Fermentation of Carrot Juice by Probiotics: Viability and Preservation of Adhesion

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Abstract: In the present work, carrot juice was investigated as an alternative carrier for probiotics. Pasteurized juice was fermented anaerobically over night at 37°C with selected probiotics and their viability was assessed after 1, 2, 4, 8 and 12 week storage. Identification was performed by species specific PCR. Mucus adhesion was assessed of carrot juice and laboratory medium grown *Lactobacillus* strains. At inoculation, the probiotic strains were present at levels of 10^6-10^7 CFU/ml and during fermentation the lactobacilli numbers increased to 10^9-10^{10} CFU/ml. After four weeks storage, *Lactobacillus* levels remained almost unchanged and after 12 weeks there were still 10^7-10^9 CFU/ml lactobacilli. Bifidobacteria, however, did not grow and started to decline after two weeks and were undetectable after 8 weeks. Adhesion of carrot juice grown lactobacilli was 50-70% less then adhesion of the strains grown in laboratory medium. The results suggest that fermented carrot juice is a promising carrier for probiotic lactobacilli but not bifidobacteria, but may alter the phenotypic properties of lactobacilli.

Keywords: Lactic acid bacteria, bifidobacteria, vegetables, identification, species specific PCR.

1. INTRODUCTION

Fermentation is a traditional way to preserve vegetables. Nowadays it is not so common to use fermented vegetables in the Western diet as food preservation utilizes pasteurization, sterilization, refrigeration/freezing and preservatives. However, such near sterile food does not contain microbes that may be valuable to our health [1]. Fermented dairy products are the main source of food microbes in the Western diet and dairy products are the most common food matrix for administering probiotics. However, some populations do not consume milk products out of principle or due to lactose intolerance. Therefore, probiotic containing products from plant origin could be a valuable alternative. Fermentation makes certain vegetable products more suitable for human consumption, e.g. through removal of anti-nutritional factors. Vegetables often contain non-digestible or slowly digestable oligosaccharides. These carbohydrates can be metabolized by the microbiota in the caecum and colon [2], this process can be supported with additional bacteria from foods [3].

The most common fermented vegetables are sauerkraut and soured gherkins which are fermented mainly by lactic acid bacteria that are important in flavor formation [4-6]. In Mediterranean countries, olives [7] and for example Brussels sprouts are fermented [8, 9]. Fermentation used to be a spontaneous process, but today also defined starters are used in the fermentation process [10]. Vegetable juices have been fermented and earlier research indicates that they may function as vehicles for probiotics delivery [11]. In the current study, 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 carrots, carrot juice and beetroot has been found to positively influence the availability of some minerals (Ca, P, Fe), β-carotene, betaine and vitamin C [12]. In fermented vegetables, lactic acid fermentation can increase levels of vitamins like folate [13] and cobalamin [14] and improve iron solubility in carrot juice. The level of improvement was strain specific and related to the produced acids rather than a simple pH effect [15].

In this study, probiotics were used to ferment carrot juice and the products were investigated for their content of probiotics during storage, pH and the adhesion of the strains after growth in carrot juice.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

The organisms used were commercial strains; *Lactobacillus plantarum* Lp-115 (Deposited by Danisco, Niebüll, Germany at Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig,

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Germany; DSM22266 and kindly provided by Danisco) is a vegetable starter, *Lactobacillus paracasei* Lpc-37 (Deposited by Danisco at the American Type Culture Collection, Manassas, US; ATCC: SD5275 and kindly provided by Danisco), *Bifidobacterium lactis* 420 (Deposited by Danisco at Deutsche Sammlung von Mikroorganismen und Zellkulturen; DSM22089and kindly provided by Danisco), *Lactobacillus rhamnosus* GG (Deposited by Valio Ltd, Helsinki, Finland at the American Type Culture Collection ATCC53103, and kindly provided Valio) and *Bifidobacterium lactis* Bb-12 (Deposited by Chr. Hansen, Hørsholm, Denmark at Deutsche Sammlung von Mikroorganismen und Zellkulturen; DSM15954, kindly provided by Chr. Hansen,) are probiotic cultures.

2.2. Producing and Fermenting of Carrot Juice

After peeling, carrots were washed and squeezed to produce juice. Fresh carrot juice was filtered through a cloth, pasteurized 30 s in a water bath in glass bottles (500 ml) at 72°C and centrifuged (16000 g, 5 min). Bacterial strains were precultured anaerobically at 37°C over night in MRS (Oxoid) broth (lactobacilli) and GAM broth (Nissui Seiyaku Co., Tokyo, Japan) (bifidobacteria). Carrot juice was inoculated separately (1% v/v; final concentration approximately 10^7 CFU/ml) with the five cultures and divided in three parallel samples. Fermentation was performed anaerobically for 18 h at 37°C.

