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Sodium citrate reduces residual levels of carbohydrates and increases bacterial counts in a fermented mixed vegetables medium

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ABSTRACT

Incomplete sugar catabolism occurs in many traditional vegetable-based lactic fermentations, such as sauerkraut and kimchi. The goal of this study was to extend sugar catabolism and improve growth of a mixed lactic starter (*Lactobacillus plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici*) by adding a citrate buffer salt or by external pH control. Fermentation of a vegetable juice medium (VJM) with external pH control (pH = 5.6) enabled utilization of 92% of total sugars after 72 h at 20 °C. When 25–100 mM sodium citrate was added to the VJM, a 3.5-fold increase in viable counts of *Leuconostoc mesenteroides* occurred in fermented VJM and lower levels of residual sugars were obtained in the fermented product. These results suggest that addition of sodium citrate into VJM prior to fermentation could increase sugar catabolism as well as viable counts of lactic bacteria with some being potentially probiotic.

1. Introduction

Most manufacturers of fermented vegetables package their products in glass jars or cans, and subsequently heat them to stabilize the product during storage. However, some companies market unheated fermented vegetables with live cultures, in the hope of offering consumers the health benefits of live lactic and probiotic bacteria, such as with yogurt. These fermented products present two challenges during storage: viability losses of the lactic/probiotic bacteria, and microbial spoilage, mainly from yeast (Savard, Beaulieu, Gardner, & Champagne, 2002). Means to avoid these two problems in probiotic-carrying fermented vegetables need to be developed.

In the lactic fermentation of vegetables, the pH drops below 4.0, which limits bacterial growth. As a result, there are residual sugars in the fermented product (Gardner, Savard, Obermeier, Caldwell, & Champagne, 2001). In industrial products that are not stabilized by a heat treatment, or that do not contain preservatives, spoilage by yeast during storage may occur (Fleming, 1982; Chap. 7; Savard et al., 2002). Thus, eliminating residual carbohydrates in the fermented products could potentially enhance the stability of unpasteurized products during storage.

Addition of acetate (Etchells, Bell, Fleming, Kelling, & Thompson, 1973), carbonate (Seo et al., 2009) or citrate buffers (Lu, Fleming, McFeeters, & Yoon, 2002) to extend the fermentation have been proposed. Citrate addition was shown to improve sugar assimilation in cucumber with *Lactobacillus plantarum* (Lu et al., 2002), but no data are available on other vegetable substrates, and with mixed starter cultures.

This study was done to determine if pH is indeed a limiting factor for complete sugar conversion in a vegetable juice medium (VJM) by carrying out a fermentation with external pH control. Second, it was ascertained if adding sodium citrate could enable the complete sugar assimilation during the lactic fermentation of a VJM with a starter composed of three strains.

2. Materials and methods

2.1. Microbial strains and media

A mixed starter culture specifically designed for the fermentation of vegetables (Gardner et al., 2001) was used. It was composed of *Lactobacillus plantarum* NK 312 (Lallemand, St-Simon, France), *Pediococcus acidilactici* AFERM 772 (Quest, Lachine, Canada) and *Leuconostoc mesenteroides* BLAC (CRDA, St-Hyacinthe, Canada) in a 1:1:1 ratio. The starter blend is marketed by Caldwell Bio Fermentation Canada (Coaticook, QC, Canada). The VJM was composed of 61% carrot juice, 12% cabbage juice, 3% onion juice and 24% brine containing 80g l^{-1} sea salt (Nature's Cargo; Tocher Trading Co. Ltd., Allenford, ON Canada) (final

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Table 1
Effect of the addition of sodium citrate on the highest viable counts reached (CFU.ml^{-1}) in the fermented vegetable juice media (VJM), and on substrate utilization levels (%).

Parameter	Traditional	External pH control at pH = 5.6	Citrate addition level				
			25 mM	75 mM	100 mM	150 mM	200 mM
Viable count: Lactobacillus plantarum	3.8 x 10^8 ab	6.3 x 10^8 ab	6.2 x 10^8 ab	9.2 x 10^8 a	2.0 x 10^8 b	7.2 x 10^6 c	5.4 x 10^6 c
Viable count: Pediococcus acidilactici	1.2 x 10^7 a	4.1 x 10^7 a	1.2 x 10^7 a	5.0 x 10^6 a	7.9 x 10^6 a	1.7 x 10^5 b	3.0 x 10^5 b
Viable count: Leuconostoc mesenteroides	3.0 x 10^8 a	9.3 x 10^8 b	1.3 x 10^9 b	1.2 x 10^9 b	3.8 x 10^8 ab	1.0 x 10^5 c	4.7 x 10^4 c
Total unfermented carbohydrate level (%)[a]	67 ab	8 c	42 b	44 b	52 b	96 a	95 a
Residual citrate level (%)[b]	ND	ND	59 b	69 b	73 b	96 a	99 a

a,b,c: For a given parameter/line (viable count or residual level), values that are followed with the same letter are not considered to be statistically different ($p \geq 0.05$).
ND = Not determined.
[a] Initial concentrations of glucose, fructose and sucrose were 21, 23 and 22 g l^{-1}, respectively. The total unfermented carbohydrate level corresponds to the fraction of sum of the three carbohydrates which remained in the medium after fermentation, as compared to the sum of these sugars at the beginning.
[b] Corresponds to the % of citrate which remained in the fermented medium as compared to the value at the beginning; this calculation takes into account the initial quantity of citrate (15 mM) and the quantity of citrate added as a buffering agent.

