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Inhibition of *Yesinia enterocolitica* in Ready to Eat Vegetable Salad by Lactic Acid Bacteria Isolated from Fermented Foods and Beverage

Angmo K[*1], Kumari A[2] and Bhalla TC[3]

[1,2,3]Department of biotechnology, Himachal Pradesh University, Summer Hill, Shimla 171005, India
*E-mail: *[1]kunzes20@gmail.com*

Abstract—*In this study, Lactobacillus sp. KJ722775 and Lactobacillus brevis KJ722775 were selected for study of antagonistic against Y. enterocolitica at refrigeration temperature. Y. enterocolitica was added to ready to eat vegetables salad and coinoculated with Lactobacillus sp. and L. brevis separately, stored at 4 °C for 14 days. In shredded vegetables salad the population of Y. enterocolitica in the control sample (no LAB isolate added) increased from 5.45 log CFU/ml (0 day) to 7.81 log CFU/ml (14 day) (Figure 4.42). On the other hand, shredded vegetables salad samples inoculated with Lactobacillus sp. PLA27 and L. brevis PLA29 found that the numbers of Y. enterocolitica decreased by 0.18 log CFU/ml and 0.43 log CFU/ml respectively over the 14 days of storage when compared to their initial count on day 0. Cell-free supernatants of selected antagonistic bacteria were studied to determine the nature of the antimicrobial compounds produced. The low pH and production of lactic acid were the main factors for inhibition of growth of Y. enterocolitica.*

Keywords*: Ready to eat vegetable, lactic acid bacteria, bio preservation, fermented food, beverage.*

1. INTRODUCTION

Freshness is considered as one of the most important issue in food preservation [1]. The people refrigerate to extend the shelf life of perishable foods as raw meat, dairy product and vegetables. During refrigeration psychrotrophic pathogen such as *Y. enterocolitica* is of major concern. Use of chemical preservatives is not usually accepted by consumer and moreover it is not compatible with the 'fresh' image of foods [2]. An alternative way to replace chemical treatments with biopreservative approaches ensure the safety of the product and fulfill consumer preferences for minimally processed foods. Biopreservation systems that use LAB to control food-borne psychrotrophic pathogens is a suitable alternative method to chemical preservatives [3]. Cultures showing antagonisms are added to meat products to inhibit pathogens and/or prolong the shelf life, while also preserving the desired sensory properties, are called protective cultures [4]. Their antagonism refers to inhibition of spoilage bacteria through competition for nutrients and/or production of one or more antimicrobially active metabolites such as organic (lactic and acetic) acids, hydrogen peroxide, antimicrobial enzymes, bacteriocins and reuterin [5]. The aim of this research was to evaluate the efficacy of lactic acid bacteria (LAB) isolated from traditional fermented foods and beverage of Ladakh as biocontrol agents against *Yersinia enterocolitica*.

2. MATERIALS AND METHODS

2.1 Bacteria

Lactobacillus sp. PLA27 (Accession number KJ722775) and *Lactobacillus brevis* PLA29 (Accession number KJ722777) were used in this study. These isolates were isolated from *chhang*, barley based traditional alcoholic beverage of Ladakh, India.

Yersinia enterocolitica MTCC 4854 was used as an indicator strain and obtained from IMTECH (Institute of Microbial Technology) Chandigarh

2.2 *In vitro* assay of antagonistic activity

For determination of antimicrobial activity agar spot method described by [6] was used. This assay was performed and described in [7].

2.3 Identification of inhibitory substance by agar well diffusion method

In order to determine the production of inhibitory substance produced during growth, the method described by [8] was performed with little modification. This test was described in [7]. *Y. enterocolitica* was cultured in CIN broth at 30°C for 18 h. The selected LAB isolates were grown in MRS broth at 4°C for 14 days in MRS broth. After 14 days of storage, samples were centrifuged (10,000 x g for 10 min at 4°C), supernatants were collected and filter sterilized using 0.22-µm pore size

syringe filters (Millipore) into sterile containers. This filter sterilized supernatant was assayed as following: Fraction A, cell-free supernatant of each LAB isolate, Fraction B, consisting of pH neutralized supernatant (pH 6.5) used for the detection of inhibition by organic acids. Inhibition by means of hydrogen peroxide was tested in fraction C, which consisted of supernatants treated with 0.5 mg catalase/ ml at 37°C for 1h. Fraction D, was used for the detection of bacteriocin-like compounds and consisted of supernatants treated separately with proteinase K (Promega) at a concentration of 0.5 U of enzyme/ml for 1 h at 37°C. The reactions with catalase and proteases were stopped by incubating the samples for 10 min at 65°C before antimicrobial activity was assayed. On the other hand, 100 µl of overnight active culture ($1x10^5$ CFU/ml) of *Y. enterocolitica* were plated on nutrient agar plate. Wells of 5 mm depth were made on the nutrient agar plate containing the lawn of *Y. enterocolitica*. 70 µl aliquot of fraction A, B, C or D was added into each well individually. The plates were held at 4°C for 2 h and incubated at 30°C for 24 h and examined for clear zones of *Y. enterocolitica* inhibition.

