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Antimicrobial susceptibility

Human-derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens

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ABSTRACT

Lactobacillus reuteri is a commensal-derived anaerobic probiotic that resides in the human gastrointestinal tract. *L. reuteri* converts glycerol into a potent broad-spectrum antimicrobial compound, reuterin, which inhibits the growth of gram-positive and gram-negative bacteria. In this study, we compared four human-derived *L. reuteri* isolates (ATCC 55730, ATCC PTA 6475, ATCC PTA 4659 and ATCC PTA 5289) in their ability to produce reuterin and to inhibit the growth of different enteric pathogens *in vitro*. Reuterin was produced by each of the four *L. reuteri* strains and assessed for biological activity. The minimum inhibitory concentration (MIC) of reuterin derived from each strain was determined for the following enteric pathogens: enterohemorrhagic *Escherichia coli*, enterotoxigenic *E. coli*, *Salmonella enterica*, *Shigella sonnei* and *Vibrio cholerae*. We also analyzed the relative abilities of *L. reuteri* to inhibit enteric pathogens in a pathogen overlay assay. The magnitude of reuterin production did not directly correlate with the relative ability of *L. reuteri* to suppress the proliferation of enteric pathogens. Additional antimicrobial factors may be produced by *L. reuteri*, and multiple factors may act synergistically with reuterin to inhibit enteric pathogens.

1. Introduction

Therapeutic microbiology is expanding and beneficial bacteria are being implemented as treatment and prevention strategies for immune disorders and infectious diseases. Important characteristics of probiotic bacteria include their abilities to suppress the proliferation and virulence of pathogenic organisms. Lactic acid bacteria (LAB) such as *Lactobacillus* spp. produce antimicrobial factors and bacteriocins including lantibiotics, small heat-stable, non-lanthionine containing membrane-active peptides, larger heat-labile proteins and complex bacteriocins containing one or more chemical moieties [1–3]. Due to the production of diverse antimicrobial agents, probiotics may be considered for the treatment and prevention of a variety of infectious diseases caused by oral, enteric and urogenital pathogens [4–8].

Lactobacillus reuteri is an established probiotic agent, the most widely distributed *Lactobacillus* species among animals and considered to be one of a limited number of indigenous *Lactobacillus* species in the human intestine [4]. A primary antimicrobial compound produced by *L. reuteri*, reuterin, lacks a peptide or protein component and is produced during glycerol fermentation. Reuterin, a β-hydroxypropionaldehyde (3-HPA) derivative of glycerol, is produced under anaerobic conditions and exhibits broad-spectrum effects against gram-positive and gram-negative bacteria [9] as well as fungi, yeasts and protozoa [10]. The broad-spectrum antimicrobial activity of *L. reuteri* has been considered an important feature conferring therapeutic potential for the prevention or treatment of infections [6,8,11–14].

In this study, we analyzed four different human-derived *L. reuteri* strains for their relative abilities to produce reuterin and inhibit a variety of bacterial species including commensal organisms and diverse enteric pathogens. We also developed high-throughput versions of existing functional assays for measuring probiotic-mediated antimicrobial activities. High-throughput strategies may be important for functional screening of many candidate probiotic strains in the future. We have demonstrated

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strain-dependent variation in reuterin production among candidate probiotics and revealed the potential for multifactorial pathogen inhibition by *L. reuteri*. These studies provide an improved understanding of probiotic-mediated pathogen inhibition, and support future applications of beneficial bacteria for the prevention of diarrheal diseases.

2. Materials and methods

2.1. Bacterial strains and media

The strains used in this study are described in Table 1. *L. reuteri* strains ATCC 55730, ATCC PTA 6475, ATCC PTA 4659 or ATCC PTA 5289 will be referred to as strains 55730, 6475, 4659 and 5289, respectively, throughout this manuscript. *Lactobacillus* strains were cultured in DeMan-Rogosa-Sharpe (MRS) media (Becton Dickinson, Sparks, MD) at 37 °C for 24–48 h in an anaerobic chamber (Model 1025 S/N, Forma Scientific, Inc., Marietta, OH). Enteric pathogen strains were cultured aerobically in Brain Heart Infusion (BHI) media (Becton Dickinson) for 24 h at 37 °C. Modifications for specific assays are detailed accordingly.

2.2. Reuterin production by bacterial cultures

Reuterin was produced in order to assess minimum inhibitory concentrations (MICs) for various bacterial strains, as done previously [10,15,16]. Briefly, MRS was inoculated with *L. reuteri* and incubated anaerobically for 24 h at 37 °C. The bacteria were collected by centrifugation and washed with 50 mM sodium phosphate buffer (pH 7.4). The bacteria were resuspended to a concentration of $\sim 1.5 \times 10^{10}$ cells/mL in 250 mM glycerol and incubated anaerobically at 37 °C for 2 h. Simultaneously, samples were taken immediately after resuspension in glycerol to obtain viable cell counts. After the 2 h incubation, the bacteria were pelleted and reuterin-containing supernatants were collected and filter-sterilized using a 0.22 μm polyvinylidine difluoride filter (Millipore, Billerica, MA). Reuterin solution was stored at 4 °C as described previously [17,18].

