 and 48 h at 30°C, but showed a stable pH during prolonged incubation at 37°C from 24 to 48 h.

Table 3. Time courses of lactic acid fermentation of roselle by *Lb. plantarum* at 37°C.

Time (h)	pH	Acidity (% lactic acid)	Sugar (mg/ml)	Cell number (cfu/ml)
0	5.32±0.03 ^a	0.17±0.01 ^a	13.09±4.5 ^a	4.5±1.5X10 ^{7a}
24	4.07±0.05 ^b	0.24±0.02 ^b	3.53±0.83 ^b	2.0±0.5X10 ^{8b}
48	4.15±0.12 ^b	0.20±0.00 ^b	3.49±0.90 ^b	1.6±0.2X10 ^{8b}
72	4.06±0.13 ^b	0.21±0.01 ^b	3.22±0.52 ^b	2.2±0.5X10 ^{8b}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Table 4. Time courses of lactic fermentation of roselle juice by *Lb. casei* at 37°C.

Time (h)	pH	Acidity (% lactic acid)	Sugar (mg/ml)	Cell number (cfu/ml)
0	4.97±0.04 ^a	0.17±0.01 ^a	20.69±4.99 ^a	2.6±0.1X10 ^{7a}
24	3.74±0.01 ^b	0.31±0.02 ^{bd}	1.67±0.13 ^b	4.2±0.8X10 ^{7a}
48	3.76±0.03 ^{bd}	0.28±0.01 ^b	0.98±0.14 ^c	2.4±0.1X10 ^{8b}
72	3.79±0.01 ^{cd}	0.32±0.00 ^{cd}	0.44±0.07 ^d	9.3±2.2X10 ^{8c}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Both *Lb. plantarum* and *Lb. casei* produced significantly more titrable acidity expressed as lactic acid at 30°C of fermentation than that at 37°C, even though at 37°C the cultures can utilize sugar in the juice more so than at 30°C (Tables 1-4). For example, *Lb. plantarum* produced only 0.2% acidity and reduced the pH to 4.0 at 72 h of fermentation at 37°C, but it produced 0.44% acidity and reduced the pH to 3.9 at 72 h of fermentation at 30°C. This was different from that found by Ostlie *et al* [15]. They reported that the amount of lactic acid produced in fermented milk by lactobacilli was highest at 37°C.

The survival of lactic acid bacteria in fermented sucrose-free roselle juice during cold storage

The data in Table 5 illustrates the effect of cold storage on the viability of both species of lactic acid bacteria in fermented roselle juice. *Lb. plantarum* grown in 30°C can survive for several weeks in the fermented roselle juice at 7°C, significantly better than that grown in 37°C. For example, the viable cell counts of *Lb. plantarum* grown at 30°C was still 1.8 X 10⁶ cfu/ml after 4 weeks of storage at 7°C, while that grown at 37°C was reduced to 2.9 X 10⁴ cfu/ml. In contrast, the growth temperature did not have much effect on the survival of *Lb. casei* during cold storage. The viable cell counts of *Lb. casei* was approximately 1.6 X 10⁶ cfu/ml after 4 weeks of storage at 7°C previously incubated at either 30°C or 37°C. For the maximum health benefits, not less than a million viable cells/ml of probiotic product have to be present for transfer of the 'probiotic' effect to consumers.

The probiotic culture should be able to multiply to reach high cell counts in the fermented product and possess a high acid tolerance to ensure high viable cell numbers during storage [5]. The viability of probiotic organisms is dependent on many factors, such as the level of oxygen in products, oxygen permeation of the package, fermentation time and storage temperature [16]. Furthermore, it is also affected by lactic acid produced during production and cold storage [17, 18].

Table 5. Effect of cold storage on the viability of lactic cultures in fermented roselle juice.

