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Full Length Research Paper

Inhibitory activity of *Lactobacillus curvatus* CWBI-B28 against *Listeria monocytogenes* and ST2-verotoxin producing *Escherichia coli* O157

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A bacteriocin-producing strain of *Lactobacillus curvatus* CWBI-B28 isolated from raw meat was shown to inhibit *Listeria monocytogenes* and pathogenic strains of *Escherichia coli* by the well diffusion assay. To confirm whether the bacteriocin was involved in *E. coli* O157 inhibition, growth of the pathogen was monitored in the neutralized cell-free supernatant (NCFS) pre-treated with pronase E or catalase. Alternatively, *E. coli* O157 (VH21) was co-cultured with *Lb. curvatus* CWBI-B28 in MRS broth. The results of the well-diffusion assay suggested that the inhibition of *E. coli* O157 (VH21) was partially due to the bacteriocin; however, growth monitoring indicated that such inhibition is exclusively due to hydrogen peroxide. In pronase-added NCFS (i.e., absence of bacteriocin) the colony forming units (cfus) of *E. coli* O157 (VHA21) declined to below the detectable limit after 24 h of incubation at 37°C. However, in presence of catalase no inhibition of the pathogen was observed and the cfus increased steadily to reach 8 log units in 24 h. Moreover, in co-culture, a significantly accelerated inhibition of *E. coli* O157 (VH21) was observed in MRS broth as compared to the NCFS without added catalase and the cfus decreased to below the detectable limit in 8 h instead of 24 h, respectively.

Key words: *Lactobacillus curvatus*, Bacteriocin, hydrogen peroxide, *Listeria monocytogenes,* enterohemorrhagic *Escherichia coli* O157.

INTRODUCTION

Lactic acid bacteria (LAB) have long been used in food fermentations where they play different roles including safety and keeping quality via the production of various antimicrobial substances including bacteriocins, hydrogen peroxide and organic acids. Production of bacteriocins by LAB has attracted increased attention in last years due to their potential as natural food preservatives. However, their efficacy in food preservation suffers from some limitations such as their narrow spectrum of action generally limited to taxonomically related bacteria (Kleanhammer, 1993). Therefore, attempts to extend the spectrum of action to Gram negative bacteria were made by combining them with other antimicrobials such as EDTA (Stevens et al., 1991) and lactoperoxidase system (Rodriguez et al., 1997) or by physical treatment to induce a sublethal injury (Kalchayanand et al., 1992; Ray 1993). Inhibition of Gram negative bacteria of health and/or spoilage significance is of paramount importance to food industry, especially for meat and poultry industries where Gram negative bacteria are common causes of gustatory and hygienic quality depreciation. In particular, *L. monocytogenes* and enterohemorrhagic *E. coli* have been frequently contaminating meat and meat products or actually involved in meat-born diseases (Farber and Peterkin, 1991; De Valk et al., 2000; Chinen et al., 2001; Griffin

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Table 1. Inhibitory activity of *Lactobacillus curvatus* CWBI-B28 against different gram positive and gram negative indicator strains by the well diffusion assay (Benkerroum et al., 2005).

Indicator strain	Source	Inhibition
Gram positive		
L. monocytogenes LMG15139	BCCM	4+
L. monocytogenes LMG16783	BCCM	3+
L. monocytogenes LMG13304	BCCM	4+
L. monocytogenes ATCC 7644	ATCC	3+
L. monocytogenes CWBI 2231	CWBI	4+
Staphylococcus aureus SAD30	IAV Hassan II	-
Staph. aureus CWBI	CWBI	-
Lactobacillus plantarum CWBI	CWBI	-
Lb. Plantarum MD1	IAV Hassan II	-
Lb. curvatus CWBI-B28	Benkerroum et al., 2005	-
Lb. brevis CWBI	CWBI	-
L. lactis LMG 21215	BCCM	3+
Gram negative bacteria		
E. coli O78:K80 (BJ2)	Benkerroum et al., 2004	3+
E. coli O157 (VH21)	Benkerroum et al., 2004	3+
Salmonella Enteri CWBI	CWBI	-

4+ : diameter of the inhibition zone >25 mm; 3+ 16 <Φ< 22 mm; 2+ 12 <Φ< 20 mm; - No inhibition.
BCCM: Belgian Coordinated Collections of Microorganisms.
ATCC: American Type collection Culture.
CWBI: Centre Wallon de Bio-Industrie (Gembloux, Belgium).
IAV Hassan II : Institut Agronomique et Vétérinaire Hassan II.

and Tauxe, 1991; Quaglio et al., 1997, Benkerroum et al., 2004).

In the present work, we report the production of bacteriocin and hydrogen peroxide by a *Lactobacillus curvatus* strain isolated from meat which inhibited pathogenic strains of *L. monocytogenes* and *E. coli* strains (O157 and K80 serotypes) *in vitro*. The bacteriocin-producing strain appears to have a good potential in food preservation as it inhibited different pathogenic strains of *L. monocytogenes* and *E. coli* by at least two different means (i.e., bacteriocin and hydrogen peroxide).

