

ORIGINAL ARTICLE

# Bacteriocin-like inhibitory activities of seven *Lactobacillus delbrueckii* subsp. *bulgaricus* strains against antibiotic susceptible and resistant *Helicobacter pylori* strains

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**Significance and Impact of the Study:** In this study, anti-*Helicobacter pylori* activity of seven *Lactobacillus delbrueckii* subsp. *bulgaricus* (GLB) strains was evaluated by four cell-free supernatant (CFS) types. The GLB strains produced heat-stable bacteriocin-like inhibitory substances (BLISs) with a strong anti-*H. pylori* activity and some neutralized, catalase- and heat-treated CFSs inhibited >83% of the test strains. Bacteriocin-like inhibitory substance production of GLB strains can render them valuable probiotics in the control of *H. pylori* infection.

## Keywords

antibiotics, bacteriocins, *Helicobacter*, *Lactobacillus*, probiotics.

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## Abstract

The aim of the study was to detect anti-*Helicobacter pylori* activity of seven *Lactobacillus delbrueckii* subsp. *bulgaricus* (GLB) strains by four cell-free supernatant (CFS) types. Activity of non-neutralized and non-heat-treated (CFSs1), non-neutralized and heat-treated (CFSs2), pH neutralized, catalase-treated and non-heat-treated (CFSs3), or neutralized, catalase- and heat-treated (CFSs4) CFSs against 18 *H. pylori* strains (11 of which with antibiotic resistance) was evaluated. All GLB strains produced bacteriocin-like inhibitory substances (BLISs), the neutralized CFSs of two GLB strains inhibited >81% of test strains and those of four GLB strains were active against >71% of antibiotic resistant strains. Two *H. pylori* strains were BLIS resistant. The heating did not reduce the CFS activity. Briefly, all GLB strains evaluated produced heat-stable BLISs, although GLB and *H. pylori* strain susceptibility patterns exhibited differences. Bacteriocin-like inhibitory substance activity can be an advantage for the probiotic choice for *H. pylori* infection control.

## Introduction

*Helicobacter pylori* is linked to severe gastroduodenal diseases, however, increasing antibiotic resistance of the bacteria is the main cause of eradication failure of the infection (Boyanova *et al.* 2016a).

Lactic acid bacteria (LAB) are widely used as starter cultures in many fermented foods and as natural biopreservatives (Montalbán-López *et al.* 2011; Kaškonienė *et al.* 2017). The LAB also possess many health-promoting activities. For instance, *Lactobacillus delbrueckii* subsp. *bulgaricus* exhibits antibacterial, immunostimulatory and antimutagenic properties (van de Guchte *et al.* 2006; Boyanova *et al.* 2009).

Anti-*H. pylori* activity of lactobacilli may involve: direct inhibition; decrease in *H. pylori* urease activity and

adhesion; secretion of lactic acid, bacteriocins, autolysins and suppression of *H. pylori*-associated IL-8 production (Ruggiero 2014). An important quality of some lactobacilli is their ability to produce bacteriocins or bacteriocin-like inhibitory substances (BLISs). The antibacterial effect of these ribosomally synthesized and extracellularly released antimicrobial peptides is due to membrane permeabilization of the bacterial cells, resulting in cytoplasmic content release, membrane potential depolarization and, finally, to cell death (Drider *et al.* 2006; Simova *et al.* 2009). Bacteriocin-like inhibitory substances can inhibit pathogenic bacteria such as *Listeria monocytogenes* (Scatassa *et al.* 2017).

*Lactobacillus delbrueckii* subsp. *bulgaricus* strains can suppress the growth of clinically important bacteria such as *Clostridium difficile*, *Escherichia coli*, *Streptococcus*

*mutans* and *Pseudomonas aeruginosa* through a competitive adhesion inhibition, organic acid production, bacteriocins production and immunomodulation (van de Guchte et al. 2006; Boyanova et al. 2009). *Lactobacillus delbrueckii* subsp. *bulgaricus* clinical benefits in *H. pylori* infection treatment have been reported. In Thailand, adding *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* either before or before and after 1-week tailored triple therapy significantly improved eradication rates (Tongtaweewee et al. 2015).

However, there are scanty reports on *L. delbrueckii* subsp. *bulgaricus* effects on *H. pylori* and only single studies on the BLIS producing strains in the subspecies (Miteva et al. 1998; Simova et al. 2009).

The aim of this study was to evaluate anti-*H. pylori* activity of cell-free supernatants (CFS) from seven *L. delbrueckii* subsp. *bulgaricus* (GLB) strains of four types against numerous clinical *H. pylori* strains and to look for CFS susceptibility variations.