2.3. Storage of Carrot Juice and Viability Determination

After fermentation, the juices were divided into polypropylene tubes (50 ml) for each sampling time point and stored dark at +4ºC. Viability of probiotics was studied by culturing lactobacilll on Rogosa–agar (Merck, Darmstadt, Germany) and bifidobacteria on reinforced clostridial medium (RCM–agar, Oxoid, Hampshire, England) anaerobically at 37°C for four days. Cultivations were made after inoculation of probiotics, immediately after fermentation (18 hours) and subsequently after 1, 2, 4, 8 and 12 weeks storage. After cultivation, ten bacterial colonies were collected from the plates, grown in broth and stored (-70°C) in 15 % glycerol until further identification. Less bifidobacteria were collected because only few colonies grew. Isolates were purified by cultivating on Rogosa and RCM agars at 37°C for two days. Three isolates of each fermentation and each collection point were identified.

In addition to viable numbers and pH–values were determined, and control cultivations from un-inoculated material were performed.

2.4. Identification of Probiotic Strains

Species specific PCR was used to identify bacterial isolates. Frozen purified isolates were cultured in MRS and GAM in broths at 37°C over night. Cultures were used as template in PCR, however, bacteria collected from *Lb. plantarum* Lp-115–fermentations needed lysis of bacteria in saline-TE buffer (0.9 % NaCl, 1 M Tris-HCl, 0.5 M EDTA). Bacterial cultures (20 µl) and saline-TE (50 µl) were added to a microfuge tube and heated at 96°C for 10 minutes, cooled and used as template (1:10).

PCR – amplifications were obtained by BioRad iCycler synthesizer. Programs used in these amplifications are described in Table **1**. Species-specific primers were used in PCR –amplifications: *Lactobacillus* GG [16], *Lb. plantarum* 115 (J. L. Vera, University of Turku), *Lb. paracasei* 37 (J. L. Vera, University of Turku) and bifidobacteria [17]. PCR–products were analyzed by agarose gel electrophoresis using Biorad PowerPac 300 (80 V, 45 min).

DNA polymerase used in amplifications was AmpliTaqGold (Roche, New Jersey, USA) 2 U. The other reagents in reactions are Taq buffer (1x), Mg_2Cl (2.5 mM), dNTP (0.2 mM) and primers which are used

Table 1: Programs Used to Amplify PCR –Products with Species-Specific Primers

cycle	*Lactobacillus* GG	*Lb. plantarum* Lp-115	*Lb. paracasei* Lpc-37	Bifidobacteria
1	95 °C 10 min	95 °C 10 min	95 °C 10 min	95 °C 10 min
2	40 x: 95 °C 15 s 58 °C 1 min 72 °C 45 s	35 x: 95 °C 15 s 38 °C 1 min 72 °C 1min	35 x: 95 °C 15 s 52 °C 30 s 72 °C 1min	35 x: 95 °C 15 s 69 °C 1 min 72 °C 45 s
3	72 °C 10 min	72 °C 5 min	72 °C 5 min	72 °C 10 min
4	4 °C ∞	4 °C ∞	4 °C ∞	4 °C ∞

for lactobacilli 0.4 µM and for bifidobacteria 0.25 µM. Template was added as 1 µl.

2.5. Other Microbes

Each fermentation was investigated for molds, yeasts and coliforms at each time point. Molds and yeasts were checked on chloramphenicol agar plates (Sabouraud dextrose agar + cloramphenicol, Pronadisa, Madrid, Spain) and coliforms on violet-red bile glucose agar plates (Pronadisa). Chloramphenicol agar plates were incubated at 30°C for five days and violet-red bile agar plates in the same temperature for 2 days.

2.6. Measuring of Adhesion of Bacterial Strains to Colon Mucus

Adhesion studies were basically performed as described by Ouwehand *et al*. [18]. In short; per well, 100 µl human colon mucus was passively immobilized on Maxisorp microtitre plates (Nunc, 96 Wells, Roskilde, Denmark) by 16 h storage at 4ºC. Mucus was diluted with Hepes-Hanks (pH 7.5) buffer to a concentration of 0.5 mg/ml.

