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LACTOBACILLUS GASSERI G7 – A BACTERIOCINOGENIC STRAIN ISOLATED FROM VAGINAL SAMPLE

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Abstract: Lactobacilli exert antagonistic activity against many microorganisms as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins. In our study, we performed species identification of strain G7 isolated from human vaginal sample; determined its antimicrobial activities against human pathogen microorganisms; analyzed the genes encoding bacteriocins and determined the primary physicochemical nature of the active substance. According to 16S rRNA gene sequence analysis our strain G7 belongs to *Lactobacillus gasseri* species. By well diffusion test using neutralized, treated with catalase, cell-free supernatant, G7 strain inhibits the growth of *Escherichia coli*. The isolate G7 was tested for the presence of genes encoding the bacteriocins with primers targeting gassericin A and gassericin T gene. The positive results were confirmed by sequencing of the PCR products. New primer pairs were designed, targeting gassericin T and gassericin A bacteriocin operons. Partial characterization of the antimicrobial substance was made. The filter-sterilized supernatant was inactivated by the proteolytic enzymes proteinase K, pepsin and trypsin, indicating the proteinaceous nature of the inhibitory substances. The use of lactobacilli as therapeutic agents for the treatment and prevention of urogenital infections is an alternative to conventional antibiotic therapy and our study could contribute to the development of this strategy.

INTRODUCTION

The bacteriocins are antimicrobial proteinaceous substances secreted by some bacteria against microorganisms that usually closely relate to the producer strain. They are separated into two distinct categories according to the classification of Cotter et al. (2005). Class I bacteriocins (lantibiotics) are

lanthionine-containing small peptides which are active through the formation of pores and efflux of small metabolites from sensitive cells or through enzyme inhibition. Class II bacteriocins are non-lanthionine-containing bacteriocins. The majority of class II bacteriocins is active by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria. The large, heat-labile antimicrobial proteins Cotter et al. (2005) separate in designation called 'Bacteriolysins'. They have a domain-type structure and mechanism of action through the lysis of sensitive cells by catalyzing cell-wall hydrolysis. Some researchers support another classification of bacteriocins according to which four distinct classes of LAB bacteriocins have been identified on the basis of biochemical and genetic characterization: lantabiotics (class I), small, heat-stable nonlanthionine peptides (class II), large heat-labile proteins (class III) and complex bacteriocins containing chemical moieties such as lipid and carbohydrate (class IV) (Klaenhammer 1993).

The aims of this study were to identify vaginal *lactobacillus* strain G7; to determine its antimicrobial activities against human pathogen microorganisms; to analyze the genes encoding bacteriocins and to determine the primary physicochemical nature of the active substance.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *Lactobacillus gasseri* G7 strain was isolated from vaginal samples of healthy reproductive-age women and was identified by classical phenotypic methods and 16S rRNA gene sequence analysis (Stoyancheva et al. 2014). The *Lactobacillus* strain was maintained as frozen stocks at -80ºC in De Man-Rogosa-Sharpe broth (MRS, Oxoid Ltd, Basingstoke, Hampshire, UK) containing 20% (v/v) glycerol. ATCC, LMG, NBIMCC reference culture and pathogenic bacteria were used as test microorganisms (Table 1).

Antimicrobial activity

Antimicrobial activity of strain G7 was determined by agar spot test and well diffusion agar test according to Hernandez et al. (2005). The *Lactobacillus* isolate was propagated twice in MRS (Oxoid) medium before use.

DNA Isolation and PCR Amplification of Bacteriocins Genes

The pure chromosomal DNA was isolated from 2 ml 24-h old cultures, using Gene JET Genomic DNA Purification Kit (Thermo Scientific). Polymerase chain reaction (PCR) was performed with primer pair described in table 2. The PCR amplification was done in a QB-96 Satellite Gradient thermal cycler (LKB Vertriebs GmbH, Vienna, Austria). The PCR products were visualized in 1% agarose gel. The obtained PCR amplification product was purified using a Gel Band Purification kit (Amersham Biosciences, Uppsala, Sweden).

Detection of the genes related to bacteriocin production

The presence of the genes associated with bacteriocin production was determined by PCR amplification: gassericin A, gassericin T (Kawai et al. 1998a; Kawai et al. 2000). The following conditions were used for our new primers GTF, GTR and GAF, GAR: denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1min, annealing 62ºC for 45s and polymerization at 72ºC for 45s. An extra final elongation step was performed at 72ºC for 8 min.