## Results and discussion

Of the 18 *H. pylori* strains evaluated, 1 (5.6%) was amoxicillin resistant, 5 (27.8%) strains were metronidazole resistant, 4 (22.2%) strains were quinolone resistant and no strain was tetracycline resistant. High (in six strains, 33.3% of the strains) clarithromycin resistance rate was found. In our prior study on 84 phenotypically resistant/intermediately susceptible to clarithromycin strains by allele-specific PCR and 3'-mismatched PCR, the resistance was confirmed in all strains and the prevalence of A2143G, A2142G, A2143G+A2142G and A2142C mutations was 64.3, 23.8, 10.7 and 1.2% respectively (Boyanova et al. 2016b). 7 (38.9%) *H. pylori* strains had single antibiotic resistance, 3 (16.7%) strains had double resistance and 1 (5.6%) strain exhibited triple resistance.

In our previous study, performed with whole *L. delbrueckii* subsp. *bulgaricus* cells and six different *L. delbrueckii* subsp. *bulgaricus* strains, three strains suppressed the growth of >86% of *H. pylori* strains at low pH values and two strains suppressed the growth of >53% of the test strains at neutralized pH values (Boyanova et al. 2009).

In the present work, the CFSs at their natural pH (CFSs1 and CFSs2) inhibited the growth of *H. pylori* strains tested in 95.6% (65/68) and 88.2% (60/68,  $P = 0.21$ ) respectively, of the CFS-*H. pylori* combinations (Table 1). Mean inhibitory zone diameters were similar and between 13.4 and 16.4 mm. The neutralized and catalase-treated CFSs3 and CFSs4 inhibited 75.9% (63/83) and 76.8% (63/82,  $P = 0.89$ ) respectively, of the CFS-*H. pylori* combinations. The highest activity (growth inhibition of >81% of the test strains with mean inhibitory

zone diameters of >13 mm) was found with both CFSs3 and CFSs4 of two (GLB44 and GLB47) *Lactobacillus* strains.

The CFSs retained their activity against *H. pylori* strains after treatment at 100°C for 10 min. The differences in the activities of the non-heat-treated CFSs1 and the heat-treated CFSs2s as well as between the non-heat-treated CFSs3 and the heat-treated CFSs4 were not statistically significant ( $P > 0.05$ ).

No *H. pylori* strain was resistant to the CFSs of all GLB strains evaluated. The growth of >71% (71.4–87.5%) of the antibiotic resistant *H. pylori* strains was suppressed by both neutralized and catalase-treated CFSs (CFSs3 and CFSs4) of four GLB strains. Only two *H. pylori* strains, both of which were antibiotic resistant, exhibited no susceptibility to all the neutralized, catalase- and heat-treated CFSs (CFSs4). One of the strains was metronidazole resistant and was susceptible to five CFSs2 but to none of the CFSs4 from the seven GLB strains. The other strain (triple resistant to amoxicillin, metronidazole and ciprofloxacin) was susceptible to only 1 CFS2 (of GLB14) and to none of the seven CFSs4 tested.

Most LAB bacteriocins are small cationic and heat-stable peptides with higher antibacterial activity at lower (<5) pH compared to higher pH values (Zacharof and Lovitt 2012). Class I and II LAB bacteriocins are heat-stable (Zacharof and Lovitt 2012). Class IIa bacteriocins are the major antibacterial peptide group of the LAB (Drider et al. 2006).

In this study, the anti-*H. pylori* activity of the non-neutralized and non-heat-treated CFSs (CFSs1) was higher (against 95.6%, 65/68 of the CFS-*H. pylori* combinations) than that (against 75.9%, 63/83,  $P = 0.001$ ) of the neutralized and non-heat-treated CFSs (CFSs3). The activity of the non-neutralized heat-treated CFSs (CFSs2) was slightly higher (against 88.2%, 60/68 of the CFS-*H. pylori* combinations) than that (against 76.8%, 63/82,  $P = 0.07$ ) of the neutralized and heat-treated CFSs (CFSs4). The results imply effects of organic acids or other bioactive substances of the lactobacilli as well as a higher BLIS activity at lower pH values (Zacharof and Lovitt 2012).

Notably, the CFSs of all seven GLB stains exhibited anti-*H. pylori* activity as well as thermostability at 100°C for 10 min, suggesting the presence of heat-stable BLISs. Simova et al. (2009) found a bacteriocin (bulgaricin BB18) with anti-*H. pylori* activity. The bacteriocin was heat-resistant (at 121°C for 15 min), stable at pH from 2 to 10 and protease sensitive.

