Probiotic properties of *Enterococcus faecium* CE5-1 producing a bacteriocin-like substance and its antagonistic effect against antibiotic-resistant enterococci *in vitro*

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ABSTRACT: A bacteriocin-like substance (BLS) producing *Enterococcus faecium* CE5-1 was isolated from the gastrointestinal tract (GIT) of Thai indigenous chickens. Investigations of its probiotic potential were carried out. The competition between the BLS probiotic strain and antibiotic-resistant enterococci was also studied. *Ent. faecium* CE5-1 exhibited a good tolerance to pH 3.0 after 2 h and in 7% fresh chicken bile after 6 h, but the viability of *Ent. faecium* CE5-1 decreased by about 2–3 log CFU/ml after 2 h incubation in pH 2.5. It was susceptible to the antibiotics tested (tetracycline, erythromycin, penicillin G, and vancomycin). The maximum BLS production from *Ent. faecium* CE5-1 was observed at 15 h of cultivation. It showed activity against *Listeria monocytogenes* DMST17303, *Pediococcus pentosaceus* 3CE27, *Lactobacillus sakei* subsp. *sakei* JCM1157, and antibiotic-resistant enterococci. The detection by polymerase chain reaction (PCR) in the enterocin structural gene determined the presence of enterocin A gene in *Ent. faecium* CE5-1 only. *Ent. faecium* CE5-1 showed the highest inhibitory activity against two antibiotic-resistant *Ent. faecalis* VanB (from 6.68 to 4.29 log CFU/ml) and *Ent. gallinarum* VanC (from 6.76 to 4.31 log CFU/ml) after 12 h of co-cultivation. The results show the future possible use of *Ent. faecium* CE5-1 as a probiotic strain for livestock to control antibiotic-resistant enterococci.

Keywords: chicken; gastrointestinal tract; bile salt; acid tolerance; *Enterococcus*

Several years ago, antibiotics were used in feed for chickens to promote growth and this caused the evolution of antibiotic resistance in both pathogen and commensal bacteria in chickens. In addition, the subtherapeutic antibiotics usage for chicken production is banned in Europe and the United States except for the use of ionophoric antibiotics in EU until 2013. In recent years, due to the increased use of antibiotics in animals and humans, enterococci have become a major concern worldwide.

Enterococci are widespread in nature and found in the gastrointestinal tract (GIT) of humans and animals (Franz et al., 2007). Although they belong to a group of microorganisms known as lactic acid bacteria (LAB) and are useful in cheeses or other fermented foods production, several of them are not considered as "Generally Recognized As Safe" (GRAS) (Ogier and Serror, 2008). Some enterococci were isolated from patients possessing virulent genes. They are the second to third most frequent bacterial genus in hospital infections. They cause endocarditis, bacteraemia, urinary tract, neonatal, central nervous system and other infections (Franz et al., 1999). Moreover, they are known to

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be resistant to most antibiotics including penicillin, aminoglycosides, glycopeptides, MLS_B antibiotics (macrolides, lincosamides, and streptogramins of the B type), chloramphenicol, tetracyclines, quinolones, oxazolidinones, and everninomicins (Klare et al., 2003). In addition, the genus *Enterococcus* can transfer the antibiotic-resistant encoding gene to pathogens and may be involved in the spreading of antibiotic resistance into the environment and food chains (Klare et al., 2003; Abriouel et al., 2005).

Probiotics is a live microbial feed supplement which affects the host animal beneficially by improving its intestinal microbial balance (Fuller, 1989). Probiotic strains are widely used in the food and feed industry to enhance the health of humans and animals because they produce various antibacterial compounds, such as organic acids, hydrogen peroxide, diacetyl, and bacteriocin (De Vuyst and Degeest, 1999). The most common probiotic strains from the GIT of chickens belong to the genera *Enterococcus* (Strompfová and Lauková, 2007; Musikasang et al., 2009), *Pediococcus* (Shin et al., 2008; Musikasang et al., 2009), and *Lactobacillus* (Souza et al., 2007). *Ent. faecalis* KT2L24, *Ent. durans* KT3L20, *Ent. faecium* KT4S13, *Ent. faecium* KT8S16, and *P. pentosaceus* KT3CE27 (Musikasang et al., 2009) are considered to be the best probiotic strains from GIT of broiler and Thai indigenous chickens. Tolerance to acid and bile salts in the GIT is usually considered one of the main criteria required for LAB strains to be used as probiotics.