DNA Sequencing and Accession Numbers

The nucleotide sequences of the PCR products were determined by Macrogen Inc. (Amsterdam, the Netherland). The sequences were blasted against the NCBI GenBank database (BLAST, http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignment was performed using CLUSTALW (Thompson et al. 1994). The sequences of the genes related to bacteriocin production were deposited in the NCBI GenBank database under accession numbers: KF724911 (for gassericin T operon of *L. gasseri* G7 strain), KF724910 (for gassericin A operon of *L. gasseri* G7 strain).

Partial characterization of the antimicrobial substance

To study the nature of the inhibitory substance, the active cell-free supernatant (CFS) was autoclaved (121°C for 15 min). After the treatment, the sample was allowed to cool down to room temperature and the remaining activity was determined.

The sensitivity of the antimicrobial substance to proteolytic enzymes was assayed by incubating the CFS (2 h at 37ºC) with: proteinase K, pepsin and trypsin. The enzymes (Sigma, USA) were used at a final concentration of 2 mg ml^{-1} for proteinase K and 1 mg ml^{-1} for pepsin and trypsin. The CFS without enzymes, as well as the enzyme solutions was exposed to the same conditions. After each treatment, the residual antimicrobial activity was determined by the well diffusion agar test. All determinations were carried out in duplicate.

RESULTS

Screening for antimicrobial activity

The inhibition spectra of the *L. gasseri* G7 isolate tested against 16 Gram (+) and Gram (–) pathogenic and non-pathogenic bacteria, are reported in Table 1 and Fig 1. Initially, the antimicrobial screening was performed by the agar spot test end well diffusion test.

Table 1. Antibacterial activity of *Lactobacillus gasseri* G7 strain determined by agar spot test and well diffusion agar test.

Indicator strains:	Agar spot test	Well-diffusion agar test
Lactobacillus acidophilus LA1 (isolate)	-	-
Lb. delbr. subsp. *bulgaricus* ATCC 11842^T	10*	-
Lb. delbrueckii subsp. *lactis* ATCC 12315	-	-
Lactobacillus plantarum ATCC 14917^T	-	-
Lactobacillus sakei LMG 9468^T	-	-
Lactobacillus curvatus LMG 9198	8	-
Lactobacillus casei A (isolate)	-	-
Lactobacillus helveticus ATCC15009^T	-	-
Escherichia coli LMG 8063	16	16
Enterococus fecalis LMG 7937^T	10	-
Enterococus sp. PO-2 (human isolate)	10	-
Staphylococus aureus LMG 8224	-	-
Klebsiella ozaenae (isolate)	-	-
Klebsiella pneumoniae (NBIMCC 8651)	4	-
Candida parapsilosis (human isolate)	-	-
Lactococus lactis subsp. *cremoris* LMG 7951	-	-

*diameters of inhibition zone (mm)

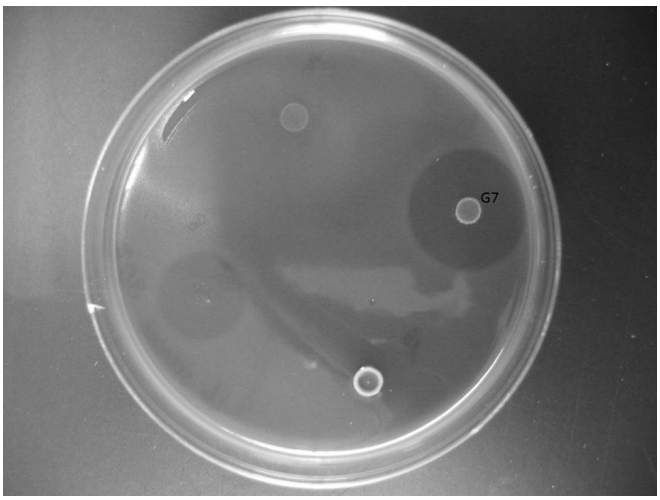

Figure 1. Antimicrobial activity produced by a *Lactobacillus gasseri* G7 strain on the growth of indicator strain *Escherichia coli* LMG 8063 by agar spot test.

Detection of genes related to bacteriocin production

L. gasseri G7 strain was tested for the presence of genes encoding the bacteriocins: gassericin A, gassericin T and acidocin LF221A. Positive results were obtained for strain *L. gasseri* G7 with primers targeting gene encoding gassericin T and with primers targeting acidocin LF221A. These results were confirmed by sequencing of the PCR products. Based on comparison of the sequences with the GenBank database new primer pairs were designed, targeting gassericin T and gassericin A bacteriocin operons (Table 2). PCR products of 700 bp for gassericin T and of 800 bp for gassericin A operon were observed with our new primers GTF, GTR and GAF, GAR. After sequencing of these amplicons, the sequences of gassericin T operon of strain G7 and gassericin A operon of strains G7 were determined.

