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Original Article

Evaluation of the probiotic and postbiotic potential of lactic acid bacteria from artisanal dairy products against pathogens

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Abstract

Introduction: Probiotic and postbiotic potential of thirty-two strains of lactic acid bacteria (LAB), obtained earlier from artisanal dairy sources in Pakistan, have been investigated against major multi-drug resistant (MDR) and food borne pathogenic bacteria.

Methodology: LAB strains were identified by 16S rRNA gene sequencing and their antibacterial activity was assessed by the microdilution method. Four LAB isolates, *Weissella confusa* PL6, *Enterococcus faecium* PL7, and *Lactobacillus delbrueckii* PL11 and PL13 were shortlisted. Their ability to degrade lactose and safety for human consumption in terms of hemolysis and antibiotic susceptibility were assessed *in vitro*. The antibacterial components in the cell-free supernatants (CFSs) of isolate cultures were characterized biochemically by HPLC.

Results: Acid neutralization but not protease treatment abolished the antibacterial activity of CFSs. Lactic, acetic and propionic acids were the main acids in the CFSs, and acid production peaked in the stationary phase of growth. The antibacterial activity of the LAB cultures resulted from secretion of organic acids that lowered the pH. The strains exhibited variable ability to degrade lactose and were non-hemolytic and susceptible to the most common antibiotics.

Conclusions: These LAB strains are probiotic candidates for further investigation of their postbiotic role in naturally preserving processed foods and for attenuation of lactose intolerance.

Key words: antagonism; multidrug resistance; organic acids; lactose intolerance; lactic acid bacteria; probiotics..

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Introduction

The recurrence of bacterial infections because of the development of antibiotic resistance has become a paramount public health concern [1]. For many years, conventional antibiotics have been at the forefront of infection treatments [2], but due to the rise in bacterial multidrug resistance (MDR), the standard antibiotic regimens have lost effectiveness [3,4]. These MDR pathogens are not only a cause of public concern in hospitals but lately, foods of animal origin have also become a conduit for their spread in communities [5]. In many MDR cases, only a few antibiotics remain effective and it is only a matter of time before these drugs also lose their potency [4]. *Salmonella* spp. is a globally recognized foodborne pathogen. The MDR phenotype has shown a high prevalence in Pakistan [6], but the incidence of extensively drug-resistant (XDR) *Salmonella* spp. has also been reported [7]. *Pseudomonas aeruginosa* is another well-known pathogen implicated in 10% of all hospital-acquired infections the world over [8]. It was found to have MDR, XDR and even pan drug-resistant (PDR) phenotypes in Pakistan [9]. *Staphylococcus aureus* is also regarded as a significant human pathogen frequently occurring in food of animal origin [10] and increasingly reported to be of the MDR variety [11]. Usually a harmless member of the gut microbiome, *Escherichia coli* can also be an opportunistic pathogen causing recalcitrant urinary tract and bloodstream infections [12,13]. Lately, its strains too have been appearing in Pakistan with purported MDR characteristics [14]. After weaning, around 75% of the world's population experiences a decline in lactase activity in the small intestine due to genetic factors [15]. Low level of lactase activity cause maldigestion of lactose and malabsorption in the small intestine [16]. Lactose maldigestion affects around 60% of Pakistan's adult population [17]. Treatment of lactose intolerance is currently restricted to medications and supplements that either have a preventive or a symptom management

function [18]. Lactose-free products and non-dairy fermented foods are also available in markets [19], but lactose is important especially for children, as it is the only disaccharide that does not appear to accentuate the risk of dental caries [20].

To replace the steadily decreasing arsenal of effective antimicrobials and to alleviate lactose intolerance, several alternatives are being tested. Foremost among these strategies are metabolites produced by LABs either during their lifetime or after death, the latter known as postbiotics [21]. Because of their broad-acting antibacterial activity and unique mechanisms of action, these products hold much promise against human pathogens [22] and are being increasingly applied in clinical and industrial settings. Several different substances, such as organic acids (lactic, phenyl lactic and acetic acid), ammonia, free fatty acids, acetaldehyde, diacetyl, hydrogen peroxide, and even peptides have been identified as the underlying antimicrobial agents [23]. Candidate bacterial strains or their postbiotics could be screened as options for treating MDR infections or eliminating pathogens from the food chain. A collection of LAB gathered from dairy foods were identified and characterized for their antibacterial activity against MDR and pathogenic bacteria and capacity to relieve lactose intolerance.