Enterococi belong to the group of LAB and are well-known producers of antimicrobial peptides called bacteriocins, with the ability to inhibit (bacteriostatic) or kill (bactericidal) the growth of similar or closely related bacterial strains (Cleveland et al., 2001). Several strains of *Ent. faecium* are reported to produce more than one enterocin. The examples include enterocin A from *Ent. faecium* CTC492 (Aymerich et al., 1996), enterocin B from *Ent. faecium* T136 (Casaus et al., 1997), enterocin P from *Ent. faecium* P13 (Cintas et al., 1997), and enterocins L50A and L50B from *Ent. faecium* L50 (Cintas et al., 1998). Several researchers dealing with the application of bacteriocins have focused on the use of bacteriocins or probiotics to control the spoilage microorganisms and foodborne pathogens, displaying a potential application in food preservation and in prevention or treatment of other diseases in hosts. The aim of the present study was to evaluate the primary probiotic properties of bacteriocin-like substance (BLS) producer *Ent. faecium* CE5-1 isolated from GIT of Thai indigenous chickens and its usage in the control of antibiotic-resistant enterococci.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Ent. faecium CE5-1 was isolated from the GIT of Thai indigenous chickens (not fed with commercial feed). The procedure is described further in the following text. Parts of organs containing crop (small intestine, caecum, and large intestine) were homogenized with sterile normal saline (0.85% NaCl) and spread onto MRS agar (MRS broth, Hi Media Laboratory Pvt. Ltd., Mumbai, India) (pH 6.5). After 24 h of incubation at 37°C, the plates were overlaid with 10 ml of soft MRS agar containing *Lb. sakei* subsp. *sakei* JCM1157 as an indicator strain and incubated at 37°C for 24 h under anaerobic conditions. Colonies exhibiting the inhibition zone were isolated and purified by 2–3 times restreaking on MRS agar. To verify bacteriocin-like substance (BLS) production, the supernatants of each strain were adjusted to pH 6.5–7.0, treated with catalase and proteolytic enzyme and used for antibacterial determination by agar well diffusion assay. Only the strain CE5-1 produced BLS and it was identified as *Ent. faecium* based on 16S rDNA sequence analysis. The 16S rDNA sequence was deposited in the DNA Data Bank of Japan (DDBJ) with accession No. DDBJ ID: AB512765.

The bacterial strains used as indicator for the inhibition study are listed in Table 1. All the LAB strains were grown in MRS broth (pH 6.5 ± 0.2) at 37°C for 24 h. Other indicator strains were grown in Brain heart infusion broth (BHI broth; Hi Media Laboratory Pvt. Ltd., Mumbai, India) at 37°C for 24 h. All the strains were maintained at –20°C in a medium containing 25% glycerol. Working cultures were grown at 37°C in appropriate media at least twice before use.

