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Application of mixture design to introduce an optimum cell-free supernatant of multiple-strain mixture (MSM) for *Lactobacillus* against food-borne pathogens

Mahmoud Yolmeh, Morteza Khomeiri*, Zahra Ahmadi

Department of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

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ABSTRACT

This study aimed to find out an optimum multiple-strain mixture (MSM) of *Lactobacillus* with the highest antimicrobial activity (AA) against common food-borne pathogenic bacteria (*Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes*, and *Bacillus cereus*) through mixture design (MD). Hence, *Lactobacillus* strains were isolated from Iranian traditional fermented olive and pickled garlic, then molecular identified. The results showed that cell-free supernatant (CFS) of the all *Lactobacillus* isolates had higher AA compared to their microbial suspensions. Therefore, CFS of the *Lactobacillus* isolates was used to find an optimum proportion through MD. A mixture containing 10% CFS of *L. brevis* isolated from fermented olive (A*), 80% CFS of *L. plantarum* isolated from fermented olive (B*), and 10% CFS of *L. brevis* isolated from pickled garlic (C*) was found as the optimum mixture against common food-borne pathogens. After incubation at 37 °C for 24 h, optical density (OD) of *E. coli*, *S. enteritidis*, *L. monocytogenes* and *B. cereus* treated with this optimum mixture were 0.131, 0.240, 0.211 and 0.483, respectively.

1. Introduction

Food-borne diseases (FBD) are often associated with the consumption of foods have been contaminated by an infectious agent or a toxin produced by microorganisms. It is estimated that 30 percent of people in developed countries suffering from FBD annually (Burt, 2004; Da Silva & Franco, 2012). Hence, the control growth of food-borne pathogens is very important, and for this purpose, researchers are looking for natural preservatives instead of synthetic types. The natural preservatives have health-promotion effects on the consumer, in addition to increase food safety. These alternatives can be provided from plant and/or microbial sources (Yolmeh, Najafi, Farhoosh, & Salehi, 2014a; Zoumpopoulou et al., 2008).

Probiotics are known as live bacteria and yeasts that have health-promotion effects on the consumers, especially on the digestive system. In recent years, the health-promotion effects of probiotics have become a major subject of lactic acid bacteria (LAB). Health-promotion effects of probiotics are included antimicrobial activity (AA) (Marianelli, Cifani, & Pasquali, 2010; Šušković et al., 2010), anticancer activity (Geier, Butler, & Howarth, 2006; Ma et al., 2010), modulation of immune system (Corthésy, Gaskins, & Mercenier, 2007), anti-inflammatory activity (Lorea Baroja, Kirjavainen, Hekmat, & Reid, 2007), modulation of allergic responses (Kalliomäki et al., 2010), reduction of lactose intolerance disease (He et al., 2008).

AA of probiotics against potential pathogens, which is known the most important property, is attributed to a variety of mechanisms. These are competitive prevention of adhesion of pathogen, decreasing luminal pH through production of organic acid (e.g. lactic acid and acetic acid), production of bactericidal proteins (e.g. bacteriocins), production of hydrogen peroxide, and the host's immune system modulating (Marianelli et al., 2010; Šušković et al., 2010). In addition to above mechanisms, it is reported that probiotics have AA in the intestinal crypts through production of defensins at this site (Boirivant & Strober, 2007).

Lactobacillus genus is the most widely studied probiotics due to its widespread distribution and its AA have been reported against several infections in-vitro and in-vivo researches (Marianelli et al., 2010; Servin, 2004). Organic acids, diacetyl, hydrogen peroxide, bacteriocins and reuterin are antimicrobial agents produced by *Lactobacillus* sp. However, the main antimicrobial agent that play

* Corresponding author.
E-mail addresses: Mahmud.yolmeh@yahoo.com (M. Yolmeh), khomeiri@gau.ac.ir (M. Khomeiri).

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main role in the AA is depended on the *Lactobacillus* strain type (homo- or hetero-fermentative) and environmental growth conditions (Marianelli et al., 2010). For example, lactic acid plays the main role in AA of *Lactobacillus rhamnosus* GG in de Mann Rogosa Sharpe (MRS) medium. It is reported that bacteriocins specially act against Gram-positive bacteria; however, organic acids are more efficient on Gram-negative bacteria (Abee, Krockel, & Hill, 1995; De Keersmaecker et al., 2006; Fayol-Messaoudi, Berger, Coconnier-Polter, Lievin-Le Moal, & Servin, 2005).

Although microbial heat-inactivation has been widely studied, there are relatively little reports on predicted studies about the non-thermal inactivation. Since there is not sufficient biochemical and/or microbial knowledge about the microbial inactivation, the major models used to predict microbial inactivation are the second and third category (Soleimanzadeh, Amoozandeh, Shoferpour, & Yolmeh, 2015; Yolmeh, Najafi, & Salehi, 2014b).

Mixture experimental designs are suitable to produce products that are composed from several components. The ratio of these components and their levels in mixture design are dependent on each other (Flores, Costa, Yamashita, Gerschenson, & Grossmann, 2010). Mixture design (MD) is useful statistical technique for multiple regression analysis using measurable results. This approach represents the relationship between the design's inputs (factors or components) and outputs (responses). Linear, quadratic, special cubic and cubic are the main models of mixture designs, which can predict an optimal condition or formulation (Dutcosky, Grossmann, Silva, & Welsch, 2006).