MATERIALS AND METHODS

Bacterial strains and media

Lb. curvatus CWBI-B28, a bacteriocin-producing (Bac+) strain previously isolated from raw meat (Benkerroum et al., 2005) and *Escherichia coli* O157 (VH21) producing St2 verotoxin isolated from ground beef (Benkerroum et al., 2004) were used in this study. Other bacteria used are listed in Table 1.

All strains were stored at –20°C in appropriate broths containing 25% glycerol. Lactic acid bacteria were stored in de Man, Rogosa and Sharp (MRS, Biokar, France) and the other strains were stored in Tryptic Soy Broth (TSB, Biokar, France). Working cultures were maintained as slant cultures in TSA or MRS agar as appropriate and sub-cultured every two to three weeks. Before experimental use, they were propagated in their respective broth media by overnight incubation at 37°C.

Well diffusion assay

Antagonistic activity of the *Lb. curvatus* CWBI-B28 against different indicator strains of Gram-negative and Gram-positive strains listed in Table 1 was tested by the well diffusion assay using the neutralized cell-free supernatant (NCFS) as described previously (Benkerroum et al., 2000). The NCFS was obtained by pelleting (4000 x g for 15 min) an overnight culture of *Lb. curvatus* CWBI-B28 in MRS broth. The supernatant was neutralized to pH 6.5 with 3 N NaOH and filter sterilized through a 0.22 µm Millipore™ membrane.

In another assay to confirm the role of the bacteriocin in the inhibition of *E. coli* O157 (VH21), the NCFS was treated with pronase E (Type XXV; Sigma) and tested for antimicrobial activity against the pathogen. The enzyme was dissolved in phosphate buffer (0.1 mol/ml, pH 6) and mixed with NCFS (1:1) to a final concentration of 1 mg/ml. Incubation was carried out at 37° C in a water bath for 2 h. Controls included sterile MRS broth, a 1:1 mixture of culture supernatant with buffer but without enzyme and buffer with only the enzyme. Each sample was then assayed for bacteriocin activity by the well diffusion method.

Growth monitoring

The antagonistic activity of *Lb. curvatus* CWBI-B28 against *E. coli* O157 (VH21) was also studied by monitoring the growth of the latter strain in NCFS in presence or absence of pronase E (Type XXV, sigma) or catalase (EC1.11.1.6, Sigma). In a series of test tubes, the NCFS (10 ml) was dispensed in each of 3 test tubes. Pronase and catalase solutions were added to two of these tubes to final concentrations of 1 mg/ml and 68 IU/ml, respectively. No enzyme was added to the third tube which served as a negative

Table 2. Diameter of inhibition zone of *E. coli* O157 (VH21) and *L. monocytogenes* LMG 13304 by NCFS treated or not with pronase E. Inhibition testing was performed by the well diffusion assay.

Strains	Neutralized Cell-Free Supernatant			Pronase-treated NCFS		
	***E. coli* O157 (VEH21)**	***E. coli* K80 (BJ1)**	***L. monocytogenes* LMG13304**	***E. coli* 157 (VEA)**	***E. coli* K80 (BJ1)**	***L. monocytogenes* LMG13304**
Inhibtion diameter (mm)	14.0 ± 0	13.0 ± 0	22±1	10 ± 0	9.5 ± 0	0 ± 0

Table 3. Counts (log cfu/ml) of *E. coli* o157 (EC O157) in neutralized cell free supernatant (NCFS) of *Lb. curvatus* CWBI-B28 in presence of protease or catalase and in co-culture with *Lb. curvatus* CWBI-B28 (LBC) in MRS broth. The counts of *Lb. curvatus* CWBI-B28 were monitored in the co-culture in MRS broth (see Material and Methods).

Hours	EC O157			Co-culture	
	NCFS	NCFS + pronase E	NCFS + catalase	EC O157	LBC
0	6.46 ± 0.24	6.40 ± 0.12	6.54 ± 0.23	6.20 ±0.24	5.69 ± 0.45
2	6.28 ± 0.32	6.23 ± 0.21	6.66 ± 0.32	6.90 ± 0.35	7.60 ± 0.30
4	6.23 ± 0.22	6.38 ± 0.32	6.90 ±0.14	7.0 ± 0.22	8.0 ± 0.25
8	6.04 ± 0.12	6.50 ±0.17	7.53 ± 0.15	7.48 ± 0.15	8.90 ± 0.30
24	5.70 ± 0.09	6.11 ± 0.13	8.0 ± 0.21	-	8.11 ± 0.15
48	1.178 ± 0.10	-	7.85 ±0.25	-	7.78 ± 0.12
72	-	-	8.0 ±0	-	7.0 ± 0.11