Characterization of primary probiotic properties of *Ent. faecium* CE5-1

The effects of simulated gastric juice and bile on the growth of *Ent. faecium* CE5-1 were tested. Briefly, 1 mm of overnight culture of *Ent. faecium* CE5-1 was centrifuged in an Eppendrof

Table 1. Antagonistic activity of cell-free supernatants of *Enterococcus faecium* CE5-1 determined by agar well diffusion assay

Species	Strain code	Source	Radius of inhibition zone (mm)
Bacillus cereus	DMST5040	DMST	0
Enterococcus durans	3L20	our strain collection	1.5
Enterococcus faecalis	2L24	our strain collection	1.0
Enterococcus faecalis	VanB*	CU	2.0
Enterococcus faecium	139*	CU	0
Enterococcus faecium	174*	CU	1.5
Enterococcus faecium	348*	CU	1.5
Enterococcus faecium	4S13	our strain collection	1.5
Enterococcus faecium	8S16	our strain collection	2.0
Enterococcus faecium	CE5-1	our strain collection	0
Enterococcus faecium	L7-45	our strain collection	2.2
Enterococcus gallinarum	VanC*	CU	2.0
Escherichia coli	DMST4212	DMST	0
Lactobacillus plantarum	D6SM3	our strain collection	0
Lactobacillus sakei subsp *sakei*	JCM1157	JCM	6.0
Listeria monocytogenes	DMST17303	DMST	5.0
Pediococcus pentosaceus	3CE27	our strain collection	3.0
Pediococcus pentosaceus	DMST18752	DMST	0
Salmonella Typhimurium	DMST16809	DMST	0
Salmonella Enteritidis	DMST15676	DMST	0
Staphylococcus aureus	DMST8840	DMST	0
Vibrio parahaemolyticus	DMST5665	DMST	0

DMST = Department of Medical Sciences, CU = The WHO Global Salm-Surv Regional Centre of Excellence: South-East Asia and Western Pacific, Faculty of Veterinary Science, Chulalongkorn University, Thailand, JCM = Japan Collection of Microorganisms

*antibiotic resistant *Enterococcus* strains

Centrifuge 5415R (Eppendorf, Hamburg, Germany) at 12 000 *g* for 10 min. Pellets were washed twice with sterile saline before being re-suspended in either simulated gastric juice containing 3 mg/ml pepsin (Fluka, Steinheim, Germany) at pH 2.5 and 3.0 for 2 h or in simulated intestinal fluid. Simulated intestinal fluid containing 1 mg/ml pancreatin (Sigma-Aldrich GmbH, Hamburg, Germany) and various concentrations of fresh chicken bile (1, 3, 5, and 7%) were used at pH 8.0 for 6 h. The viable counts were determined by the drop plate method on MRS agar (modified from Madureira et al., 2005).

Survival of *Ent. faecium* CE5-1 in simulated intestinal juice after incubation in simulated gastric juice was tested. 1 mm of the selected strains was centrifuged in an Eppendrof centrifuge at 12 000 *g* for 10 min. This was then washed twice with sterile saline before being re-suspended in simulated gastric juice with a pH value of 3.0. The tubes were incubated at 37°C for 2 h. After incubation, viable bacterial counts were determined by the drop plate method on MRS agar. After gastric digestion, cells were harvested and suspended in simulated intestinal fluid which contained 7% of fresh chicken bile at pH 8.0. The suspension was incubated at 37°C for 6 h and the viable counts were determined by the drop plate method on MRS agar (modified from Madureira et al., 1993).

Antibiotic resistance of *Ent. faecium* CE5-1

The antibiotic resistance of *Ent. faecium* CE5-1 and *Enterococcus* strains is listed in Table 4. This was detected by broth microdilution assay following the method described by Parente et al. (1995).

A two-fold dilution of each antibiotics listed in Table 4 was prepared in a 96-well microtiter plate by using MRS broth as the diluents (final volume was 100 ml). Then, 100 ml of culture broths of each enterococci were added into each well (final concentration was 10^4 CFU/ml). The microtiter plate cultures were incubated at 37°C and after 24 h the growth inhibition of the indicator strain was measured by optical density at 660 nm (OD_{660}) in a microplate reader Powerwave X (BioTek Instruments, Winooski, USA). A positive control was obtained by the use of 100 ml of MRS broth and 100 ml of each culture broth. A negative control contained 200 ml of MRS broth without the LAB strain. The percentage of inhibition was expressed by the following equation:

Inhibition (%) = [(OD_{660} in the positive control group – OD_{660} in the group)/OD_{660} in the positive control group] × 100

Minimal inhibitory concentration (MIC) is defined as the lowest concentration of the sample tested that could restrict bacterial growth at an inhibition rate higher than 90%. To determine minimal bactericidal concentration (MBC), aliquots (10 ml) of each well at MIC and higher than the MIC were subcultured on MRS agar. MBC is defined as the lowest concentration that allowed no visible growth on the MRS agar (Almeida et al., 2008).