2.4 Evaluation of selected LAB isolates for inhibition of *Y. enterocolitica* in ready to eat vegetables salad

Vegetables i.e. carrots (*Daucus carota*) and cabbage (*Brassica oleracea*) were purchased on the day of processing from a local retailer. They were processed as follows: outer leaves of cabbage were removed and inner leaves were shredded manually using sharp knives to approximately 10 mm stripe. After washing with distil water, the carrots were peeled, shredded into 5 to 10 mm wide strips and exposed to the UV lamp in a laminar flow cabinet for 30 min on both sides to minimize interference of the natural bacterial flora.

Using sterile forceps, five pieces of vegetable stripe were dipped into 250 ml of sterile water for 30s and then placed into four individual sterile bags. These served as controls for the background flora on the inoculated samples (uninoculated background controls). The other 15 pieces were dipped for 30s in water which had been inoculated with an appropriate amount of *Y. enterocolitica* to yield a population in the water of 1.0 X 10^5 CFU/ml. Five of these pieces inoculated with *Y. enterocolitica* were then dipped into water containing 5.0 X 10^7 CFU/ml of isolate PLA27, five into water containing 1.0 X 10^8 CFU/ml of isolate PLA29, and five into sterile water. The pieces inoculated with *Y. enterocolitica* and dipped in sterile water served as treatment controls. Each individual vegetables piece was placed into a sterile bag and stored at 4^oC. One bag for each treatment was removed from storage for microbial analysis on 0^{th}, 3^{rd}, 6^{th}, 9^{th}, 12^{th} and 14^{th} day. The experiment was repeated three times. The total numbers of *Y. enterocolitica* and LAB present were determined by pour plating of appropriate dilutions onto CIN agar and MRS agar, respectively.

2.5 pH of sampling

The pH of sampling (0, 3, 6, 9, 12 and 14 days) was taken by uniformly mixed sample with distilled water (1:1) ratio (w/v) was measured using digital pH meter (MAC) after standardization at 25°C using buffers of pH 4.0 and 7.0.

2.6 Statistical analysis

Results were expressed as the mean and standard error mean (SEM) of three experiments with triplicate determinations. Statistical analysis was performed on the data by SPSS 19.0 Bivariate Correlation Analysis (SPSS Inc., Chicago, Ill., U.S.A.) with statistical significance determined at $P < 0.05$. Data were subjected to ANOVA and the differences between treatments versus control were assessed by a Tukey's HSD.

3. RESULTS

3.1 Antagonistic activity of lactic acid bacteria

On the agar spot test isolates PLA27 and PLA29 were antagonistic towards *Y. enterocolitica* at 4 ˚C for 28 days having inhibitory zone of 6mm (PLA27) and 8mm (PLA29) and were further processed as potential bio-control agents.

The low pH i.e. acidification of the medium was the major reason for antimicrobial effect produced by LAB isolates. It was found that pH-neutralized supernatants (fractionB) did not show any zone of inhibition towards *Y. enterocolitica* whereas in case of fraction C (supernatant treated with catalase) and fraction D (supernatant treated with proteinase k) showed zone of inhibition. This study confirms that inhibitory substance was not susceptible toward catalase or proteolysis. However, in in-vitro antagonistic test, organic acids were the substances produced by the selected LAB that were capable of inhibiting growth of *Y.enterocolitica*.

3.2 Inhibition of *Y. enterocolitica* in ready to eat vegetable salad by the selected LAB isolates

In shredded vegetables salad the population of *Y. enterocolitica* in the control sample (no LAB isolate added) increased from 5.45 log CFU/ml (0 day) to 7.81 log CFU/ml (14 day) (Figure 1). On the other hand, shredded vegetables salad samples inoculated with *Lactobacillus* sp. PLA27 and *L. brevis* PLA29 and found that the numbers of *Y. enterocolitica* increased by 0.92 log CFU/ml and 1.14 log CFU/ml, over the 6 days of storage when compared to their initial count on day 0. The numbers of the *Y. enterocolitica* showed no significant increase or reduction in count on 9 and 12 day in test samples inoculated with *Lactobacillus* sp. PLA27 and *L. brevis* PLA29. On day 14 there was little dropped in the number of *Y. enterocolitica* by 0.18 log CFU/ml (*Lactobacillus* sp. PLA27), 0.43 log CFU/ml (*L. brevis* PLA29). Overall, the growth inhibition was significantly different ($P< 0.05$) when compared to the control samples; hence, the inhibitory activity

of LAB against *Y. enterocolitica* in this case can be considered bacteriostatic.

The number of LAB isolates were significantly (P >0.05) increased from 7.31 to 8.46 (*Lactobacillus* sp. PLA27) and 7.05 to 8.37 (*L. brevis* PLA29) during the period storage at 4 °C (Table 1).

Changes in pH were significantly different (P >0.05) between the control sample and the treatments containing LAB isolates (Figure 2) over the 14 days storage period. The pH decreased from 6.66 to 5.02 in control sample while in test samples it decreased from 6.59 to 4.70 (*Lactobacillus* sp. PLA27) and 6.69 to 4.54 (*L. brevis* PLA29).