The discovering a multiple-strain mixture (MSM) of *Lactobacillus* is important due to various *Lactobacillus* strains probably have made different organoleptic properties in food and have differed range of AA against pathogens. However to date, a study has not been done on AA of an identified MSM of *Lactobacillus*, and a research to find a MSM with the highest AA (optimum).

Hence, the present study aimed to find out an optimum MSM of *Lactobacillus* with the highest AA against food-borne pathogenic bacteria (*Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (ATCC 49594), and *Bacillus cereus* (ATCC 70876)) form the *Lactobacillus* strains isolated from Iranian traditional pickled garlic and fermented olive through MD.

2. Materials and methods

2.1. Materials and microorganisms

Iranian traditional fermented olive and pickled garlic (9-year-old) were prepared from local market (Gorgan, Iran). Food-borne pathogenic bacteria (*E. coli* (ATCC 25922), *S. enteritidis* (ATCC 13076), *L. monocytogenes* (ATCC 49594), and *B. cereus* (ATCC 70876)) were obtained from Infectious Research Centre and Microbiology Department, Golestan University of Medical Sciences, Gorgan, Iran. All chemicals and mediums used in this study were analytical grade and obtained from Merck (Germany) and Sigma-Aldrich (USA) Companies.

2.2. Isolation of lactic acid bacteria

LABs were isolated using the method described by Angmo, Kumari, and Bhalla (2016) with some modification. Briefly, a sample (50 g) of fermented olive and pickled garlic was homogenized with 10 mL sterile peptone solution (1% w/v) using a grinder. Then, serial dilutions (10^{-2}-10^{-5}) were prepared in 1% peptone solution and plated on MRS agar (Merck, Germany) supplemented with sodium azide (0.2 g/L, Sigma-Aldrich, UAS) as selective medium for LAB iolation under anaerobic condition (GasPak kit, Merck Millipore, Germany) and incubated at 37 °C for 48 h. Some colonies grown on MRS were selected and subcultured to obtain a pure colony. LAB isolates were prepared and stored at −80 °C.

2.3. Molecular identification of the LAB isolates

The finally LAB isolates were identified by 16S rDNA sequencing. The amplifying of 16S rDNAs were done PCR using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primers. DNA sequencing of DNA amplified fragments were sequenced by Macrogen DNA sequencing service (Seoul, Korea). BioEdit 7.2.5 software was used to assemble and edit. Basic Local Alignment Search Tool 136 (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare DNA consensus sequences with those reported in the GenBank DNA database (Angmo et al., 2016).

2.4. Supernatant preparation

Cell-free supernatant (CFS) of the LAB isolates were collected from fresh overnight culture (MRS, Merck, Germany) by centrifugation at 12,000 rpm and 4 °C for 15 min, followed by adjusting pH at 6.5 and filtering (0.22 μm Millex Syringe Filter, Millipore, USA) (Zoumpopoulou et al., 2008).

2.5. Antimicrobial activity

2.5.1. Agar diffusion

AA of the LAB isolates was studied using agar diffusion technique. Briefly, 0.1 mL (~10^8 cells) of pathogenic bacteria in the late exponential phase was spread on Mueller-Hinton agar (Merck, Germany). Working cultures of LAB isolates were supplied by adjusting with 0.5 McFarland turbidity and serially diluted to acquire 10^6 CFU/mL. Sterile paper disks (Merck, Germany) containing 40 μL of the LAB isolates were placed on the plate and the diameter (mm) of inhibition zone was measured after incubation at 37 °C for 24 h (Fooks & Gibson, 2002).

2.5.2. Microdilution plate technique

AA of MSM of the all *Lactobacillus* was evaluated by employing an ELISA reader (CLX800−BioTek Instruments). Briefly, MSMs were prepared according to proportions applied by MD, then diluted with MRS broth (100 μL), and distributed in 96−well plate. Pathogenic bacteria (20 μL, 0.5 mcfarland standard) were added to the wells, and the total volume was brought to 220 μL. As well as, a sterility control (without pathogenic bacteria) and a growth control (without supernatant of the LAB isolates) were considered. After incubation at 37 °C for 24 h, bacterial growth was evaluated by optical density (OD). All experiments were performed in triplicate. The results were expressed in ΔOD (optical density before incubation − optical density after incubation) (Valgas, Souza, Smânia, & Smânia, 2007).

2.6. Experimental design

Design-Expert software (7.0.0) and three-component simplex-centroid MD were employed to find the optimum proportion of MSM of *Lactobacillus* isolates. CFS of *L. brevis* isolated from fermented olive (*L. brevis* (1)) (A*), CFS of *L. plantarum* isolated from fermented olive (B*), and CFS of *L. brevis* isolated from pickled garlic (*L. brevis* (2)) (C*) were considered as the mixture components, which these components had higher AA compared to their cells. The proportions of components were expressed as fractions of the mixture with a sum of one ($A^* + B^* + C^* = 1$).