It was observed that bacteria develop antibiotic resistance. The following may be considered in terms of resistance to the antibiotics: when MICs are < 8 mg/ml, the bacteria may be classified as "susceptible"; when the MICs are ≥ 8 mg/ml, they may be classified as "moderately resistant"; and when the MICs are above 32 mg/ml, they may be classified as "clinically resistant" (D'Aimmo et al., 2007).

Growth curve and BLS production of *Ent. faecium* CE5-1

1 l of MRS broth was inoculated with 2.5% of an overnight culture of *Ent. faecium* CE5-1 and incubated at 37°C with agitation at 100 rpm. At 3 h intervals, pH and growth by absorbance at 660 nm were measured. The antimicrobial activity was calculated as arbitrary units (AU/ml) defined as the inverse of the highest twofold dilution showing a growth inhibition divided by the sample volume.

Effect of antibacterial activity of BLS against bacterial indicators

Ent. faecium CE5-1 was grown in MRS broth at 37°C for 15 h. Cells were removed by centrifugation at 12 000 *g* at 4°C for 10 min and supernatant was collected. The cell-free supernatants (CS) of *Ent. faecium* CE5-1 were adjusted to pH 6.5–7.0 with 0.1N NaOH and used for the determination of the antibacterial activity of the BLS using the method of agar well diffusion assay as described by Schillinger and Lücke (1989).

PCR detection of enterocin structural gene

The genomic DNA of *Ent. faecium* CE5-1 was extracted and purified using the Genomic DNA Mini Kit (Geneaid, Tao-Yuan, Taiwan). The sequences of primers used for PCR-amplification of the structural genes of enterocin A (*ent*A), enterocin B (*ent*B), enterocin P (*ent*P), enterocin L50B (*ent*L50B), and

Table 2. Primers used in the study

Primers		Sequence	Reference
EntA	F	5'-GGTACCACTCATAGTGGAAA-3'	Aymerich et al. (1996)
	R	5'-CCCTGGAATTGCTCCACCTAA-3'	
EntB	F	5'-GCTACGCGTTCATATGGTAAT-3'	Casaus et al. (1997)
	R	5'-TCCTGCAATATTCTCTTTAGC-3'	
EntP	F	5'-CAAAATGTAAAAGAATTAAGATCG-3'	Cintas et al. (1997)
	R	5'-AGAGTATACATTTGCTAACCC-3'	
EntL50B	F	5'-ATGGGAGCAATCGCAAAATTA-3'	Cintas et al. (1998)
	R	5'-TAGCCATTTTTCAATTTGATC-3'	
EntIIa	F	5'-TAYGGIAAYGGIGTITAYTG-3'	Yi et al. (2010)
	R	5'-CYTCDATNGCRTTRTC-3'	

F = forward primer, R = reverse primer

enterocin IIa (*ent*IIa) are shown in Table 2. The PCR reaction of *ent*A detection was composed of the following steps: initial denaturation at 95°C for 5 min and then performed in 30 cycles of denaturation at 95°C for 30 s; annealing at 58°C for 30 s and extension at 72°C for 30 s; and a final extension at 72°C for another 5 min, then immediate cooling at 4°C. The same profile was used for *ent*B, *ent*P, and *ent*L50B detection, but the annealing temperature was 56°C (Lauková et al., 2008). For *ent*IIa, the amplification program was as follows: 94°C for 5 min; 30 cycles at 94°C for 1 min; and 51°C for 40 s, 72°C for 3 min with a final extension at 72°C for 10 min (Yi et al., 2010). After PCR amplification, PCR products were separated by 2% agarose gel electrophoresis and visualized under UV light after staining with SYBR® Gold nucleic acid gel stain (Invitrogen, Eugene, USA). The PCR product was then gel-purified before sequencing. Finally, the nucleotide sequences were compared to the sequence database of GenBank through a BLAST search (http://www.ncbi.nlm.nih.gov).

