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Research Article

Development of a Novel Probiotic Yogurt "PENTOYO" with a Fully Sequenced *Lactobacillus pentosus* KCA1 and its Survival during Storage at 4 ^{o}C

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Abstract

Purpose: To determine whether *L. pentosus* KCA1 can be used to create a new probiotic yogurt and the organism's duration of survival when stored at 4 ^{o}C.
Methods: Mother cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* and *L. pentosus* KCA1 were prepared and subsequently added to a suspension of pasteurized milk. It was then incubated at 37 ^{o}C for 6 h, after which it was removed and placed in a refrigerator at 4 ^{o}C. Survival test was determined in MRS agar plate supplemented with 30 µg of tetracycline for the selective enumeration of *L. pentosus* KCA1 at predetermined intervals over a period of 63 days at 4 ^{o}C.
Results: pH decreased both in normal yogurt and probiotic yogurt and there was no significant difference ($p > 0.05$) in the pH of the two preparations. The strain showed higher viability for 49 days, indicating the presence of a sufficient number of viable bacterial cells at 4 ^{o}C. There were only 3 log cycle losses in the number of cells surviving from day 1 (5.6×10^9 cfu/ml) to day 49 (5.5×10^6 cfu/ml).
Conclusion: This study shows that yogurt has the potential to deliver biotherapeutic benefits associated with probiotic bacteria to consumers adequately.

Keywords: Probiotics, *Lactobacillus pentosus* KCA1, Yogurt, Health benefit

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INTRODUCTION

There has been a shift in fermented food production and intake in many developing countries due to the introduction of Western foods designed with long shelf-life rather than meeting nutritional needs. We have previously identified a lactic acid bacteria probiotic strain that holds great promise, especially as it has genomic capabilities for inhibiting pathogens [1].

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. Various probiotic bacteria have been shown to provide biotherapeutic benefits such as faster relief from diarrhea, modulation of immune system, alleviation from lactose intolerance, reduction in cholesterol and prevention of urogenital infections [3]. Although the mechanisms of action of probiotics are not fully understood, the ability of probiotic organisms to compete for adherence site, and nutrients, as well as production of antimicrobial substances such as bacteriocins have been demonstrated [4].
In our previous study, the *Lactobacillus pentosus* KCA1 (formally *L. plantarum* KCA1) strain isolate was found to produce huge amounts of biosurfactants and hydrogen peroxide, and also inhibited the growth of intestinal and urogenital pathogens [1]. In addition, the isolate exhibited varying degrees of acid and bile tolerance [5]. To deliver probiotic organisms to consumers requires a shuttle. and milk has been found to be useful in this regard. However, other probiotic bacteria generally do not grow rapidly in cow milk [6] and, in addition, the normal yogurt starter cultures, *L. delbreukii* sub-species *bulgaricus* and *Streptococcus thermophilus*, are not bile resistant. Yogurt in reality is not a probiotic, as the organisms are designed to ferment the milk, and most of them die when consumed due to stomach acid pH and bile salt.

However, in order to stimulate the process of delivering the potential beneficial effects of *Lactobacillus pentosus* KCA1 into various consumer products, we sought in this study to, first, determine whether *L. pentosus* KCA1 can be used to create a new probiotic yogurt here coined "PENTOYO" and, second, to assess how long the organism can survive during storage in a refrigerator at 4 ^oC.

EXPERIMENTAL

Starter cultures

Lyophilized yogurt starter culture (2 g) containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* was purchased locally (Lyo-San, Ca) and added directly to 10 ml sterilized de Man Rogosa and Sharp (MRS) broth (MicroMaster, India). The broth was incubated micro-aerophilically using Gas Pak (BBL Gas Park, BD &Co. Sparks, MD) at 37 ^oC overnight. Stocks were prepared routinely in MRS broth during the study period.

Preparation of *Lactobacillus pentosus* KCA1 culture

Frozen stock cultures of *L. pentosus* KCA1 (20 % glycerol in MRS broth) were re-constituted by plating out in MRS agar and incubated at 37 ^oC for 18 h and thereafter a colony was picked and added to 10 ml fresh MRS broth and incubated micro-aerophilically for 18 h at 37 ^oC.