- Not detected in 1 ml sample.

control. All tubes were each inoculated with 100 µl of an overnight culture of *E. coli* O157 (VH21) and incubated at 30°C. At regular time intervals, a 1-ml sample was withdrawn to enumerate *E. coli* O157 (VH21) in Eosin Methylen-Blue (Oxoid, England). In parallel, 10 ml of sterile MRS broth were inoculated with an overnight culture of *E. coli* O157 (VH21) and *Lb. curvatus* CWBI-B28 (100 µl each) to study the associative growth. The viable counts of both of the strains was monitored by enumerations after serial dilutions in saline solution (0.85%) on MRS agar and on EMB at 37 and 44°C, respectively, after 48 h of incubation.

Statistical analysis

The experiment was repeated twice and each determination was done in duplicate. Statistical analysis (analysis of variance α = 0.05% and Student t-test) of data was done by computations using Excel software.

RESULTS AND DISCUSSION

Table 1 summarizes the results of the antimicrobial activity testing by the well-diffusion assay. The NCFS of *Lb. curvatus* CWBI-B28 inhibited the five strains tested of *L. monocytogenes*, *Lc. lactis* subsp. *lactis* LMG 21215 and both strains of *E. coli*. The inhibition of *L. monocytogenes* and *Lc. lactis* strains was not surprising as sensitivity of Gram positive bacteria to bacteriocins of LAB, especially those taxonomically related to the producer strain, was well documented (Klaenhammer, 1993). However, few studies have reported on the inhibition of Gram negative bacteria by bacteriocins (Skyttä et al., 1991; Malik et al., 1995; Todorov et al., 2004). In addition, the inhibition of Gram negative bacteria by spent broth cultures of bacteriocin-producing LAB was shown not to be directly related to bacteriocins as evidenced by the loss of the inhibitory activity upon purification (Gomez et al., 1997). Antimicrobial substances other than bacteriocins or ecological factors existing in the spent broth were shown to sensitize Gram negative bacteria to bacteriocins (Blachburn et al., 1989; Stevens et al., 1991; Kalchayanand et al., 1992; Rodrigez et al., 1997; Gänzle et al., 1999). To confirm whether or not the bacteriocin of *Lb. curvatus* CWBI-B28 was involved in the inhibition of *E. coli* O157 (VH21), the NCFS was treated with pronase E or catalase and subjected to the well diffusion assay against *L. monocytogenes* and *E. coli* O157 (VH21). The results (Table 2) suggest that the bacteriocin was, at least partially, responsible for the inhibition of *E. coli* O157 (VH21) as suggested by the significant ($p < 0.05$) reduction in the diameter of inhibit-tion zones of the pronase-treated NCFS compared to that of the crude NCFS. In addition, the activity of the prona-se-treated NCFS against *L. monocytogenes* was comple-tely lost.

For further evidence regarding the implication of the bacteriocin in the inhibition of *E. coli* O157 (VH21), were the counts of the pathogen which were monitored in the NCFS after treatment with pronase or catalase, or in co-culture with the bacteriocin-producing strain. No signif--cant ($p > 0.05$) difference was observed between the counts of *E. coli* O157 (VH21) in the NCFS with or without added pronase E during the first 24 h of incubation (Table 3). Furthermore, the bacterium grew well in the catalase-treated NCFS to reach 8 log units in 24 h whereas in presence of pronase E (i.e. absence of the bacteriocin) the counts declined to below the detectable

limit in 24 h suggesting that the inhibition of *E. coli* O157 (VH21) was mainly due to hydrogen peroxide. Production of hydrogen peroxide by lactobacilli is well documented (Marty-Teysset, 2000; Kot et al., 1996) and some strains were shown to produce sufficient amounts to inhibit pathogenic or spoilage micro-organisms including Gram-negative bacteria (Collins and Aramaki 1980; Zalán et al., 2005).

In the associative growth experiment, the counts of *E. coli* O157 (VH21) declined to below the detectable limit in 8 h while those of *Lb. curvatus* CWBI-B28 increased steadily to exceeded 8 log units in 24 h (Table 3) indicating that the lactic acid bacterium has an evident competitive advantage over *E. coli* O157 (VH21).

Conclusion

In addition to the production of an anti-*Listeria* bacteriocin, *Lb. curvatus* CWBI-B28 appears to produce hydrogen peroxide thereby extending the inhibitory effect to pathogenic strains of *E. coli*. Furthermore, the inhibitory effect of *Lb. curvatus* CWBI-B28 against the shiga-toxin producing *E. coli* O157 (VH21) was significantly ($p < 0.05$) enhanced in associative growth as compared to the NCFS. Therefore, the application of live cells *Lb. curvatus* CWBI-B28 in starter cultures or as an adjunct starter would be more advantageous to food preservation than the purified bacteriocin.

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