Methodology

LAB strains and culture conditions

The thirty-two LAB isolates were obtained from the culture collection of the National Probiotic Lab, National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan (Table 1) and were regrown on de Man, Rogosa and Sharpe (MRS, Merck, Darmstadt, Germany) agar plates with LQFXEDWLRQ IRU KRXUV DW &. KH LGHQWLWLHV RI WK partial 16S rRNA gene sequences of the isolates previously submitted to GenBank were confirmed by a %/67 GDWDEDVH VHDUFK RQ 1&%, 6HTXHQFHV ZLWK •

Table 1. Detail of LAB strains used in present study.

Strain code*	Source	Accession number	Strain identification
PL1	Raw cow milk	KT626385	*Lactobacillus plantarum*
PL3	Homemade yogurt	KT626387	*Lactobacillus rhamnosus*[1]
PL4	Homemade yogurt	KT626388	*Lactobacillus delbrueckii*
PL5	Raw cow milk	KT626389	*Lactobacillus Paracasei*[1]
PL6	Raw cow milk	KT626390	*Weissella confusa*
PL7	Raw cow milk	KT626391	*Enterococcus faecium*
PL8	Raw cow milk	KT626392	*Enterococcus faecium*
PL9	Homemade yogurt	KT626392	*Lactobacillus delbrueckii*
PL10	Raw cow milk	KT626394	*Weissella paramesenteroides*
PL11	Homemade yogurt	KT626395	*Lactobacillus delbrueckii*
PL12	Homemade yogurt	KT626396	*Lactobacillus delbrueckii*
PL13	Homemade yogurt	KT626397	*Lactobacillus delbrueckii*
PL14	Raw cow milk	KT626398	*Lactobacillus sakei* subsp. *sakei*
PL15	Raw cow milk	KT626399	*Lactobacillus sakei* subsp. *sakei*
PL16	Local cheese	KT626400	*Streptococcus lutetiensis*
PL17	Local cheese	KT626401	*Enterococcus faecium*
PL18	Local cheese	KT626402	*Lactobacillus paracasei*[1]
PL19	Local cheese	KT626403	*Leuconostoc lactis*
PL20	Local cheese	KT626404	*Lactobacillus fermentum*[1]
PL21	Raw goat milk	KT626405	*Weissella confusa*
PL22	Raw sheep milk	KT626406	*Lactobacillus fermentum*
PL23	Raw buffalo milk	KT626407	*Streptococcus lutetiensis*
PL24	Raw cow milk	KT626408	*Lactobacillus fermentum*[1]
PL25	Raw goat milk	KT626409	*Lactobacillus fermentum*[1]
PL26	Raw goat milk	KT626410	*Lactobacillus fermentum*[1]
PL27	Raw goat milk	KT626411	*Lactobacillus fermentum*[1]
PL28	Raw sheep milk	KT626412	*Lactobacillus fermentum*[1]
PL29	Raw cow milk	KT626413	*Lactobacillus fermentum*[1]
PL30	Raw sheep milk	KT626414	*Lactobacillus fermentum*[1]
PL31	Raw cow milk	KT626415	*Lactobacillus fermentum*[1]
PL32	Raw cow milk	KT626416	*Lactobacillus fermentum*[1]
PL33	Raw cow milk	KT626417	*Lactobacillus fermentum*[1]

***** stocks from NPL culture collection[1] isolation first reported in an earlier study [67].

99% similarity to the previously published sequences were used as the criteria to confirm species identity. The sequences were aligned using the CLUSTAL W program of the software MEGA 7.0.2. A phylogenetic tree was created using forty-five 16S rDNA sequences comprising the thirty-two sequences of LAB used in this study. Thirteen sequences were obtained from GenBank that were firmly related to the different species obtained in this study. The sequence of *Alkalibacterium olivapovliticus* (AB294175) was also recorded and used as an outgroup.