Table 2. Primers for genes related to bacteriocin production.

Bacteriocin	Primer	Sequence (5'-3')	Annealing temperature (C°)	$MgCl_2$ (mM)	Reference
gassericin A	gaAF	GACCACAGCGAACATT	50	2.5	Kawai et al. (1998)
	gaAR	AATGAGGCACCAGAAG			
gassericin T	gaTF	GGAGTAGGTGGAGCGACAGT	65	2.5	Kawai et al. (2000)
	gaTR	TCCACCAGTAGCTGCCGTTA			
acidocin LF221A	LFAF	GTTGCAGGATCATGTG	50	2.5	Canzek Majhenic et al. (2003)
	LFAR	TGTTGCAGCTCCGTTA			
gassericin T	GTF	GGTGGGAGAAATAATTGGGC	62	2.5	This study
	GTR	CCTATTACAAACGATATGGCC			
gassericin A	GAF	GAACAGGTGCACTAATCGGT	62	2.5	This study
	GAR	CAGCTAAGTTAGAAGGGGCT			

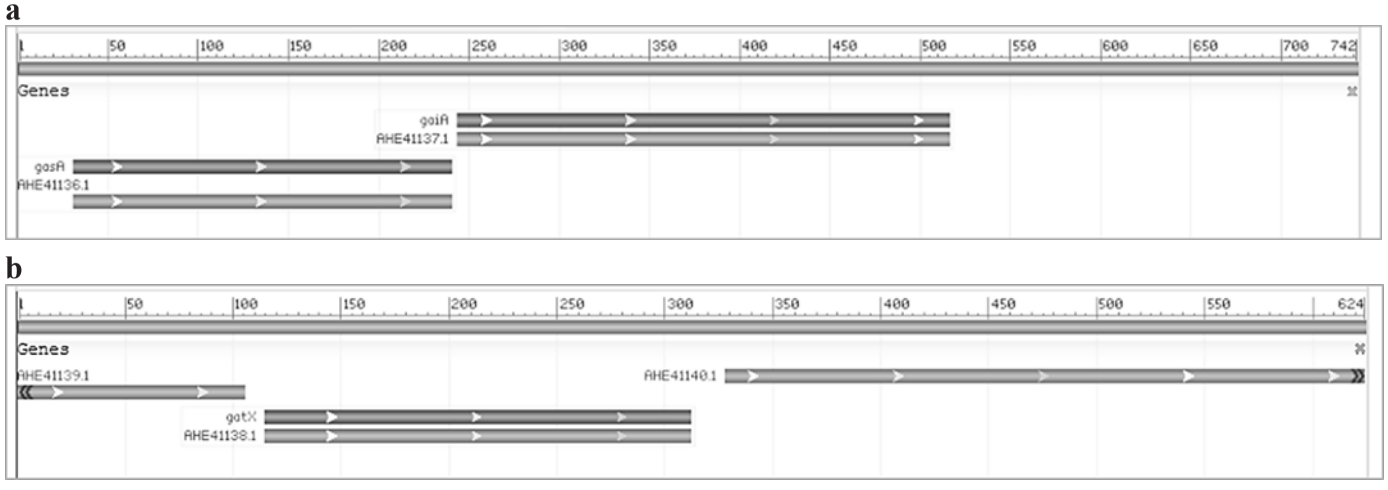

Figure 2. a) Graphics of *L. gasseri* G7 strain gassericin A region: gassericin A (gasA) and gassericin A immunity protein (gaiA) genes, complete cds and b) Graphics of *L. gasseri* G7 strain gassericin T gene region: bacteriocin (gatA) gene, partial cds; bacteriocin (gatX) gene, complete cds, putative immunity protein gene, partial cds.

Effect of different treatments on the antimicrobial substance

Partial characterization of the antimicrobial substance for *L. gasseri* G7 was made. The antimicrobial activity of CFS was completely lost after autoclave treatment. The filter-sterilized CFS of *L. gasseri* G7 strain was inactivated by the proteolytic enzymes proteinase K, pepsin and trypsin, indicating the proteinaceous nature of the inhibitory substances.

DISCUSSION

In this study vaginal *L. gasseri* G7 strain was recovered from vaginal samples obtained from healthy women in reproductive age. According to research conducted by Verhelst et al. (2004), Pavlova et al. (2002), El Aila et al. (2009) *L. gasseri* is one of the major phylotypes detected in healthy women with a lactobacillus-dominant vaginal microflora. *L. gasseri* is a widespread bacterium that inhabits human mucosal niches and demonstrates potential probiotic applications by fulfilling many desirable probiotic attributes (Selle et al. 2013).

