Food Bioscience 13 (2016) 26–31

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Antagonistic activities of lactic acid bacteria from fermented foods and beverage of Ladakh against *Yersinia enterocolitica* in refrigerated meat

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ARTICLE INFO

Article history:
Received 12 June 2015
Received in revised form
14 November 2015
Accepted 8 December 2015
Available online 8 December 2015

Keywords:
Fermented food
Beverage
Ladakh
Yersinia enterocolitica
Lactic acid bacteria
Inhibition
Meat
Biopreservation

ABSTRACT

This study evaluated the efficacy of lactic acid bacteria (LAB) isolated from traditional fermented foods and beverage of Ladakh as biocontrol agents against *Yersinia enterocolitica.* The antagonistic activities of 46 LAB isolates were screened for their ability to inhibit the growth of *Y. enterocolitica* at refrigeration temperatures using agar spot tests. On the basis of the 16S rDNA sequences, two selected inhibitory isolates were identified as *Lactobacillus* sp. and *Lactobacillus brevis* and their accession number were KJ722775 and KJ722777, respectively. The growth of *Y. enterocolitica* in mixed cultures coinoculated with *Lactobacillus* sp. and *L. brevis* separately in de Man Rogosa Sharpe (MRS) broth at 4 °C for 28 days was investigated. It was found that the growth of *Y. enterocolitica* on 28th day decreased to 1.22 log CFU/ml (*Lactobacillus* sp.) and 2.17 log CFU/ml (*L. brevis*) in mixed culture. *Y. enterocolitica* was added to meat samples and coinoculated with *Lactobacillus* sp. and *L. brevis* separately, stored at 4 °C for 28 days. *Y. enterocolitica* count was dropped to 4.90 log CFU/ml (*Lactobacillus* sp.) and 4.44 log CFU/ml (*L. brevis*) on 28th day of storage indicating bactericidal activity. It was observed that the inhibition was higher as the incubation period progressed. 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.*

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1. Introduction

Fresh and minimally processed fresh cut food products provide a favorable environment for proliferation of spoilage organisms and microorganisms (Francis, Thomas, & O'Beirne, 1999). A number of human infections are caused due to the consumption of food which is contaminated with these spoilage or pathogenic microorganisms. *Yersinia enterocolitica* is one of the psychrotrophic foodborne pathogen, capable of significant growth in foods stored at refrigeration temperatures without apparent signs of spoilage (Estrada, Velazquez, Favier, Genaro, & Escudero, 2012). It is ubiquitous in nature and has been isolated from a variety of foods including meat (Bonardi et al., 2010), milk (Okwori, Martinez, Fredriksson-Ahomaa, Agina, & Korkeala, 2009) seafoods, vegetables (Siddique, Sharma, & Al-Khaldi, 2009) and ready to-eat foods (Xanthopoulos, Tzanetakis, & Litopoulou-Tzanetaki, 2010). This pathogen is transmitted by contaminated water and foods including meats, dairy products, seafood and vegetables (Bottone, 1999). Infection may cause gastrointestinal problems such as acute enteritis with fever, bloody diarrhoea and pseudo appendicitis, which frequently leads to unnecessary laparotomy (abdominal surgery) in humans (Vlachaki, Tselios, Tsapas, & Klonizakis, 2007).

Fermented foods are produced by the activities of lactic acid bacteria (LAB) which play a role in enhancing flavour, increased digestibility, improving nutritional value and pharmacological values. LAB inhibits spoilage causing, toxin producing or food poisoning microorganisms during growth and refrigerated storage in associative cultures and in food products (Amezquita & Brashears, 2002). Their antagonism towards spoilage bacteria is through competition for nutrients and/or production of one or more antimicrobially active metabolites such as organic acids, hydrogen peroxide and bacteriocins (Holzapfel, Geisen, & Schillinger, 1995). In particular, by producing lactic acid and thus lowering the pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens and sometimes even kill them (Vandenbergh, 1993). Most lactic acid bacteria are tolerant to antagonistic effects of lactic acid than *Y. enterocolitica*, and produced lactic acid can cause a negative influence on the growth of *Y. enterocolitica* (Janssen et al., 2006). The bactericidal activity of lactic acid is due to their undissociated forms, which target the metabolic functions of microorganisms, such as protein production, the inhibition of ATP and an increase in osmotic pressure (Lues & Theron, 2011).

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http://dx.doi.org/10.1016/j.fbio.2015.12.004
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Increasing the acidity of foods, either through fermentation process or by addition of weak acids has been used as a preservation method since ancient times. Due to these preservative properties, use of LAB is an interesting substitute for chemical and/or physical preservatives. Moreover, LAB are generally regarded as safe (GRAS) and usually fit all recommendations, including a lack of evidences of pathogenicity to humans, to be used in food products (Stiles & Holzapfel, 1997). These characteristics make LAB the ideal candidates for the development of bioprotective agents, providing a good antagonistic activity towards target organisms (Trias, Baneras, Badosa, & Montesinos, 2008).