Identified isolates from fermented carrot juice, three from each fermentation, were cultivated first in MRS – medium overnight 37°C. The original *Lactobacillus* strains were used as control. Strains were grown in pasteurized carrot juice at 37°C 16-20 h. Radioactive tritium-thymidine (TRK 328, Amersham Biosciences, UK), 5 µl/ml, was added to the cultivation media. Bacteria were collected by centrifugation (2 100 g 7 min) and washed twice with Hepes-Hanks buffer (pH 7.5) in equal volume and suspended in same buffer. Bacterial suspensions were diluted to absorbance 0.25 ± 0.01. Suspensions were added (four parallels) to a mucus coated microtitre plate and incubated at 37°C for one hour. The plate was washed twice by HEPES-Hanks buffer and NaOH-SDS (0.1 M and 1 % w/v respectively) was added to the wells and incubated one hour at 60°C to release the adhered bacteria. The material was transferred to microfuge tubes filled with 1 ml scintillation liquid (Optiphase 'HiSafe' 3, Perkin Elmer, UK). Radioactivity was measured by scintillation counter Microbeta 1450 (Wallac, Turku, Finland). Change in adhesion results was calculated as percents of radioactivity recovered from the wells with carrot juice grown bacteria compared to the radioactivity of MRS grown bacteria. *Salmonella typhimurium* (ATCC 14028) strain was used as negative control of adhesion and *Lb. rhamnosus* GG as positive control.

2.6. Statistical Analysis

The results are the average of 3 independent adhesion experiments. Data are expressed as the mean value with standard deviation. Differences were calculated by student's-t test. p-values less than 0.05 were considered significant.

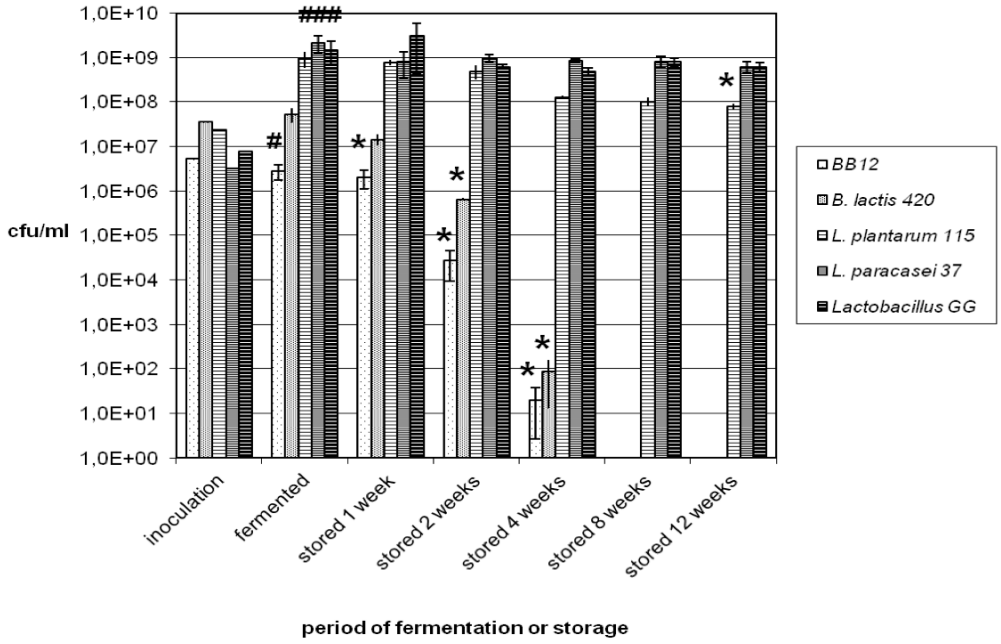

Figure 1: Viable counts of tested bacteria in fermented carrot juice, at inoculation, after fermentation and after refrigerated storage. Viable counts were determined by growth on Rogosa (lactobacilli) and reinforced clostridial agar (bifidobacteria), species were confirmed by PCR. #Significantly different from Inoculation. *Significantly different from Fermented $p<0.05$.

3. RESULTS

3.1. Viability

After fermentation, lactobacilli were present in higher numbers (10^8-10^9 CFU/ml), increasing from 10^6-10^7 CFU/ml at inoculation. This increase exhibited a strong trend for significance with all *Lactobacillus* strains tested *Lb. paracasei* Lpc-37 and *Lb. rhamnosus* GG and *Lb. plantarum* Lp-115 (t-test; p=0.06, 0.09 and 0.05 respectively) Figure **1**.