Preparation of Mother cultures

In preparing mothers of the yogurt starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, 10 g of skimmed powered milk was dissolved in 100 ml sterile water and pasteurized at 82 ^oC for 30 min and cooled to 37 ^oC. The starter cultures grown in MRS broth were centrifuged and washed twice with sterile PBS (phosphate buffered saline) to remove MRS broth and thereafter the bacterial pellets were reconstituted in 2 ml of PBS. Out of this, a 100 ul aliquot containing 2.5 x 10^8 cfu/ml was added to 10 ml of the pasteurized milk and incubated for 18 h at 37 ^{o}C.

Developing probiotic yogurt

The mother culture (10 ml) of *Lactobacillus delbrueckii*, subsp. *bulgaricus* and *Streptococcus thermophilus*, was added to the final milk volume containing 230 g of powered milk and 5 % granulated sugar, suspended in 1 L of sterile water. *Lactobacillus pentosus* KCA1 culture, previously prepared and washed twice with PBS, was added at a level of 10 % final milk volume. The suspension was pasteurized as stated above, incubated at 37 ^{o}C for 6 h, after which it was removed and placed in a refrigerator at 4 ^{o}C. The control yogurt did not contain *L. pentosus* KCA1 strain.

Measurement of pH

The pH of the preparations at 0 h and after fermentation was measured using a digital microprocessor pH meter. (pHep®3, Hanna Instruments, USA). The pH meter was standardized using reference pH 4.0 and 7.0 buffer solutions.

Survival test of probiotic bacteria

For the survival test, the prepared probiotic yogurt was stored at 4 ^{o}C for 65 days; weekly, the stored product was diluted serially 10^{-1} to 10^{-7} in sterile PBS and plated out in triplicate in MRS agar plate supplemented with 30 ug of tetracycline for the selective enumeration of *L. pentosus* KCA1. Tetracycline was used based on both the phenotypic and genomic properties of *L. pentosus* KCA1 as the strain encodes chromosomal genes for multidrug resistance, including a gene locus for tetracycline resistance. Viable numbers of *L. pentosus* KCA1 were evaluated after 1, 7, 14, 21, 28, 35, 42, 49, 56, and 63 days of storage at 4 ^{o}C.

Statistical analysis

Student *t*- test was used when comparing the means of continuous data between two independent groups. Two-sided Fisher's Exact Test was used for significant associations between two categorical variables in 2 by 2 contingency tables. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was carried out using GraphPad Prism version 4 (GraphPad Software Inc, California, USA).

RESULTS

The addition of *Lactobacillus pentosus* KCA1 in the yogurt mixture was successful as the preparation yielded a complete process. There was no difference in the texture of the probiotic yogurt and the yogurt preparation without *L. pentosus* KCA1. The pH decreased both in the normal yogurt and probiotic yogurt and there were no significant differences ($p = 0.8250$) in the pH of the two preparations.

The mean pH value at 0 h (immediately after adding the mother starter culture and *L. pentosus* KCA1) was 6.4 and it decreased to 4.22 after 6 h of fermentation (Table 1)

Table 1: Changes in pH of regular yogurt and probiotic yogurt prepared with *L. pentosus* KCA1

	pH	
Storage (days) at 4 ^{o}C	**Probiotic Yogurt (*L. pentosus* KCA1)**	**Regular Yogurt (*L. bulgaricus/S. thermophilus***
0*	6.40	6.41
1	4.22	4.30
7	4.21	4.25
14	4.20	4.23
21	4.20	4.23
35	4.15	4.20
42	4.10	4.20
49	4.05	4.15
56	3.98	4.12
63	3.96	4.10
Mean pH (±SD)	**4.35±0.28**	**4.42±0.70**

**Before fermentation*

The final pH at the end of the 63 days storage of probiotic yogurt was not significantly lower (pH 3.96) than that of the regular yogurt (pH 4.10) (p = 0.8244)

Enumeration of *Lactobacillus pentosus* KCA1

Figure 1 shows the survival of *Lactobacillus pentosus* KCA1 in yogurt over time. There were only 3 log cycle losses in the number of cells surviving from day 1 (5.6 x 10^9 log10 cfu/ml) to day 49 (5.5 x 10^6 log10 cfu/ml).