Table 1
Listing of bacterial strains included in this study

Strains	Description	Source
Probiotic *Lactobacillus* strains		
L. reuteri ATCC 55730	Isolate from Peruvian mother's milk	Biogaia AB
L. reuteri ATCC PTA 6475	Isolate from Finnish mother's milk	Biogaia AB
L. reuteri ATCC PTA 4659	Isolate from Finnish mother's milk	Biogaia AB
L. reuteri ATCC PTA 5289	Oral isolate from Japanese female	Biogaia AB
L. acidophilus ATCC 4356	Probiotic strain, human isolate	ATCC[a]
L casei ATCC 334	Probiotic strain isolated from Emmental cheese	ATCC
L. gasseri ATCC 33323	DSM 20243, Probiotic strain, human isolate	ATCC
L. johnsonii ATCC 33200	VPI 7960, Human blood isolate	ATCC
L. plantarum 42/6	Fecal isolate from healthy Thai woman	S. Tumwasorn
Enteric pathogen strains		
EHEC	Clinical isolate of enterohemorrhagic *E. coli*	TCH[b]
ETEC	Clinical isolate of enterotoxigenic *E. coli*	TCH
Salmonella enterica	Clinical isolate	TCH
Shigella sonnei	Clinical isolate	TCH
Vibrio cholerae	Clinical isolate	TCH

EHEC, enterohemorrhagic *E. coli*; ETEC, enterotoxigenic *E. coli*.
[a] American Type Culture Collection (ATCC).
[b] Microbiology Laboratories, Department of Pathology, Texas Children's Hospital (TCH).

2.3. Quantification of reuterin by HPLC

The chromatographic separation of reuterin was performed using modified methods of Catassi et al. [19] and Talarico et al. [20]. In brief, the filter-sterilized reuterin solution was diluted 1:10 in sterile double-distilled water (ddH_2O), and a 20 μL aliquot was injected into an Aminex HPX 87C 300 7.8 mm cation-exchange column protected with a "Deashing Guard Cartridge" (Cat # 1250118, BioRad Laboratories, Richmond, CA) and eluted with degassed pure water at a flow rate of 0.6 mL/min at 85 °C. The column effluent was monitored with a differential refractometer (Dionex Corperation, Sunnyvale, CA) having an internal temperature of 50 °C. This method was calibrated with 1 mg/mL (10.87 mM) glycerol, and the assay's limit of detection was 1 μM of glycerol. The conversion of glycerol to reuterin occurs at a 1:1 ratio [20], and the concentration of reuterin (aldehyde form, 74 g/mol) in each sample was determined by subtracting the remaining amount of glycerol from the HPLC-determined concentration of the starting material.

2.4. Reuterin quantification by absorbance spectrophotometry

Reuterin samples were analyzed colorimetrically as done previously [16,21], with modifications to a 96-well plate format as follows. Reuterin samples were serially diluted in sterile ddH_2O, mixed with 10 mM tryptophan (Fluka) solution (0.01 M in 0.05 M HCl, stabilized with a few drops of toluene) followed by addition of 12 M HCl (Fisher) and incubation at 37 °C for 30 min. The optical density of each reaction was measured at 560 nm using a Spectramax 340PC (Molecular Devices Corporation, Sunnyvale, CA). A standard curve was generated using HPLC-quantified reuterin. The accuracy of this assay was confirmed by HPLC analyses of multiple reuterin samples from four different *L. reuteri* strains.

2.5. Minimum inhibitory concentration (MIC) assay for reuterin

The effects of reuterin on various bacterial strains were tested in a MIC assay. These experiments were performed three times in duplicate. This assay was described previously by Chung et al. [10], but it has been modified to accommodate specific quantities of reuterin in a 96-well format. Enteric pathogen strains were grown aerobically for 16–18 h in BHI broth at 37 °C and subsequently diluted to a concentration of $\sim 1 \times 10^6$ cells/mL. Aliquots (1 mL) were deposited into individual wells of 96-well, 2.5 mL deep-well plates (Cat # 37001–520, VWR International, West Chester, PA). Reuterin solution was serially diluted, and 1 mL of each dilution was added per well. The deep-well plates were incubated aerobically at 37 °C for 18–24 h, and aliquots from each well were transferred to individual wells of a 96-well microtiter plate (VWR, West Chester, PA). Optical densities were measured at 600 nm in a Spectramax 340PC (Molecular Devices Corporation). Positive growth controls were assayed using 1 mL BHI broth without reuterin.

2.6. Agar spot method of pathogen inhibition

An agar spot test was used to detect antimicrobial activities of *L. reuteri* against various bacterial strains. These assays were