According to some authors vaginal lactobacilli play an important role in controlling the health of the host. For example, they can positively influence and stabilize the host's vaginal microbiota via the production of acidic and antimicrobial compounds or exert a direct inhibiting action toward pathogenic bacteria (Boris and Barbe´s 2000; Witkin et al. 2007; Al Kassaa et al. 2014). The production of bacteriocins is one of the main antagonist mechanisms against colonization of undesirable bacteria (Collado et al. 2005).

The antimicrobial activity of the lactobacilli is a phenomenon which depends on many factors. In our experiments, inhibition caused by hydrogen peroxide and organic acids was ruled out by culturing the producer strains were anaerobically, neutralizing the culture supernatants and treating with catalase before assessing the antimicrobial activity.

The inhibitory spectrum of bacteriocins produced by different species of lactobacilli varies greatly - many inhibit other lactobacilli or closely related Gram-positive bacteria (Tahara et al. 1997; Yamato et al. 2003; Chumchalova et al. 2004, Chen et al. 20014), whereas others, are active against a wide spectrum of Gram-positive, Gram-negative bacteria and yeasts (Miteva et al. 1998; Ayeni et al. 2009; Simova et al. 2009; Messaoudi et al. 2011; Gerbaldo et al. 2012). According to some authors (Collado et al. 2005), the production of bacteriocins is one of the main antagonist mechanisms against colonization of undesirable bacteria. Some LAB strains need inducing factor of antimicrobial activity (close contact of competing microorganisms, cell wall-associated protein etc. (Maldonado et al. 2004). For this reason we performed co-cultivation of four *Lactobacillus* isolates with some potential inducing strains (the results not shown). Unfortunately, we could not provoke antimicrobial activity in liquid medium by co-cultivation.

In our research *L. gasseri* G7 strain show inhibitory activity against *E. coli*, similar to the activity of *L. gasseri* strains in the work of Al Kassaa et al (2014).

There are many studied proteins with antimicrobial activity of the genus *Lactobacillus* but only some of the responsible genes are sequenced. The genetic organization of Gram-positive bacteriocins is very diverse (Riley and Wertz 2002; Drider et al. 2006).

Studying the strain *L. gasseri* G7 we found and sequenced two operons for different bacteriocins: gassericin A (similar to gassericin K7 A and acidocin LF221A) and gassericin T (similar to gassericin K7 B and acidocin LF221B). Our sequence with accession number KF724910 includes orfA1 putative complemental factor partial cds, orfA2 gassericin K7 A and orfA3 putative immunity protein complete cds. It showed 99% similarity with nucleotide sequences EF392861.1 (for gassericin K7 A) and AY295874.1 (for acidocin LF221A). It is important to note that gassericin K7 A and acidocin LF221A are two bacteriocins with similar nucleotide sequences from two different strains *L. gasseri*, described from Majhenic et al. (2003, 2004). Strains *L. gasseri* K7 and *L. gasseri* LF221 are of human origin like our isolates.

Kawai et al. purify gassericin A from the supernatant by reverse-phase chromatography and sequenced its structural gene by the cloning for the first time (1998a). The complete primary structure revealed that gassericin A has a cyclic structure and has a molecular weight (Mr) of 5652 by mass spectrometry (Kawai et al. 1998b).

As mentioned above, the gassericin T region (including gatA and gatX genes) of *L. gasseri* strain G7 was sequenced. Our sequence with accession number KF724911 show 100% similarity with nucleotide sequences for the gassericin T gene region (AB710328.1 from *L. gasseri* SBT2055 and AB029612.1 from *L. gasseri* LA158) (Kawai et al. 2000). The sequence showed 99% similarity with acidocin LF221B (AY297947.1, *L. gasseri* LF221) and gassericin K7 B (AY307382.1) (Majhenic et al. 2004) as well.

In conclusion: This study proves that some *Lactobacillus* strains obtained from human vaginal samples and able to produce active bacteriocins can affect the development of unwanted bacterial species in the human ecosystem.

The genes related to bacteriocin production of *L. gasseri* G7 strain were sequenced. Most inhibitory spectra of vaginal isolates are specific at the strain level, which suggests the use of a combination of bacteriocinogenic strains to inhibit diverse undesired microorganisms.

The study of the hemolytic activity, antibiotic sensitivity, and hydrophobicity and auto-aggregation properties of this strain will be the object of our future research, which could lead to the development of new probiotic therapeutic preparation.

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