Bifidobacteria-like organisms, however, did not grow during the fermentation. The viable numbers of *B. lactis* 420 remained similar to the level at the beginning of the fermentation 10^6-10^7 CFU/ml. Levels of *B. lactis* Bb-12, however, were significantly decreased after fermentation (p=0.047). Identification showed that there were substantial numbers of lactobacilli in the fermentations. Upon storage, the counts of both bifidobacteria decreased rapidly. After 1 week storage, bifidobacteria levels decreased substantially (p>0.05 for *B. lactis* Bb-12 and p=0.048 for *B. lactis* 420). This decrease continued until after 8 weeks storage no culturable bifidobacteria could be detected.

Lactobacilli levels remained stable for the first 4 weeks of storage. After 8 weeks of storage, only *Lb. plantarum* Lp-115 fell significantly (p=0.05) until they were below 10^8 CFU/ml after 12 weeks of storage, Figure **1**.

Neither yeasts or molds, nor coliforms could be detected in the carrot juice fermentations at any of the sampling times (detection limit 10 CFU/ml for yeasts and molds and 1 CFU/ml for coliforms).

3.2. Change of pH

Before fermentation, the pH-value of carrot juice was 6.7. Carrot juice fermented with lactobacilli had lower pH–values (3.8-3.9) than juice fermented with bifidobacteria (4.8-4.9). During storage, there was some increase in pH–values in the bifidobacteria fermented carrot juices (pH 5-5.2; p<0.05) and a reduction in the lactobacilli fermented juice (3.5-3.7; p<0.05) so pH values in fermented carrot juice by lactobacilli during storage reduced but values in juices fermented bifidobacteria raised.

3.3. Identification of Probiotics

Only few bifidobacteria could be identified from fermented carrot juices just after fermentation. After one week storage no *B. lactis* Bb-12 could be identified. *B. lactis* 420 could be identified at low levels after one week storage. The majority of the viable counts were therefore bacteria other than those inoculated. After four weeks storage, no bifidobacteria could be detected (<10 CFU/ml).

Lactobacilli were identified successfully. All the bacteria which were isolated from juices fermented with *Lb. rhamnosus* GG were identified as this strain. Also *Lb. plantarum* Lp-115 and *Lb. paracasei* Lpc-37 were identified successfully from fermentations. Three isolates were not identified as *Lb. paracasei*-37. These samples had been stored one week (two isolates) and 12 weeks. One isolate was not identified as *Lb. plantarum* Lp-115. This sample had been stored 12 weeks.

3.4. Changes in Adhesion of Probiotics to Colon Mucus After Fermentation in Carrot Juice

Adhesion of probiotics decreased after fermentations in carrot juice. The results were variable for all the strains. *Lactobacillus* GG showed the reduction of adhesion more than other bacteria used after fermentation of carrot juice. However, this reduction did not reach statistical significance. *Lb. plantarum* Lp-115 adhesion was 0.98 % (isolates grown in carrot juice) compared to 2.73 % of the original strain grown in MRS (p=0.007). Fermentation in carrot juice also caused some decrease in adhesion of isolates of *Lb. paracasei* Lpc-37 to 9.94 % of the original strain (16.7 %), though this did not reach statistical significance, Figure **2**. Adhesion of the negative control, *Salmonella typhimurium* (ATCC 14028) was 1.43 % (SD 1.06) on average.

4. DISCUSSION

The current investigation shows that specific probiotics, in particular lactobacilli, can ferment carrot juice well. However, bifidobacteria were not observed to grow and did also not survive in the fermented product for more than two weeks. Bifidobacteria do therefore not appear to be suitable for commercial production of fermented carrot juice by the methods used in this study. The lack of growth may be explained by a limited presence of easily fermentable carbohydrates as is also suggested by the limited reduction in pH. Alternatively, carrot juice may lack other essential nutrients required for *Bifidobacterium* growth. This could be improved by addition of a fermentable carbohydrate source, or the addition of