Time (Wks)	cfu/ml (30°C)		cfu/ml (37°C)	
	<i>Lb. plantarum</i>	<i>Lb. casei</i>	<i>Lb. plantarum</i>	<i>Lb. casei</i>
0	4.6±0.5X10 ^{8a}	2.7±0.2X10 ^{8a}	2.2±0.5X10 ^{8a}	9.3±2.2X10 ^{8a}
1	2.9±1.0X10 ^{7a}	4.6±0.5X10 ^{6b}	1.3±0.2X10 ^{7b}	1.4±0.4X10 ^{7b}
2	2.1±0.1X10 ^{6b}	2.3±0.04X10 ^{6c}	9.1±0.9X10 ^{5c}	1.2±0.08X10 ^{7b}
3	1.8±0.2X10 ^{6b}	1.9±0.1X10 ^{6d}	7.0±0.6X10 ^{4d}	6.4±0.3X10 ^{6c}
4	1.8±0.1X10 ^{6b}	1.8±0.06X10 ^{6d}	2.9±0.3X10 ^{4e}	1.6±0.03X10 ^{6d}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Growth and acid production of lactic acid bacteria in sucrose-added roselle juice during 72-h fermentation period

When sucrose was added to the roselle juice and fermentation undertaken at 30°C, both *Lb. plantarum* and *Lb. casei* produced significantly more titrable acidity expressed as lactic acid than the sucrose-free juice (Tables 1, 2, and 6-10). The increase of acidity at 72-h fermentation was approximately 3 times higher in sucrose-added roselle juice fermented with *Lb. plantarum* (Table 1, 6 and 7) and nearly 2.5 times higher in sucrose-added juices fermented with *Lb. casei* (Table 2, 8 and 9), compared with the sucrose-free juice. Greater reduction of pH for the first 24 h of fermentation in the sucrose-added roselle juice was also observed. After 48 h, the pH of all fermented sucrose-added roselle juice was reduced below 3.5. Haddadin [19] also reported the significant effect of sugar on the acidification of milk. He found that the addition of glucose and galactose caused the faster acidification of milk fermented by *Lb. casei* and *Lb. plantarum*, compared with the control. On the other hand, the addition of sucrose did not support good growth of *Lb. plantarum*, same as the results reported by Shamala *et al* [13]. However, sucrose supported the growth of *Lb. casei* in this study. Its cell number at 72-h of fermentation was 4.7 – 6.4 X 10⁹ cfu/ml in sucrose-added roselle juice, compared with 2.7 X 10⁸ cfu/ml in sucrose-free juice.

Table 6. Time courses of lactic fermentation of roselle juice by *Lb. plantarum* at 30°C with the addition of 7% sucrose.

Time (h)	pH	Acidity (% lactic acid)	Cell number (cfu/ml)
0	4.80 ± 0.00 ^a	0.24 ± 0.02 ^a	1.1 ± 0.90 X 10 ^{8 a}
24	3.52 ± 0.02 ^b	0.75 ± 0.04 ^b	5.2 ± 0.8 X 10 ^{9 b}
48	3.28 ± 0.00 ^c	1.17 ± 0.00 ^c	2.7 ± 0.2 X 10 ^{8 c}
72	3.26 ± 0.01 ^c	1.19 ± 0.05 ^c	3.6 ± 0.6 X 10 ^{8 c}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Table 7. Time courses of lactic fermentation of roselle juice by *Lb. plantarum* at 30°C with the addition of 14% sucrose.

Time (h)	pH	Acidity (% lactic acid)	Cell number (cfu/ml)
0	4.79 ± 0.02 ^a	0.24 ± 0.02 ^a	2.6 ± 0.75 X 10 ^{8 a}
24	3.49 ± 0.02 ^b	0.72 ± 0.03 ^b	6.0 ± 0.6 X 10 ^{9 b}
48	3.24 ± 0.01 ^c	1.17 ± 0.00 ^c	7.4 ± 0.5 X 10 ^{8 c}
72	3.19 ± 0.00 ^d	1.40 ± 0.00 ^d	6.1 ± 0.8 X 10 ^{8 c}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Table 8. Time courses of lactic fermentation of roselle juice by *Lb. casei* at 30°C with the addition of 7% sucrose.

Time (h)	pH	Acidity (% lactic acid)	Cell number (cfu/ml)
0	4.80 ± 0.00 ^a	0.25 ± 0.00 ^a	8.9 ± 0.90 X 10 ^{7 a}
24	3.80 ± 0.03 ^b	0.54 ± 0.03 ^b	7.9 ± 0.5 X 10 ^{9 b}
48	3.39 ± 0.01 ^c	0.90 ± 0.00 ^c	3.9 ± 0.6 X 10 ^{9 c}
72	3.30 ± 0.01 ^d	1.13 ± 0.04 ^d	4.7 ± 0.4 X 10 ^{9 c}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Table 9. Time courses of lactic fermentation of roselle juice by *Lb. casei* at 30°C with the addition of 14% sucrose.