In this study, the activity of the neutralized and catalase-treated CFSs (CFSs3 and CFSs4) of all GLB strains was similar with regard to the mean *H. pylori* inhibitory diameters (11.8–15.0 mm). It is important, however, that the CFSs3 and CFSs4 of both GLB44 and GLB47

**Table 1** *Lactobacillus delbrueckii* subsp. *bulgaricus* (GLB) cell-free supernatant (CFS) activity against *Helicobacter pylori* strains

GLB strain	Filtrate type*	No. of <i>H. pylori</i> strains tested	No. of strains inhibited	% of strains inhibited	95% CI†	Mean diameter of inhibition‡ (mm)	Range of diameters of inhibition (mm)
GLB14	CFS1	10	10	100.0	67.9–100.0	14.4	10.0–17.0
GLB23		10	9	90.0	57.4–99.9	13.4	7.0–20.0
GLB60		10	10	100.0	67.9–100.0	14.6	11.0–18.0
GLB35		10	9	90.0	57.4–99.9	14.0	7.0–18.0
GLB44		9	8	88.9	54.3–99.9	14.8	7.0–18.0
GLB47		9	9	100.0	65.5–100.0	14.7	12.0–17.0
GLB55		10	10	100.0	67.9–100.0	14.8	12.0–18.0
GLB14	CFS2	10	10	100.0	67.9–100.0	16.4	10.0–23.0
GLB23		10	8	80.0	47.9–95.4	14.7	7.0–24.0
GLB60		10	9	90.0	57.4–99.9	15.6	7.0–22.0
GLB35		10	9	90.0	57.4–99.9	15.5	7.0–24.0
GLB44		10	8	80.0	47.9–95.4	16.3	7.0–26.0
GLB47		10	9	90.0	57.4–99.9	16.4	7.0–26.0
GLB55		8	7	87.5	50.8–99.9	15.5	7.0–22.0
GLB14	CFS3	12	8	66.7	38.8–86.4	11.8	7.0–17.0
GLB23		12	9	75.0	46.2–91.7	13.0	7.0–19.0
GLB60		12	9	75.0	46.2–91.7	12.2	7.0–17.0
GLB35		12	9	75.0	46.2–91.7	12.8	7.0–17.0
GLB44		11	9	81.8	51.2–96.0	13.6	7.0–17.0
GLB47		12	10	83.3	54.0–96.5	13.4	7.0–16.5
GLB55		12	9	75.0	46.2–91.7	12.7	7.0–17.0
GLB14	CFS4	12	9	75.0	46.2–91.7	13.8	7.0–23.0
GLB23		12	9	75.0	46.2–91.7	13.8	7.0–20.0
GLB60		12	9	75.0	46.2–91.7	12.8	7.0–18.0
GLB35		12	9	75.0	46.2–91.7	13.2	7.0–20.0
GLB44		12	10	83.3	54.0–96.5	15.0	7.0–24.0
GLB47		12	10	83.3	54.0–96.5	14.5	7.0–20.0
GLB55		10	7	70.0	39.2–89.7	13.7	7.0–22.0

\*CFS1-non-neutralized and non-heat-treated CFS, CFS2-non-neutralized and heat-treated CFS, CFS3-neutralized and catalase-treated but non-heat-treated CFS, CFS4-neutralized, catalase- and heat-treated CFS4.

†95% confidence interval.

‡Diameter of the well, 7 mm.

suppressed the growth of the highest (>81%) proportion of *H. pylori* strains tested.

Similarly, according to Dr. Andrew B. Onderdonk, Ph.D., Director of Clinical Microbiology Laboratory and Professor of Pathology at Harvard Medical School, GLB 44 (Genesis Laboratories Ltd., Sofia, Bulgaria), isolated from *Galanthus nivalis* leaves, reduced the growth of most pathogenic bacteria and could be added to unpasteurized juices as a natural preservative (<http://glb44.org/>).

Bacteriocin-like inhibitory substance susceptibility within different strains of the same species has seldom been evaluated, although it is highly important (Kjos *et al.* 2009). In the present work, two strains (11.1% of all test strains and 18.2% of the 11 *H. pylori* strains resistant to antibiotics) were BLIS resistant, showing no susceptibility to the neutralized, catalase- and heat-treated CFSs (CFSs4). In the case of the strain with triple resistance, either mutations or a possible efflux pump may be present and may affect the BLIS activity.

