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Clinical Microbiology 2018 : In Vitro Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Some Traditionally Fermented Ethiopian Food Products- Guesh Mulaw-Addis Ababa University

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Probiotics are live microorganisms which when consumed in large number together with a food promote the health of the consumer. The aim of this study was to evaluate in vitro probiotic properties of lactic acid bacteria (LAB) isolated from traditional Ethiopian fermented Teff injera dough, Ergo, and Kocho products. A total of 90 LAB were isolated, of which 4 (4.44%) isolates showed 45.35–97.11% and 38.40–90.49% survival rates at pH values (2, 2.5, and 3) for 3 and 6 h, in that order. The four acid-tolerant isolates were found tolerant to 0.3% bile salt for 24 h with 91.37 to 97.22% rate of survival. The acid-and-bile salt-tolerant LAB isolates were found inhibiting some food-borne test pathogenic bacteria to varying degrees. All acid-and-bile-tolerant isolates displayed varying sensitivity to different antibiotics. The in vitro adherence to stainless steel plates of the 4 screened probiotic LAB isolates were ranged from 32.75 to 36.30% adhesion rate. The four efficient probiotic LAB isolates that belonged to Lactobacillus species were identified to the strain level using 16S rDNA gene sequence comparisons and, namely, were Lactobacillus plantarum strain CIP 103151, Lactobacillus paracasei subsp. tolerans strain NBRC 15906, Lactobacillus paracasei strain NBRC 15889, and Lactobacillus plantarum strain JCM 1149. The four Lactobacillus strains were found to be potentially useful to produce probiotic products.

1. Introduction

Worldwide, a variety of fermented food products are produced, which contribute significantly to the diets of many people. Fermented food products are used to describe a special class of the food products characterized by various kinds of carbohydrate breakdowns in the presence of probiotic microorganisms, but seldom is carbohydrate the only constituent acted upon. Fermented food and beverage products have emerged as not only the source of nutrition but also as functional and probiotic foods, which besides nutritional value have health effects or provide protection against food-borne diseases.

2. Materials and Methods

2.1. Sample Collection

Traditionally fermented food products (Teff dough, Kocho, and Ergo) were obtained from Addis Ababa and its surroundings, Ethiopia. Each sample (200 g) was aseptically collected by using sterilized containers. The samples were brought to the laboratory with an ice box and stored in a refrigerator at +4°C until further analysis was carried out. Ergo is a locally fermented milk product. Teff dough is made by fermenting teff (Eragrostis tef) flour which is used to prepare a thin pancake-like product with many eyes known as injera. Kocho is a product which is prepared from decorticated and pounded pulp of enset plant (Ensete ventricosum), which is further mixed and kneaded into a mash and fermented in a pit.

2.2. Isolation and Purification of LAB from Traditional Fermented Foods

For isolation of LAB, 25 ml or 25 g of each sample of traditionally fermented foods (Teff dough, Kocho and Ergo) was mixed with 225 ml of separate sterile peptone water (0.1% W/V). Then, a sequential decimal dilution of the homogenate was obtained. From the appropriate dilutions, 0.1 ml aliquots were

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spread plated on duplicate predried surfaces of MRS (de Man, Rogosa, and Sharp) agar (Oxoid, Basingstok, Hampshire, England) plates. The inoculated plates were incubated under anaerobic condition using an anaerobic jar (BBL, Gas Pak Anaerobic Systems) at 37°C for 48 hours.

2.3. Confirmation Tests of LAB Isolates

2.3.1. KOH Test

The KOH test was used to determine the gram reaction of LAB isolates. LAB cultures were grown on MRS agar at 37°C for 24 h under anaerobic conditions. A drop of 3% aqueous KOH was placed on a clean slide. Using a sterile loop, visible cells from fresh cultures were transferred to the drop of 3% KOH. The cells and KOH were mixed thoroughly on the slide and stirred constantly over an area about 1-2 cm2. The isolates, which did not give a viscid product, were selected since lactic acid bacteria (LAB) are known as Gram-positive cells.

2.3.2. Catalase Test

Overnight cultures of isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. The catalase test was conducted by dripping two drops of hydrogen peroxide (3%) on 24 h-old cultures on a glass slide. The catalase test showed positive reaction characterized by the formation of oxygen bubbles that indicate the production of catalase enzyme by the test bacterium. Therefore, the isolates, which did not give gas bubbles, were selected for subsequent activities.

2.3.3. Spore Staining

Gram-positive and catalase-negative isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. The spore-staining procedure was applied [20]. After the spore-staining technique, the endospore formulation was examined under light microscopy using oil immersion objectives. The isolates which did not form endospores were selected for further analysis.

2.4. In Vitro Characterization of Probiotic Properties

The common methods for in vitro analysis of probiotic properties include tolerance to low pH, tolerance against bile salt, antibiotic susceptibility, antimicrobial activity, and bacterial adherence to stain steel plates.

2.4.1. Tolerance to Low pH

The isolates were grown separately overnight in 5 ml MRS broth at +37°C under anaerobic conditions. A volume of 1 ml of log 7 CFU/ml of each overnight-grown culture was inoculated into 10 ml of MRS broth to give an initial inoculum level of log 6 CFU/ml. The culture was then centrifuged at 5000 rpm for 10 min at +4°C. The pellets were washed twice in phosphate buffer (pH 7.2). The pellets were re suspended in 5 ml sterile MRS broth which was adjusted to pH values of 2.0, 2.5, and 3.0 using 1 N•HCl to simulate the gastric environment. initial bacterial concentration: where N1 is the viable count of isolates after incubation and N0 is the initial viable count.