Table 1: Count of *Lactobacillus* sp PLA27 and *Lactobacillus brevis* PLA29 in vegetable salad samples coinoculated with *Yersinia enterocolitica* for 14 days of storage at 4 °C.

Days	*Lactobacillus* sp. PLA27 (CFU/ml)	*L. brevis* PLA29 (CFU/ml)
0 hrs	7.31±0.04[a]	7.05±0.03[a]
3 hrs	7.40±0.05[a]	7.16±0.03[a]
6 hrs	7.98±0.09[b]	7.57±0.04[b]
9 hrs	8.04±0.05[b]	7.75±0.13[b]
12 hrs	8.27±0.08[bc]	8.24±0.07[c]
14 hrs	8.46±0.29[c]	8.37±0.11[c]

Background controls showed no counts of any microorganisms on either CIN or MRS agar plates during the 14 days storage, demonstrating that there was no influence of background microbiota on the inhibition of the pathogen.

4. DISCUSSION

Decontamination of *Y. enterocolitica* in vegetable salad samples during refrigerated was studied. The count of *Y. enterocolitica* in vegetable salad samples inoculated with LAB isolates *Lactobacillus* sp. PLA27 and *L. brevis* PLA29 were observed to be increased to 6.09 log CFU/ml (*Lactobacillus* sp. PLA27) and 5.81 log CFU/ml (*L. brevis* PLA29) on 28 days of storage at 4 °C. Overall, the count of *Y. enterocolitica* was not either decreased or increased in sample inoculated with selected LAB isolates with the progressed in incubation time. The inhibitory activity of selected LAB isolates against *Y. enterocolitica* in this case can be considered bacteriostatic. It suggests that the acidic environments due to the production of organic acid by selected LAB isolates are not favourable for the growth of *Y. enterocolitica.*

Rodriguez et al. (1994) found that *L. sake* 23 (stronger lactic acid producer strain was strong inhibitor of *Y. enterocolitica* and reductions in the growth of *Y. enterocolitica* became more pronounced with the decrease in pH which suggests that lactic acid is the major factor contributing for antagonism [9]. During the low-temperature fermentation of Turkish dry sausage (sucuk) *Y. enterocolitica* was inhibited by *L. sake* and *Pediococcus acidilactici* and pH decreased from 6.3 to about 4.7 [10]. Vereecken et al., 2003 proposed model supported that the inhibition of *Y. enterocolitica* in mono- and coculture with *L. sake* is due to the lactic acid production which caused an early induction of the stationary phase [11].

In conclusion the application of such cultures could be an alternate measure to improve the shelf life and safety of products and thus reducing the use of chemical additives for food preservation and storage.

5. ACKNOWLEDGEMENTS

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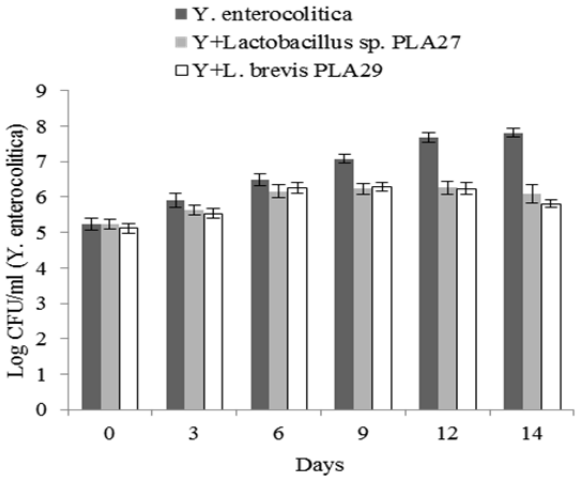

Fig. 1: Inhibition of *Yersinia enterocolitica* in vegetable salad by *Lactobacillus* sp. and *Lactobacillus brevis* during the storage at 4 °C. Control sample was without lactic acid bacteria.

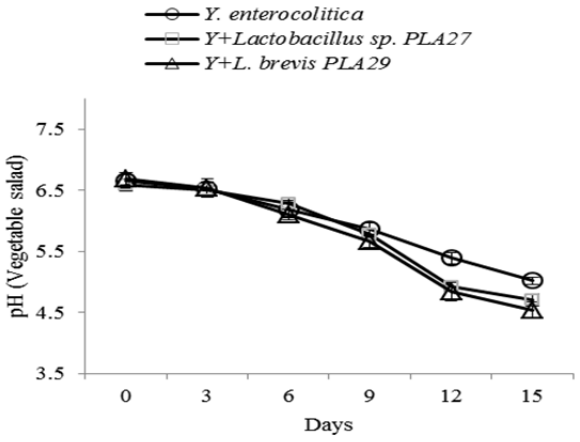

Fig. 2: Changes of pH in vegetable salad samples inoculated with *Y. enterocolitica* only and coinoculated with *Y. enterocolitica* + *Lactobacillus* sp. and *Y. enterocolitica* + *L. brevis* at 4 °C for 14 days.

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