Antimicrobial Activity of *Lactobacilli* and *Bifidobacteria* Isolates

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ABSTRACT

Three isolates of lactic acid bacteria produced different antibacterial agents against pathogens in their culture cell filtrates. The lactic acid bacteria were isolated from a traditional Egyptian dairy product (Soft cheese). The antimicrobial agents were bacteriocin and organic acids. The isolates were identified as *Lactobacillus rhamnosus* HM2, *Bifidobacterium bifidum* B11 and B12. HPLC analysis indicated that the isolates produced lactic and acetic acids. Propionic acid was produced only by *bifidobacteria* isolates. Inhibition zones diameters were affected by the addition of Proteinase K and / or neutralization of cell filtrates.

INTRODUCTION

Lactic acid bacteria (LAB) are widely used as starter cultures in dairy fermentations. One of the major reasons for their wide use is the wide range of antimicrobial substances they are able to produce which efficiently contribute to the preservation of the fermented products *(De Vuyst and Vandamme, 1994 and Dodd and Gasson 1994).*

Some of lactic acid bacteria are described as probiotics, which were defined as live microorganisms and when consumed in appropriate amounts in the food, confer a health benefit on the host *(FAO/WHO 2001).* The probiotic strains that are currently most often being investigated are *Lactobacillus* and *Bifidobacterium* spp. The effectiveness of selected *Lactobacillus* strains used as probiotics to prevent and treat infectious bacterial and viral diarrhea, Helicobacter pylori gastroenteritis and urovaginal infections has been demonstrated in well-designed *in vitro* and *in vivo* experimental studies and double-blind, placebo-controlled clinical trials *(Reid et al., 2003 and Servin 2004).*

The mechanism(s) of the antibacterial activity of probiotic *Lactobacillus* strains appears to be multifactorial *(Servin, 2004).* In particular, by producing metabolites such as acetic and lactic acid and thus lowering the pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens *(Richard et al., 2006).* The anti-Salmonella enterica serovar typhimurium killing activity of probiotic *Lactobacillus* and *Bifidobacterium* strains has been previously investigated using an *in vitro* method in which the activity was measured in the presence

of Luria broth (LB) or phosphate-buffered saline *(Bernet-Camard et al., 1997; Coconnier et al., 1997 and Lievin et al., 2000).* Using a new *in vitro* method, we were able to distinguish between the lactic acid- and non-lactic acid-dependent anti-Salmonella activities of *Lactobacillus* strains known to be probiotic. Of these antimicrobial substances, bacteriocins are one of the most promising natural food preservatives produced by LAB *(Daeschel 1993; McMullen and Stiles 1996; Ray and Daeschel 1994 and Ruiz-Barba et al., 1994).* This preservation potential could be achieved either by using a bacteriocin-producing starter culture or by applying the bacteriocin itself as a food additive. Bacteriocins produced by lactic acid bacteria (LAB) have received considerable attention as food preservatives and as potential replacements of antibiotics *(Vandenbergh 1993).*

The aim of this study was to examine the antagonistic activity by the *Lactobacillus* and *bifidobacteria* isolates from traditional Egyptian fermented dairy products against some pathogenic indicator strains.

MATERIALS AND METHODS

Isolation:

Lactobacillus and *Bifidobacterium* were isolated from a traditional Egyptian fermented dairy product (Soft cheese). Samples were serially diluted from 10^{-1} to 10^{-8}. The dilutions from 10^{-5} to 10^{-8} were plated on MRS medium. The plates were incubated at 40°C for 48 h. *Lactobacillus* and *Bifidobacterium* colonies were isolated on MRS agar

and TPY agar medium, respectively. MRS medium was used to grow *lactobacilli* and TPY medium was used for *bifidobacteria*. Isolates were stored at −80° C in medium supplemented with 25% (v/v) glycerol as cryoprotectant. Pathogen strains (Table 1) were propagated at 37°C in Mueller-Hinton medium (Difco, USA) and grown aerobically.

To prepare cell-free culture filtrate, *Lactobacillus* and *Bifidobacterium* isolates were grown at 37°C for 72 h. Bacterial suspension was centrifuged at 7000 xg for 5 min at 4°C and the supernatants were filtered through a 0·22 µm pore-size disposable sterile filter. The sterile, cell-free filtrate was stored at -20°C until use.