Bacteriocin activity depends on many factors of the test strains such as the bacterial cell envelope/membrane composition, reduction in bacteriocin binding/insertion, bacteriocin sequestering and degradation or efflux pumping (Drider *et al.* 2006, 2016). Different expression of the genes encoding bacteriocin receptors can cause variations in BLIS susceptibility within the test strains of the same species (Kjos *et al.* 2009). This can explain the present differences in CFS susceptibility in the different *H. pylori* strains. A drawback of this study is the evaluation of CFSs against dissimilar numbers of test strains. However, an advantage of the study is the assessment of CFS activities against many clinical *H. pylori* strains, including those with multidrug antibiotic resistance.

Bacteriocin-like inhibitory substance producing GLB strains can have an important role as adjunctive treatment in *H. pylori* infections and perhaps for prophylaxis. Moreover, the BLISs have a growing importance in the age of increasing antibiotic resistance (Simova *et al.* 2009).

In this study, the seven selected GLB strains were BLIS producers with good activity against the *H. pylori* strains tested, including many antibiotic resistant strains. Importantly, both neutralized and catalase-treated CFSs of four GLB suppressed the growth of most (>71%) antibiotic resistant *H. pylori* strains.

In conclusion, as the antimicrobial properties of the Bulgarian lactobacilli are highly strain-specific, a careful selection of probiotic strains is required. The seven selected GLB strains produced heat-stable BLISs with considerable anti-*H. pylori* activity and may be useful for prophylaxis or as a treatment adjuvant for *H. pylori* infection control. Notably, different *H. pylori* BLIS susceptibility patterns were found, but some neutralized, catalase- and heat-treated CFSs inhibited up to 83.3% of the test strains. BLIS production of GLB strains can be a strong advantage of the *L. delbrueckii* subsp. *bulgaricus* strains used as probiotics.

## Materials and methods

### *Helicobacter pylori* strains

A total of 18 randomly selected *H. pylori* strains isolated from gastric biopsy specimens of patients with gastro-duodenal diseases were evaluated in December 2016 to February 2017. There were eight children (three boys and five girls, aged 11–15 years) and 10 adults (five men and five women aged 31–76 years), 14 patients with chronic gastritis, three patients (children) with acute gastritis and one patient with gastroesophageal reflux disease. Seventeen patients were untreated and one patient had previously been treated for *H. pylori* infection. Strain isolation and identification were performed during the laboratory diagnostic work as previously reported (Boyanova *et al.* 2016a). The patients and the parents of the children filled informed written consent. The study was approved by the Ethical Committee of Medical University of Sofia, Sofia, Bulgaria.

### Antimicrobial susceptibility test

*Helicobacter pylori* strain susceptibility to amoxicillin, metronidazole, clarithromycin, tetracycline and ciprofloxacin (purchased from Sigma-Aldrich/Merck, Darmstadt, Germany) was tested by a breakpoint susceptibility testing method as previously described (Boyanova *et al.* 2008, 2016a). The method is a simplified version of agar dilution method using several concentrations of the antibiotic agent. In our prior work, the breakpoint susceptibility testing method exhibited a very good (93.3–100%) category agreement with the results of E test or the agar dilution method (Boyanova *et al.* 2008). Briefly, *H. pylori*

suspensions (2–3 McFarland turbidity standard), Mueller Hinton agar (Oxoid, Basingstoke, UK) containing 5% sheep blood and the following agents were used: metronidazole 4, 8 and 16 mg l<sup>-1</sup>, clarithromycin 0.25, 0.5, 1, 2 and 4 mg l<sup>-1</sup>, amoxicillin 0.12, 0.25, 0.5, 1 and 2 mg l<sup>-1</sup>, ciprofloxacin 1 and 10 mg l<sup>-1</sup>, and tetracycline 1 and 2 mg l<sup>-1</sup>. Resistance breakpoints were: >8 mg l<sup>-1</sup> metronidazole, >0.5 mg l<sup>-1</sup> clarithromycin, >0.125 mg l<sup>-1</sup> amoxicillin, >1 mg l<sup>-1</sup> tetracycline and >1 mg l<sup>-1</sup> ciprofloxacin (EUCAST 2015).

### *Lactobacillus delbrueckii* subsp. *bulgaricus* (GLB) strains

Seven *L. delbrueckii* subsp. *bulgaricus* (labeled by the provider as GLBs) strains were chosen and kindly provided by Genesis Laboratories Ltd., and were evaluated for BLIS production. The strains involved: GLB14, GLB23, GLB35, GLB44 (the strain also used in the commercially available probiotic Proviotic<sup>®</sup>), GLB47, GLB55 and GLB60. The GLB strains were obtained in sterile 10% skim milk solutions and were inoculated onto De Man, Rogosa, Sharpe-MRS agar (Oxoid). The plates were incubated in anaerobic atmosphere (AnaeroGen gas generating sachets, Oxoid) at 37°C for 48 h. The GLB strains were kept at –70°C in MRS broth. The filtrates were kept at –20°C for up to 60 days.