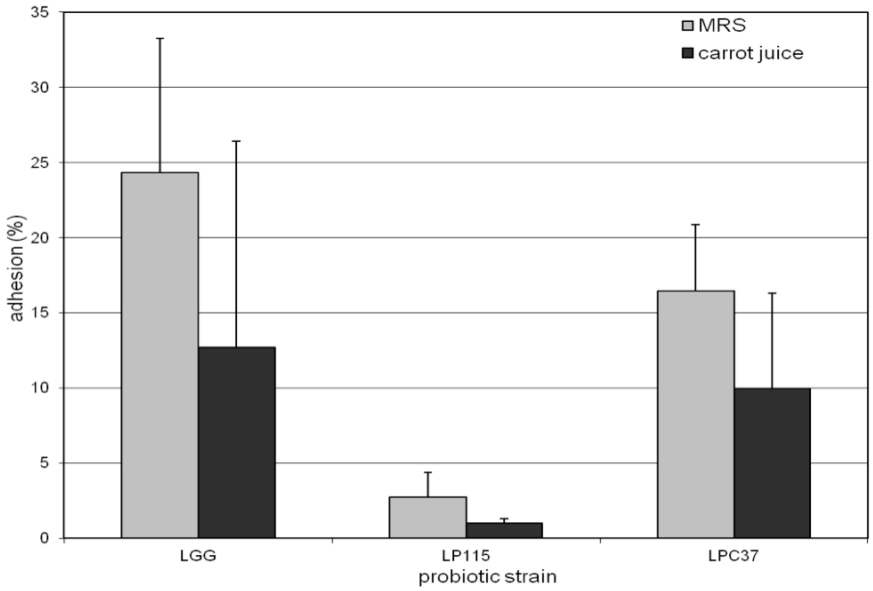

Figure 2: Adhesion of probiotic *Lactobacillus* strains to human colon mucus after growth in carrot juice. Control strains were cultured in MRS broth. Bacterial strains collected from fermented carrot juice and grown in carrot juice prior to the adhesion assay. *Significantly different from MRS grown bacteria, p=0.007.

enzymes that would make fermentable carbohydrates or other growth factors available. On the other hand, carrot juice has been demonstrated to be a suitable base for the *B. lactis* and *Bifidobacterium bifidum* strains [19] and also *Lb. rhamnosus* and *Lactobacillus bulgaricus* strains [20]. The poor survival, however, was not expected. Both tested *B. lactis* strains are known to survive well at low pH in dairy products [1,2] and *B. lactis* is known for their relatively high oxygen tolerance. A possible explanation could be the presence of antimicrobial activity in the carrot juice [21]. Furthermore, these carrot juices were pasteurized and apparently a substantial number of non-starter bacteria remained which out-competed the inoculated bifidobacteria. If bifidobacteria are to be used in carrot juice fermentation, the juice should be sterilized before fermentation. However, autoclaving causes coagulation and changing of color of the carrot juice and is therefore not an advisable way to sterilize the juice.

Lactobacilli could compete well with the remaining bacteria in the carrot juice and the pH –values were low, below pH 4. There were only few isolates which were not identifiable with species-specific PCR. Decrease of pH–values during storage of the juice indicates active metabolism of micro-organisms under low temperatures. During the 12 week following-up, *Lactobacillus* levels did not decrease significantly in the fermented carrot juice. Yoon *et al*. [22] reported on the development of probiotic cabbage juice. In their study *Lb. plantarum* C3, *Lb. casei* A4, and *Lb. delbrueckii* D7 were used as starters of fermentation. *Lb. casei*, *Lb. delbrueckii*, and *Lb. plantarum* reached nearly 10^9 CFU/ml after 48 h of fermentation at 30°C. After 4 weeks of cold storage at 4°C, the viable cell counts of *Lb. plantarum* and *Lb. delbrueckii* were 4.1×10^7 and 4.5×10^5 CFU/ml, respectively. *Lb. casei* lost cell viability completely after 2 weeks. When these results are compared with those of the current investigation, carrot juice and the *Lactobacillus* strains used have considerable potential in a vegetable based probiotic product.

Fermentation of carrot juice often causes coagulation of material but not as much as with for example autoclaving. The problem is not always the same and it would be useful to know the components contributing to this reaction. It may be possible to use certain ingredients, for example pectin or other thickeners, to prevent this coagulation.

Isolates collected after carrot juice fermentation exhibited a considerable reduction of their adhesion ability, although this was only significant for *Lb. plantarum* Lp-115. Adhesion of bacteria can change after cultivation in different broths. In a matrix with low pH, bacterial adhesion has been shown to be reduced [18]. The influence of the food matrix therefore deserves more attention as it could affect the functionality of selected probiotics.

In conclusion, lactobacilli fermented carrot juice well and survived in the fermented carrot juice for up to 12

weeks. Bifidobacteria, on the other hand, did not survive as well as lactobacilli. This suggests that fermented carrot juice is a promising alternative food matrix for probiotic lactobacilli but not for bifidobacteria.

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