2.4.2. Tolerance to Bile Salts

To estimate bile tolerance of acid-tolerant LAB (those only were grown in pH 2.0, 2.5, and/or 3.0), the isolates were separately grown overnight in MRS broth at 37°C under anaerobic conditions. Each culture with the initial concentration of 106 CFU/ml was then centrifuged at 5000 rpm for 10 min at 4°C. The pellets were washed twice in the phosphate-saline buffer (PBS at pH 7.2). Cell pellets were resuspended in sterile MRS broth supplemented with 0.3% (w/v) bile salt (Oxgall, USA).

2.4.3. Antimicrobial Activity

Antibacterial activity of the acid-bile-tolerant LAB strains against some food-borne pathogens was determined using the agar-well diffusion method with some modifications of the protocol indicated by Fontana et al. The test organisms (Staphylococcus aureus ATCC 25923, Listeria mono cytogenes (clinical isolate), Salmonella enterica Typhimurium, and

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Escherichia coli ATCC 25922) were obtained from the Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia.

2.4.4. Antibiotic Susceptibility Tests

Each of acid-bile-tolerant and antagonistic lactic acid bacteria isolates was assessed for its antibiotic resistance by the disc diffusion method as described by Zhangetal. against some antibiotics that included ampicillin (10 µg/ml), erythromycin (15 µg/ml), streptomycin (10 µg/ml), kanamycin (25 µg/ml), and tetracycline (30 µg/ml). Thus, a volume of 100 µl of actively growing cultures of each acid-bile-tolerant and antagonistic lactic acid bacteria was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. After drying, the antibiotic discs were placed on the solidified agar surface, and the plates were left aside for 30 min at 4°C for the diffusion of antibiotics and then anaerobically incubated at 37°C for 24 to 48 h.

2.4.5. Bacterial Adhesion to Stainless Steel Plates

The adherence assay of acid-bile-tolerant, antagonistic, and antibiotic-sensitive lactic acid bacterial isolates was determined on stainless steel plates with some modifications of the protocol given by El-Jeni etal. Briefly, LAB were cultured in sterile MRS broth. Thereafter, the overnight bacterial culture (500 µl) was deposited in a test tube, which was then filled with 450 µl of MRS broth, wherein the sterile stainless steel plate was deposited, and the test tubes were then incubated for 24 h at 37°C. The stainless steel plate was removed under aseptic conditions, washed with 10 ml of sterile 1% peptone water, and left for 5 min in a sterile 1% peptone water tube.

2.5. Morphological, Biochemical, and Physiological Tests

The probiotic LAB isolates were identified according to their morphological, physiological, and biochemical characteristics based on Bergey's Manual.

2.5.1. Cell Morphology

Overnight cultures were wet mounted on microscopic slides and examined under a light microscope using oil immersion objectives. Cellular morphological criteria considered during the examination were cell shape and cell arrangements.

2.5.2. Growth at Different Temperatures

Each of the overnight LAB cultures of 50 µl was transferred into four separate tubes that contained 5 ml medium (modified MRS broth) containing bromecresol purple indicator at a concentration of 0.12 g/l. After inoculation, two of the inoculated test tubes were incubated for 7 days either at 15°C and the other two test tubes at 45°C. During this incubation time, growth at any temperature was observed by the change of the growth medium (cultures) from purple to yellow.

2.5.3. Growth at Different NaCl Concentrations

LAB isolates were tested for their tolerance to different NaCl concentrations. For this purpose, 4% and 6.5% NaCl concentrations were used for testing. Similarly, test tubes with 5 ml of modified MRS broth containing bromecresol purple indicator were prepared according to the appropriate concentrations. Four test tubes with 4% NaCl and the other four test tubes with 6.5% NaCl were inoculated separately with 50 µl of 1% of each overnight culture of LAB and incubated at +37°C for 7 days. The change of the color from purple to yellow was considered as proof of cell growth.

2.6. Identification of Probiotic LAB Isolates Using 16S rRNA Gene Sequencing

2.6.1. Genomic DNA Extraction

Genomic DNA was extracted from pure cultures (n = 4) of potential probiotic LAB. Separately, 1 ml of each pure liquid culture was centrifuged for 3 min at 10000 rpm. The supernatant was removed, and the cells were resuspended in 300 µl buffer (10 mM Tris-HCl, pH 8.0; 50 mM glucose, and 10 mM EDTA).

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2.6.2. PCR Amplification of 16S rDNA

For the amplification of the 16S rDNA gene, the specific primers AMP_F 5'- GAG AGT TTG ATY CTG GCT CAG -3' and AMP_R 5'- AAG GAG GTG ATC CAR CCG CA -3' were used. PCR reaction mixture was prepared by mixing 25 µl of the Tag 2x Master mix (buffer, polymerase and dNTPs), forward primer 1 µl, reverse primer 1 µl, and UPH2O 22 µl. Then, 49 µl of the mixtures was added to a sterile PCR tube, and 1 µl of the gDNA was used as a template; the amplification reactions were carried out in a thermal cycler (Bio-Rad Mycycler).

2.6.3. DNA Electrophoresis

PCR products were separated in a 1% agarose gel and stained with ethidium bromide followed by examination on a UV illuminator, and images were captured by a digital camera.

3. Results

Isolation of Potentially Probiotic Lactic Acid Bacteria from Traditional Fermented Foods A total of 90 (30 from each sample) lactic acid bacteria were isolated from three different traditionally fermented Ethiopian food products (Teff dough, Ergo, and Kocho). Among them, 56 (62.22%) isolates were found Gram-positive, endospore-negative, and catalase-negative.

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