Indicator strains and culture conditions

The pathogenic bacterial strains used in this study were obtained from the Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. *Staphylococcus aureus* N1, *Escherichia coli* SS1, and *Salmonella enterica* ser Typhi D1 were previously isolated from infected patients and all exhibit an MDR phenotype [24-26]. A non-MDR strain of *Pseudomonas aeruginosa* ATCC 9027 was chosen because it is regarded as a reference strain for evaluating antimicrobial effectiveness in standardized testing protocols worldwide (ATCC® MP16™). All of these indicator strains were cultivated for 18-24 hours in QXWULHQWEURWK0HUFNŠUPVWHGWHPSDQGDW& with shaking at 200 rpm.

Cell-free culture supernatant preparation

The LAB strains were cultured in enriched media (MRS broth) DW&RU KRXUVDQGVXE-cultured in fresh media of the same type until the stationary phase was attained (Figure 1). The cell-free supernatants (CFS) were obtained by centrifugation of the LAB FXOWXUHV DW J IRU PLQXWHV DW &DQG ILOWHU sterilized by passing through a syringe filter (FilterBio®, Nantong, China) of 0.22 µm pore size.

Antibacterial activity of CFS by microdilution method

Overnight cultures of pathogenic bacteria were inoculated into fresh liquid medium (1:100 dilution in nutrient broth) and grown to an OD_{630} of 0.1. For finding out the minimum percentage of CFS that inhibits the growth of target pathogens, the pathogenic cultures were incubated without CFS (control) or with concentrations of CFS ranging from 1 to 18% (v/v). Aliquots of 200 µL from each culture were immediately transferred to a 96-well microtiter plate (Nunc®, Roskilde, Denmark), which was incubated in a microtiter plate reader (Spectramax 384 PLUS®, 6XQQ\YDOH&DOLIRUQLD&RUKRXUV7KHJURZWK of the pathogenic bacteria was monitored by measuring absorbence at 630 nm every 15 min. The antimicrobial activity was expressed as percent inhibition of pathogen growth relative to controls grown without CFS. All experiments were performed twice with readings taken in triplicate. The MIP (minimal inhibitory percentage) of CFS is the lowest percentage of CFS that resulted in • UHGXFWLRQ LQ JURZWK DQG ZDV GHWHUPLQHG E\ subtracting the CFS/culture OD_{630} from that of the cell-free medium [27].

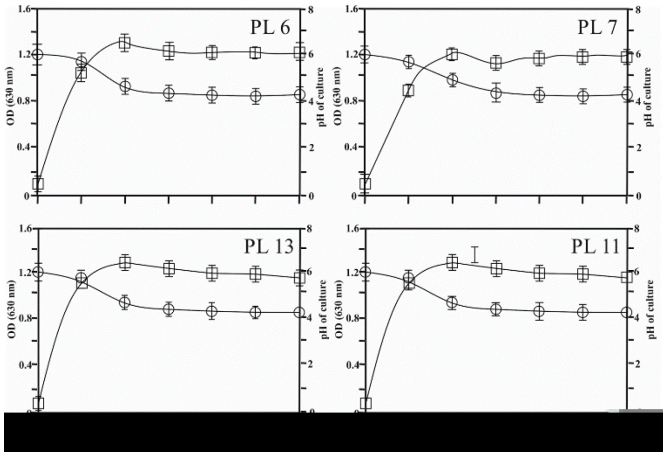
Agar well diffusion assay

The capacity of the shortlisted isolates to inhibit MDR pathogenic bacteria was also examined using indicator organisms in an agar well diffusion assay [28]. Twenty-five mL of liquefied 0.8% (w/v) nutrient agar was mixed with an active overnight culture of indicator isolate (1%, v/v), poured into sterile petri dishes and allowed to solidify. Wells were made with a sterile 8 mm diameter borer and filled with CFS from the LAB isolates. The dishes were incubated for 2 hours at 4°C to permit CFS diffusion into the agar, and then all the plates were incubated at 37°C for 24 hours. The diameter (mm) of the inhibition zone was a measure of the extent of antimicrobial activity. The assays were carried out three times independently.

Nature of the antibacterial metabolite

To investigate the chemical nature of the potentially inhibitory substances secreted by *Weissella confusa* PL6, *Enterococcus faecium* PL7, *Lactobacillus delbrueckii* PL11, and PL13 strains, the CFSs were subjected to a variety of tests. To determine if

Figure 1. Reduction in pH of the culture media during 24 hours growth of select LAB strains.



Data are the mean ± SD of at least three independent experiments. (Squares indicates the growth; circles indicates pH).