concentration of $20 g\,l^{-1}$). The juices were prepared at the research centre as described by Gardner et al. (2001). The vegetables were placed individually in a Santos juice extractor (Vaulx-en-Velin, Lyon, France) and the resulting liquids were centrifuged ($3000 \times g$/15 min) (Beckman, Palo Alto, CA, USA; JLA series 8.100 rotor) to separate pulp and juice. Due to the variability in pH between lots and types of vegetables, the VJM was standardized by adjusting to pH 6.0 with 0.1 mol l^{-1} NaOH. The medium was then sterilized (110 °C/10 min) as described by Gardner et al. (2001). However, heating of the vegetable base to eliminate their wild microbiota is not done commercially. It was nevertheless carried out in these assays to better control the variability in microbiological counts of raw material to obtain more accurate data on the growth of the starter strains.

2.2. Fermentations

In the production of lactic starters, there are two methods to reduce or prevent excessive acidification. The first is to systematically add an alkali to the broth throughout the fermentation to maintain the pH at a given value (Peebles, Gilliland, & Speck, 1969). This is considered to be "external pH control". The second method is to add buffering ingredients to the growth medium to delay the drop in pH. This approach is referred to as "internal pH control" (Ustunol, Hicks, & O'leary, 1986).

For the experiments, the VJM medium (1 l) was inoculated with the mixed starter and placed into 1.5-L fermentation vessels (Biostat M, B. Braun Instruments, Münster, Germany). The inoculation level was set at 3 x 10^6 CFU.ml^{-1} (1 x 10^6 CFU.ml^{-1} of each strain) and the medium was incubated at 20 °C for 72 h.

In one series of fermentations, identified as the "Traditional process", no external or internal form of pH control was carried out.

In fermentations under external pH control, the pH was allowed to reach a value of 5.60, at which point it was maintained at that level with 5 N NH_4OH. During these fermentations, the VJM was gently stirred (75 rpm). Ammonia was selected over NaOH because it generates higher biomass yields (Peebles et al., 1969).

In assays with sodium citrate (internal pH control), the fermentations were carried out in six double-jacketed stainless steel vessels containing 200 ml of VJM to which 25–300 mM of sodium citrate were added. The pH (Radiometer Red Rod electrodes; Radiometer Analytical, Villeurbanne, France) was recorded every 15 min and the temperature was kept at 20 °C by circulating hot or cold water in the jackets of the fermentation vessels using a BioGénie ID control system (Quebec City, QC, Canada). Agitation was provided by magnetic bars, maintained at about 75 rpm/min.

2.3. Analyses

HPLC analysis of VJM for sucrose, glucose, and fructose was carried out as described by Gardner et al. (2001). Briefly, 10 µl samples were injected in a 30 cm Aminex HPX-87H column (Bio-Rad Laboratories Canada Ltd., St. Laurent, QC, Canada) coupled to a refractive index monitor (Model 419, Waters, Montreal, QC, Canada). The column was kept at 45 °C, and the eluent was 0.08 HCl with a flow rate of 0.4 ml min^{-1}.

Viable counts of the three strains were obtained on differential and selective media. All three strains grew on Bacto Lactobacilli MRS® with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), but the only colonies having a white color were *Pediococcus* (Savard, Champagne, & Beaulieu, 2000). The *Lactobacillus* and *Leuconostoc* cultures gave blue-colored colonies on MRS + X-gal; thus, *Pediococcus* colony-forming-units (CFU) were obtained by counting the white colonies on MRS + X-gal. The medium of Mayeux, Sandine, and Elliker (1962) contained sodium azide, and only the *Leuconostoc* strain developed colonies on this medium. The *Lactobacillus* population was calculated by subtracting the *Leuconostoc* CFU obtained on the Mayeux medium from the total number of blue colonies on the MRS + X-gal medium.

The experiments consisted of two independent trials, each done in duplicate. When the two groups were compared, an "unpaired *t*-test" was carried out. When means of 5–7 groups were compared (Table 1) a one-way ANOVA was done. These statistical analyses were carried out with InStat software (GraphPad, San Diego, CA, USA) at an acceptance level of $p < 0.05$.

3. Results and discussion

The initial intention was to use calcium citrate to improve the healthfulness of the fermented food, e.g., as a potential source of calcium. The addition of calcium citrate resulted in a white opaque VJM. This was considered to be undesirable, and the calcium salts were rejected on this basis. Turbidity problems with calcium salts have also been reported by Seo et al. (2009) in kimchi. Thus, the sodium form of citrate was used in the study.

3.1. Fermentation with external pH control

The hypothesis was that sugar assimilation by the lactic acid bacteria in fermented vegetables is inhibited due to reaching a pH below 4.0. To test this hypothesis, the experiments were designed to determine if greater assimilation of the main sugars was possible with external pH control during the fermentation. At a constant pH of 5.6, it took 72 h to complete the fermentation. Compared to the "Traditional" treatment, higher viable counts of the three strains were obtained in the product with external pH control (Table 1). However, the only organism for which this increase was statistically significant ($p < 0.05$) was for *Leuconostoc mesenteroides*.

Initial glucose, fructose and sucrose levels in the VJM are shown in Table 1. Without any form of pH control, the metabolism of carbohy-