Time (h)	pH	Acidity (% lactic acid)	Cell number (cfu/ml)
0	4.75 ± 0.02 ^a	0.24 ± 0.02 ^a	8.2 ± 1.6 X 10 ^{7a}
24	3.77 ± 0.03 ^b	0.54 ± 0.03 ^b	6.4 ± 0.8 X 10 ^{9b}
48	3.37 ± 0.01 ^c	0.91 ± 0.01 ^c	5.0 ± 0.4 X 10 ^{9b}
72	3.26 ± 0.01 ^d	1.24 ± 0.06 ^d	6.4 ± 0.5 X 10 ^{9b}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

The survival of lactic acid bacteria in fermented sucrose-added roselle juice during cold storage

Both species were not able to survive well at the low pH and high acidity conditions in fermented sucrose-added roselle juice at 7°C. As can be seen from Table 10, the viable cell counts of both species was reduced to approximately 10⁴ cfu/ml in fermented sucrose-added juice after 3 weeks of cold storage at 7°C, compared with 10⁶ cfu/ml in sucrose-free juices (Table 5). Nevertheless, the fermented juices with sugar had more acceptable taste and flavour than the fermented sucrose-free juice (personal observation). According to Luckow and Delahunty [7], the sensory characteristics of probiotic blackcurrant juice was 'perfumey' and 'dairy' in odour and 'sour' and 'savory' in flavour. These off-flavours also occurred in roselle juice. However, when sucrose was added at the beginning of fermentation, these flavours seemed to be reduced and the taste was more acceptable (personal observation).

Table 10. Effect of cold storage on the viability of lactic cultures in fermented roselle juice at different sugar concentrations.

Time (Weeks)	cfu/ml (7% sucrose)		cfu/ml (14% sucrose)	
	<i>Lb. plantarum</i>	<i>Lb. casei</i>	<i>Lb. plantarum</i>	<i>Lb. casei</i>
0	3.6 ± 0.6 X 10 ^{8a}	4.7 ± 0.4 X 10 ^{9a}	6.1 ± 0.8 X 10 ^{8a}	6.4 ± 0.5 X 10 ^{9a}
1	3.9 ± 0.9 X 10 ^{6b}	3.0 ± 0.2 X 10 ^{6b}	3.6 ± 0.6 X 10 ^{6b}	2.6 ± 0.1 X 10 ^{6b}
2	1.1 ± 0.5 X 10 ^{5c}	8.8 ± 3.4 X 10 ^{4c}	3.5 ± 0.7 X 10 ^{4c}	1.2 ± 0.6 X 10 ^{4c}
3	5.2 ± 0.3 X 10 ^{4c}	8.1 ± 3.4 X 10 ^{4c}	1.9 ± 0.3 X 10 ^{4cd}	2.5 ± 1.3 X 10 ^{4c}
4	4.4 ± 0.4 X 10 ^{4c}	3.7 ± 0.3 X 10 ^{4c}	1.3 ± 0.1 X 10 ^{4d}	5.0 ± 0.9 X 10 ^{3d}

Mean and standard deviation for n = 3. Means with different letters within the same row are statistically significant (p<0.05) according to t-test range.

Conclusions

Two lactic acid bacteria, *Lb. casei* and *Lb. plantarum* were examined for their ability to utilize roselle juice for cell synthesis and lactic acid production without nutrient supplement. These lactic cultures grew better in roselle juice at 30°C than at 37°C and the viable cell counts reached 10⁹ cfu/ml at 24 h of fermentation at 30°C. Both *Lb. plantarum* and *Lb. casei* were capable of surviving the low pH and high acidic conditions in fermented roselle juice during cold storage at 7°C. On the other hand, when sucrose was added to the roselle juice at the beginning of fermentation, both cultures could not survive well at the low pH and high acidity in fermented sucrose-added roselle juice. The cell number was reduced to approximately 10⁴ cfu/ml after 3 weeks of cold storage at 7°C. From the results of this study, it is concluded that both *Lb. plantarum* and *Lb. casei* are suitable for use as probiotic cultures for production of a healthy beverage from roselle calyces for vegetarians or consumers who are allergic to lactose present in probiotic dairy products.

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