Lactobacillus (isolate HM1 and HM2) and *Bifidobacterium* (isolates B11 and B12) isolated from traditional Egyptian fermented dairy product were used in this study.

Identification:

Bifidobacteria and *lactobacilli* isolates were identified by morphological, physiological parameters by using API manual (BioMérieux, USA). Genomic DNA was purified from the isolates and amplified with T7 and SP6 sequencing primers. PCR products were purified and applied for sequencing.

Antibacterial activity:

Growth was carried out in 50 ml medium, supernatants were collected by centrifugation at 7000 xg for 5 min at 4°C, then the supernatant was divide to 3 parts; 1) without adjusted pH, 2) adjusted to neutral pH 7.0 and 3) digest the proteins with proteinase K and boiled at 95°C for 10 min for determination of antibacterial substances. The study was achieved as triplicates.

The antibacterial spectrum of the supernatants from the isolates was determined using disc fusion method. The samples were filter sterilized by passage through 0.22 µm pore size membrane. 10 µl aliquots of sterile samples were added to the discs (Oxoid, UK) that had been arranged on the Mueller-Hinton sloppy agar plates previously seeded with the indicator bacteria (Table 1) and incubated overnight at 37°C. After 12-18 h of incubation, the diameters of the growth zones were measured.

Table 1: Indicator strains.

Indicator strains	Source
Listeria monocytogenes	DSM 12464
Enterococcus faecalis	ATCC 29212
Escherichia coli	ATCC 11775
Staphylococcus aureus	ATCC 6538
Pseudomonas aeruginosa	DSM1117
Enterobacter aerogenes	ENT 77/403

RESULTS

DNA sequence analysis showed that the 4 isolates are identified as *Bifidobacterium bifidum* (B11 and B12) and *Lactobacillus rhamnsosus* (HM1 and HM2). Isolates *B. bifidum* B11, B12 and *L. rhamnosus* HM2 showed their antimicrobial activity against the used pathogen's strains by using cell filtrates. Only *Pseudomonas aeruginosa* DSM1117 was resistance to antimicrobial substances in the cell filtrates. *L. rhamnosus* HM1 did not show any antimicrobial activity against all used pathogens as shown in Table (2).

Table 2: Inhibition zone diameter (cm) using culture cell filtrate of *B. bifidum* B11, B12, HM1 and *L. rhamnosus* HM2 strains without adjust the pH.

Indicator strains	B11	B12	HM1	HM2
L. monocytogenes DSM 12464	3.0	2.7	0	3.2
Ent. faecalis ATCC 29212	1.7	2.4	0	2.4
E. coli ATCC 11775	1.8	1.9	0	1.6
S. aureus ATCC 6538	3.1	3.7	0	3.7
P. aeruginosa DSM1117	0	0	0	0
Ent. aerogenes ENT 77/403	1.5	1.6	0	1.8

Our isolate strains showed different diameters of inhibition zones. The inhibition zone's diameters were ranged between 1.3 to 3.5 cm according to the isolate and the indicator strains. *Listeria monocytogenes* DSM 12464 and *Staphylococcus aureus* ATCC 6538 were highly affected with antimicrobial substance in *lactobacilli* and *bifidobacteria* cell filtrates. The inhibition zone's diameter of *L. monocytogenes* ranged between 2.7 to 3.0 cm by *B. bifidum* B12 and B11, respectively and 3.2 cm by cell filtrate of *L. rhamnosus* HM2. The inhibition zones of *S. aureus* were 3.7, 3.1 and 3.7 cm by the cell filtrate of *L. rhamnosus* HM2 and *B. bifidum* B11 and B12, respectively, as shown in Table (2).

Growth of *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 11775 was inhabited by the same cell filtrates, but the inhabation zones' diameters were samller and ranged between 2.4, 1.7 and 2.4 cm against *Ent. faecalis* and 1.6, 1.8 and 1.9 cm against *E. coli*, by the cell filtrate of *L. rhamnosus* HM2 and *B. bifidum* B11 and B12, respectively. However the inhabation zones against *Enterobacter aerogenes* ENT 77/403 were smaller than other indicator strains, as 1.8, 1.5, 1.6 cm by the cell filtrate of *L. rhamnosus* HM2, *B. bifidum* B11 and B12, respectively as shown in Table (2).