Bacteriocin-like inhibitory substance extraction was carried out according to previously reported methods (Simova *et al.* 2009) with some modifications. Suspensions of the GLB strains were prepared to obtain a density of 4 McFarland turbidity standard in De Man, Rogosa, Sharpe-MRS broth (Oxoid) and were inoculated (1 : 10 v/v) into 30 ml of MRS broth. The suspensions were incubated anaerobically (as above) at 37°C for 18–24 h. After the incubation, the cells were removed by centrifugation (5000 g, 10 min, 4°C). The supernatants were filter-sterilized (0.22 µm pore size; Bonna-Agela Technologies Ltd., Tianjin, China) to eliminate the possible presence of viable cells.

Four types of cell-free supernatant (CFS) of each GLB strain were prepared as follows: non-neutralized and non-heat-treated (CFSs1), non-neutralized and heat-treated (CFSs2), neutralized and catalase-treated but non-heat-treated (CFSs3), or neutralized, catalase- and heat-treated (CFSs4) CFSs. The natural pH of CFSs was between 4.0 and 5.0 (assessed by a pH meter, model: 3320; Jenway Ltd., Felsted, Dunmow, Essex, UK), while the pH of the neutralized CFSs was adjusted to 6.5–7.5 (measured by a pH-meter) with 1 mol l<sup>-1</sup> of NaOH to eliminate the inhibitory effects of organic acids. Then the neutralized CFSs were treated with catalase (Sigma-Aldrich/Merck, final concentration of 1 mg ml<sup>-1</sup>) for 2 h at 25°C to remove the inhibitory effect of the hydrogen peroxide



(Simova *et al.* 2009) because H<sub>2</sub>O<sub>2</sub> production due to a NADH oxidase has been detected in *L. delbrueckii* subsp. *bulgaricus*, especially in aerated cultures (Marty-Teyssset *et al.* 2000).

A part of the obtained non-neutralized (CFSs1) or neutralized and catalase-treated (CFSs3) CFSs was additionally treated by heating at 100°C for 10 min in order to assess thermal stability of the antimicrobial compounds.

#### Agar-well diffusion method

For the BLIS assay, *H. pylori* suspensions corresponding to 1–2 McFarland turbidity standard were prepared in Mueller-Hinton broth (National Centre of Infectious and Parasitic Diseases, NCIPD, Sofia, Bulgaria) and were subcultured onto nonselective Mueller-Hinton agar plates (Oxoid) containing 5% sheep blood, and then were incubated in microaerophilic (CampyGen gas generating sachets, Oxoid) conditions at 37°C for 48–72 h before the experimental use.

An agar-well diffusion method was used to assess CFS effect on *H. pylori* growth as previously published (Boyanova *et al.* 2009). The method was carried out by nonselective Mueller-Hinton agar (Oxoid) plates containing 5% sheep blood. *Helicobacter pylori* inocula were prepared in Mueller-Hinton broth (NCIPD) to correspond to 1–2 McFarland turbidity standard and were inoculated onto the blood Mueller-Hinton agar plates. Wells (diameters, 7 mm) were drilled into the Mueller-Hinton agar plates by a sterile stainless steel borer. The wells were filled with 80 µl of different CFSs or sterile MRS broth (pH adjusted for CFS1/CFS2 testing and catalase-treated for CFS3/CFS4 testing) as a control. In addition, the CFSs were subcultured onto blood agar to make sure the lack of bacterial growth. The plates were incubated in microaerophilic atmosphere (CampyGen gas generating sachets, Oxoid) at 37°C for 48–72 h. Inhibitory zone diameters were measured in millimeters.

Different combinations between *H. pylori* strains and the CFS types were evaluated. The activity of CFS types against eight *H. pylori* strains was tested twice and the corresponding mean inhibitory zone diameters were considered. Because of the scanty or no growth of some strains during the AWDM performance, the number of *H. pylori* strains tested was limited to 12 for the different CFS types (Table 1).

#### Statistical analysis

Differences between groups were assessed with chi-square test or Fisher's exact test, when appropriate (<https://graphpad.com/quickcalcs/contingency1.cfm>). Statistically significant differences ( $P < 0.05$ ), and 95% confidence intervals were evaluated.

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#### Conflict of Interest

The authors declare that they have no conflict of interest.

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