This study was aimed to isolate and characterize LAB from fermented foods and beverage of Ladakh with inhibitory activity toward *Y. enterocolitica* at refrigeration temperature. The selected LAB isolates were examined for antagonistic activity against *Y. enterocolitica* in meat sample and to identify the predominant inhibitory substances produced by them.

2. Materials and methods

2.1. Isolation of lactic acid bacteria from fermented products

The samples of fermented products such as dried cottage cheese (*chhurphe*), mixed vegetable pickle and barley based beverage (*chhang*) were collected from different regions (Nyemo, Saspol, Sankar, Thiksay, Zanskar) and Leh town of Ladakh, India in sterile vials and stored at 4 °C till further processing. Ten gram of sample was homogenized with 90 ml of physiological saline and serial dilutions were prepared. The diluted sample was spread plated onto de Mann Rogosa Sharpe (MRS) agar (Himedia, Mumbai, India) and incubated at 30 °C for 48 h. Individual colonies (different in morphology i.e. size, shape and colour) were picked and streaked on MRS agar plate. Pure cultures were stored at −80 °C in 20% (v/v) glycerol. Gram stain reaction, cell morphology and catalase reaction were studied for preliminary identification of isolates.

2.2. Pathogen

Y. enterocolitica strain 4854 used as an indicator strain was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. It was maintained by subculturing in Cefsulodin Irgasan Novobiocin (CIN) broth (Himedia, Mumbai, India) and incubation at 30 °C for 18 h. It was subcultured twice before it was used in experiments. Stock cultures were prepared in CIN broth containing 20% (v/v) glycerol and stored at −80 °C.

2.3. In vitro assay of antagonistic activity

Screening was done using an agar spot method described by Trias, Baneras, Badosaet al. (2008). LAB isolates were spotted on MRS agar plates and incubated 24 h at 30 °C. 0.5 ml of a *Y. enterocolitica* cell suspension containing 10^7 CFU/ml were mixed in 4.5 ml of MRS soft agar (0.7% agar) and overlaid on the plate containing previously grown colonies of LAB. Plates were incubated and diameters of the inhibition areas were measured. Two different incubation conditions were studied: 30 °C for 24 h and 4 °C for 28 days. Isolates exhibiting inhibition zones greater than 1 mm toward the *Y. enterocolitica* were considered to possess antimicrobial activity.

In addition, isolates displaying activity against *Y. enterocolitica* at low temperature were included in the selection and used for further experiments. Two LAB isolates PLA27 and PLA29, active against *Y. enterocolitica* at refrigeration temperatures were selected for further analysis.

2.4. 16 S rDNA sequencing

The two selected isolates were identified by 16S rRNA gene sequencing. The nucleic acids of each isolate were extracted by alkaline lysis method according to Sambrook, Fritsch, and Maniatis (1989). 16 S rRNA gene was amplified by PCR using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR reaction was performed in 50 µl of reaction mixture containing 1 µl of DNA, 5.0 µl of PCR buffer (10X) (Promega, Madison, USA), 1.0 µl of 5 Mm dNTPs (Promega, Madison, USA), 1.0 µl each of forward and reverse primers 5 mM, 1 µl of Dynazyme (Promega, Madison, USA) and 33 µl of autoclaved deionized water (MilliQ, Millipore, Mumbai, India). The amplification was performed with a total of 34 cycles in a thermal cycler (Eppendorf, Hamburg, Germany). The cycling program was started with an initial denaturation at 94 °C for 5 min followed by 34 cycles of denaturation at 94 °C for 1 min and annealing at 57 °C. The PCR reaction was ended with a final extension at 72 °C for 7 min. PCR products were purified by using the Promega QIA quick PCR purification kit (Qiagen, Hilden, Germany). All amplicons were sequenced bi-directionally at Xcelris Labs Limited, Ahmedabad, India. Sequences obtained were analyzed and aligned using software BioEdit 7.2.5, and homology search was done using Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) at National Center for Biotechnology Information (NCBI) database (Alstchul, Gish, Miller, Meyers, & Lipman, 1990). The sequences were submitted to NCBI.

2.5. 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 Trias, Baneras, Montesinos, and Badosa (2008) was performed with little modification. *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 × *g* for 10 min at 4 °C), supernatants were collected and filter sterilized using 0.22-µm pore size syringe filters (Millipore, Mumbai, India) 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 1 h. 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 protease 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 (1×10^5 CFU/ml) of *Y. enterocolitica* were plated on nutrient agar (Himedia, Mumbai, India) 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.

2.6. Evaluation of selected LAB isolates for inhibition/killing of Y. enterocolitica in mixed cultures

Mixed cultures were prepared as described by Rodriguez et al. (1994) with little modification. The strain of *Y. enterocolitica* was grown in 200 ml of CIN broth at 30 °C for 18 h. The culture was centrifuged at 10,000 × *g* for 10 min at 4 °C, washed twice and

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