The mechanism(s) of the antibacterial activity of probiotic *Lactobacillus* strains appears to be multifactorial by producing metabolites such as acetic and lactic acid and thus lowering the pH. For this reason, we adjusted the pH of the cell filtrates to pH 7.0 to mask the pH effect. Table (3) shows the effect of neutral pH of cell filtrate on indicator strains; Neutral pH of culture cell filtrate of *B. bifidum* B11 and *L. rhamnosus* HM2 strains

reduced inhibition zones' diameters of *L. monocytogenes* DSM 12464. However, neutralized pH of culture cell filtrates of *B. bifidum* B11, B12 and *L. rhamnosus* HM2 strains reduced inhibition zones' diameters of *S. aureus* ATCC 6538, *E. coli* ATCC 11775 and *Ent. aerogenes* ENT 77/403, respectively, reduction in inhabation zones' diameters was ranged by 0.2 to 0.3 cm.

Table 3: Effect of neutral pH culture cell filtrate of *B. bifidum* B11, B12 and *L. rhamnosus* HM2 strains on the inhibition zone diameter (cm).

Indicator strains	B11	B12	HM2
L. monocytogenes DSM 12464	2.8	2.7	3.0
Ent. faecalis ATCC 29212	1.7	2.4	2.4
E. coli ATCC 11775	1.8	1.5	1.6
S. aureus ATCC 6538	2.9	3.7	3.7
Ent. aerogenes ENT 77/403	1.5	1.6	1.5

HPLC analysis for organic acids showed that the isolates produced lactic, acetic and propionic acids. The isolates were cultured for 2 days in MRS broth medium for *lactobacilli* isolate and TPY medium for *bifidobacteria* isolates to investigate substrate effects on the production of organic acids. In all cases, the isolates of *lactobacilli* produced 11.1 to 11.4 g/l lactic acid. *L. rhamnosus* HM2 produced 0.132 g/l acetic acid, whereas, *L. rhamnosus* HM1 produced only lactate. Both *B. bifidum* B11, B12 produced lactic, acetic and propionic acids, which ranged between 21-23, 0.25-0.31 and 0.12-0.14 g/l, respectively. The cell filtrates of *L. rhamnosus* HM2, *B. bifidum* B11 and B12 inhibited the growth of the indicator strains as a result of lactic, acetic and propionic acids production.

Our results indicate that the differences in the size of inhibition zones revealed to the strain's production of antimicrobial proteins in addition to the production of acids, which decreased the pH value. To insure these results, the neutral pH cell filtrates were applied to digest their protein contents with proteinase K. The digestion with proteinase K was for 2 hours at 37°C and reaction was stops by incubating at 95°C for 10 min. Table 4 and Figure 1 show the effect of proteinase K on the inhibition zones' diameters. Observed inhibition zones' diameters were ranged from 0.7 to 3.7 cm. These differences in inhibition zones' diameters indicated that the cell filtrates contained different substances produced by the isolates as shown in (Table 2, 3, 4 and Figure 1).

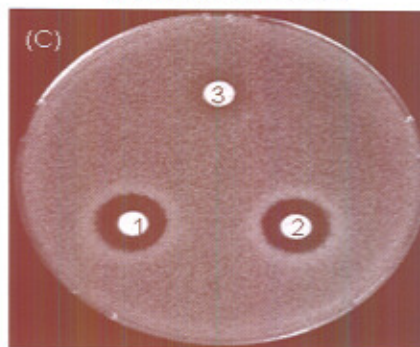**Table 4:** Effect of the digestion of cell filtrate protein contents with proteinase K.