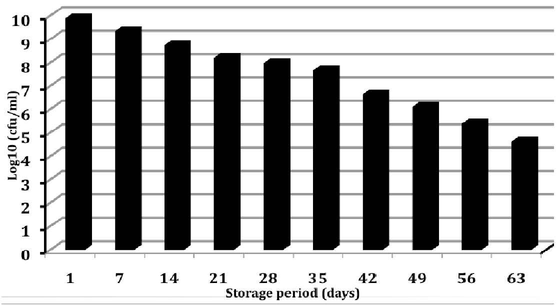

Fig 1: Mean survival of *L. pentosus* KCA1 strain in yogurt stored at 4 ^{o}C.

DISCUSSION

To the best of our knowledge, this is the first study to produce probiotic yogurt with *L. pentosus* KCA1 strain of human origin. Both the starter cultures alone and addition of *L. pentosus* KCA1 led to increased production of lactic acid and other products of fermentation as indicated by the decrease in pH. Low pH has been shown to trigger extracellular polysaccharide (EPS) production by *L. bulgaricus* [7], and increasing acid resistance. There are conflicting reports on probiotic strains with respect to survival in acidic niches, such as in milk products as a result of increase in the sensitivity of the strains to post-acidification during storage [8]. Although pH decreased slightly from 4.22 to 3.96 in the *L. pentosus* KCA1 product, compared with the conventional yogurt (pH 4.30 - 4.10), this did not affect overall survivability of *L. pentosus* KCA1. The poor survival of some probiotic strains in yogurt has been reported to be mainly due to the intrinsic properties of yogurt such as reduced pH [9]. A recent genomic study has shown that *L. pentosus* KCA1 contain acid-resistant loci and in addition *L. pentosus* KCA1 encodes seven genes for Na+/H+ antiporter which could also be involved in acid stress response as has been reported for similar genes in *L. plantarum* WCFS1 [10].

The characteristic yogurt flavour, which is mainly due to conversion of lactose to lactic acid, acetic acid, acetaldehyde, acetone, and diacetyl [11], was not affected by the addition of *L. pentosus* KCA1. This suggests that the strain might have potentiated activities in the conversion process, as predicted in the genome, which encodes 21 genes for lactose and galactose uptake and utilization, including a novel β-galactosidase 3 [12]. A recent transcriptome study has revealed the molecular basis of mixed-culture growth of the conventional yogurt starter cultures indicating that interactions between these bacteria are primarily related to purine, amino acid, and long-chain fatty acid metabolism [13].

In the *L. pentosus* KCA1 survival study, the strain showed higher viability for 49 days, indicating the presence of a sufficient number of viable bacterial cells, which appears to be a *sine qua non* to provide biotherapeutic benefits [14]. In contrast, other studies have reported poor survivability of *L. acidophilus* and *B. bifidum* in yogurt due to its low pH. It was shown that *L. acidophilus* lost 90 – 99 % of its viability after three to five days of storage on addition to yogurt [15].

It has been shown in the present study that *L. pentosus* KCA1 viability after 56 days improved with bacterial counts over 10^5 cfu/ml contrary to the findings of Shah *et al* [8] in which viable counts of *L. acidophilus* and *B. bifidum* decreased to less than 10^5 and 10^3 bacterial cells, respectively, in commercial yogurt during storage.

Other methods of increasing probiotic viability in yogurt have been proposed, such as microencapsulation [16] which facilitates the manufacture of fermented dairy products by making the bacteria more stable during storage.

CONCLUSION

We have succeeded in developing a novel probiotic yogurt with *L. pentosus* KCA1 having both the genomic and functional capability of maintaining acceptable high viable counts needed to confer health benefits on the host. Clinical studies are needed to confirm this.

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