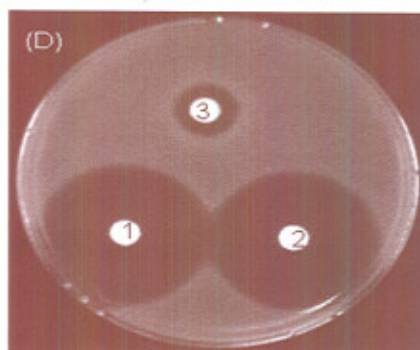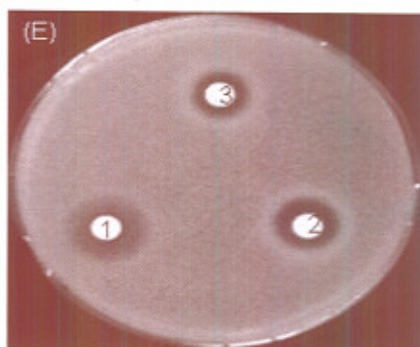| Indicator strains	B11	B12	HM2
L. monocytogenes DSM 12464	1.0	2.3	2.3
Ent. faecalis ATCC 29212	0.7	2.4	2.4
E. coli ATCC 11775	0.7	1.4	1.4
S. aureus ATCC 6538	1.3	3.7	3.7
Ent. aerogenes ENT 77/403	1.5	1.3	1.1
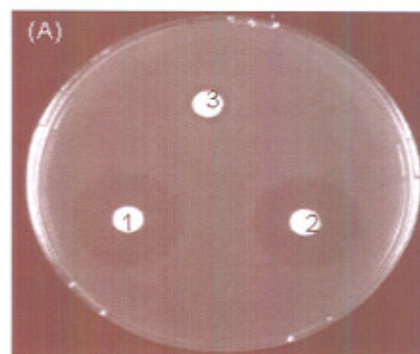
A) *L. monocytogenes* DSM 12464
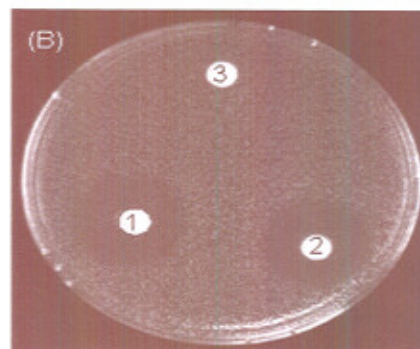
B) *En. faecalis* ATCC 29212

C) *E. coli* ATCC 11775

D) *S. aurous* ATCC 6538

E) *Ent. aerogenes* ENT 77/403

Figure1: Effect of addition of proteinase K and neutralization of pH on the inhibition zones' diameter; 1) cell filtrate; 2) neutral pH cell filtrate and 3) digested cell filtrate with proteinase K

These substances inhibited the growth of used pathogenic strains. Some of these substances are acids, which reduce the pH of culture. After proteinase k digestion, some inhibition zones diameters were reduced, however the isolate *B. bifidum* B11 did not produced active antimicrobial peptides against *Ent. Aerogenosa. B. bifidum* B12 and *L. rhamnosus* HM2 did not produce antibacterial peptides against *S. aureus* and *Ent. faecalis* as the inhibition zones' diameters did not show any differences after the digestion with proteinase K. Results of digested cell filtrates revealed that the cell filtrates contained antimicrobial proteins. *L. rhamnosus* HM2 and *B. bifidum* B11 and B12 are producing organic acids and bacteriocins which inhibited the growth of used indicator strains.

DISCUSSION

During this study, four isolates were identified as *Bifidobacterium bifidum* isolate (B11 and B12) and *Lactobacillus rhamnsosus* isolate (HM1 and HM2). Isolates *B. bifidum* B11, B12 and *L. rhamnosus* HM2 showed their antimicrobial activity against the used pathogen's strains by using cell filtrates. Only *Pseudomonas aeruginosa* DSM1117 was resistance to antimicrobial substances in the cell filtrates. *L. rhamnosus* HM1 dose not show any antimicrobial activity against all used pathogens. It was previously demonstrated that probiotic lactobacilli can develop antagonistic activity against human pathogens *(Silva et al., 1987; Bernet et al., 1994 and Bernet-Camard et al., 1997).*

The genus *Bifidobacterium* has been extensively studied because of its beneficial effects on health, especially the protection of the intestinal tract from microbial infection *(Kaur et al., 2002; Hamilton-Miller, 2003; Mack and Lebel 2004 and Asahara et al., 2004).* Several mechanisms have been proposed to explain the efficacy of bifidobacteria in preventing infection. Our isolated strains showed different diameters of inhibition zones. The inhibition zone's diameters varied according to the isolate and the indicator strains. *L. monocytogenes* DSM 12464 and *S. aureus* ATCC 6538 were highly affected with antimicrobial substance in *lactobacilli* and *bifidobacteria* cell filtrates. Despite its low incidence in Western countries, *L. monocytogenes* is ranked first among the food-borne pathogenic bacteria in terms of death rate (40%). *L. monocytogenes* has to cross the intestinal barrier in order to produce central nervous system or feto-placental unit infections *(Velge et al., 1997).* *L. monocytogenes* is a facultative intracellular, gram-positive bacterium responsible for severe food-borne infections leading to meningitis, encephalitis and gastroenteritis *(Velge et al., 1997 and Doganay, 2003).* Many of the *lactobacilli* and *bifidobacteria* strains are capable of inhibiting growth of some gram-positive bacteria, such as pathogenic species like *Listeria monocytogenes*, by secreting antimicrobial compounds *(Schillinger et al., 1996 and Toure' et al., 2003).*

Lactic acid bacteria have potential applications as biopreservatives in the food industry *(O'Sullivan et al., 2002).* It inhibits the growth of some food spoilage and food-borne pathogens, such as *Listeria monocytogenes, Staphylococcus aureus* and *Clostridium botulinum (Cintas et al., 1997).*

Growth of *Ent. faecalis* ATCC 29212, *E. coli* ATCC 11775 and *Ent. aerogenes* ENT 77/403 was inhabited by the same cell filtrates of B1, B2 and HM2, but the inhibation zones' diameters were samller than other indicator strains.

The mechanism(s) of the antibacterial activity of probiotic *Lactobacillus* strains appears to be multifactorial *(Servin 2004).* In particular, by producing metabolites such as acetic and lactic acid and thus lowering the pH, *Lactobacillus* strains inhibit the growth of bacterial pathogens and sometimes even kill them *(Vandenbergh 1993).* In this study, pH of the cell filtrates was adjusted to pH 7.0 to mask the pH effect; no reduction of inhibition zone diameter was observed against *E. coli* and *Ent. faecalis* by using the neutral cell filtrate of isolates. Reduction in inhibation zones were obsevered by using *L. monocytogenes, S. aureus* and *Ent. aerogenes.*

Certain lactobacilli synthesize antimicrobial compounds against human pathogens; these compounds are related to the bacteriocin family *(Klaenhammer 1993).* In our study, HPLC analysis for organic acids showed that the isolates produced lactic, acetic and propionic acids. All of *lactobacilli* and *bifidobacteria* isolates produced lactic acid. Acetic acid was produced by *lactobacillus* HM2 and *bifidobacteria* isolates B11 and B12. Both *bifidobacteria* isolates B11 and B12 produced propionic acids. Metabolic end products of lactic acid fermentation are well known *(Vandenbergh 1993).*

The genus *Bifidobacterium* and *Lactobacillus* have several mechanisms in preventing infection. These mechanisms include production of antimicrobial agents, such as organic acids *(Vandenbergh 1993 and Roche et al., 2001).*

Our results indicated that the differences in the size of inhibition zones revealed to the strain's production of antimicrobial proteins in addition to the production of acids, which decreased the pH value. To insure these results, proteinase K had been used with the neutral pH cell filtrates to digest their protein contents. Treatment with proteinase K affected the inhibition zones' diameters. These differences in inhibition zones' diameters indicated that the cell filtrates

contained different substances produced by the isolates. These substances inhibited the growth of used pathogenic strains. Some of these substances are acids, which reduce the pH of culture. After proteinase k digestion, the inhibition zones diameters were reduced, however the isolate *B. bifidum* B11 revealed no production of active antimicrobial peptides against *Ent. Aerogenosa*. *B. bifidum* B12 and *L. rhamnosus* HM2 did not produce antibacterial peptides against *S. aureus* and *Ent. faecalis* as the inhibition zone diameter did not show any differences after the digestion with proteinase K. However, other results of the same digested cell filtrates revealed that *L. rhamnosus* HM2 and *B. bifidum* (B11 and B12) are producing organic acids and bacteriocins which inhibited the growth of used *L. monocytogenes, E. coli* as indicator strains.

Lactic acid bacteria are known to have an antagonistic activity toward a variety of microorganisms. They produce well known metabolic end products of organic acids, hydrogen peroxide and bacteriocins *(De Vuyst and Vandamme 1994 and Servin 2004)*. Bacteriocin production is one of the properties responsible for the antibacterial activity against closely related species and possibly gram-positive food spoilers and pathogens *(Klaenhammer 1993)*. Large numbers of bacteriocin producers have been found among different genera of the lactic acid bacteria *(Piard and Desmazeaud 1992.; Hoover and Steenson 1993; Dodd and Gasson 1994; Jack et al., 1995)*.

The three *B. bifidum* (B11 and B12) and *L. rhamnosus* HM2 strains used in this study should be considered as probiotic strains with interesting potential for preventing contamination in food and enteric infections in humans.

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