Effect of co-culturing of *Ent. faecium* CE5-1 with antibiotic-resistant *Enterococcus* in MRS broth

The overnight cultures of BLS producer (*Ent. faecium* CE5-1) and each antibiotic-resistant *Enterococcus* (*Ent. faecalis* VanB or *Ent. gallinarum* VanC) were added to 200 ml of MRS broth. The initial cell density of each strain was approximately 10^6 CFU/ml. Then the mixture of cultures was incubated at 37°C. Samples were taken at 6 h intervals. The viability of the antibiotic-resistant enterococci was assessed by using the drop plate method in triplicate onto MRS agar containing 100 mg/ml of tetracycline and incubated at 37°C for 24–48 h. The total LAB of the mixture cultures were determined in MRS agar. Similarly, the control consisted of the separate strain in 200 ml of MRS broth and incubated under the same conditions.

RESULTS AND DISCUSSION

Characterization of primary probiotic properties of *Ent. faecium* CE5-1

The GIT is the major location affecting the viability of LAB cells. Most bacteria do not survive well at low pH values (Lin et al., 2006). Consequently, the application of LAB as feed additives should be tested for the probiotics profiles such as tolerance to acid and bile salt conditions, adhesion to the intestine epithelium of the hosts, and antimicrobial activity against pathogenic bacteria. In this sense, the low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective

Table 3. Survival of *Enterococcus faecium* CE5-1 in the simulated gastric and intestinal juice

Treatment		Viable count (log CFU/ml)				
		simulated gastric juice		simulated intestinal juice		
		0 h	2 h	0 h	3 h	6 h
pH	pH 7.0	9.14 ± 0.10	9.38 ± 0.04	nd	nd	nd
	pH 3.0	9.18 ± 0 .08	9.38 ± 0.04	nd	nd	nd
	pH 2.5	9.14 ± 0.09	7.46 ± 0.08	nd	nd	nd
Fresh chicken bile concentration (%)	0	nd	nd	8.93 ± 0.05	9.04 ± 0.03	9.06 ± 0.02
	1	nd	nd	8.73 ± 0.11	9.20 ± 0.13	9.16 ± 0.02
	3	nd	nd	9.32 ± 0.03	9.45 ± 0.03	9.39 ± 0.03
	5	nd	nd	9.47 ± 0.03	9.39 ± 0.04	9.27 ± 0.02
	7	nd	nd	9.41 ± 0.04	9.28 ± 0.02	9.14 ± 0.03
Sequential incubation in the simulated gastric and intestinal juice	control*	9.25 ± 0.02	9.36 ± 0.05	9.36 ± 0.05	9.44 ± 0.02	9.42 ± 0.01
	7% FCB	9.28 ± 0.03	9.21 ± 0.01	9.21 ± 0.01	8.84 ± 0.07	8.78 ± 0.06

*sequential incubation in the simulated gastric juice at pH 3.0 and in the intestinal juice without fresh chicken bile (FCB)
nd = not detected

barrier against the entry and survival of bacteria in the intestinal tract (Huang and Adams, 2004).

In the present study, the viability of the *Ent. faecium* CE5-1 was determined after a 2 h incubation in simulated gastric juice (pH 2.5 and 3.0) containing pepsin. *Ent. faecium* CE5-1 exhibited good survival (9.38 log CFU/ml) at pH 3.0 and retained a moderate rate of survival (7.46 log CFU/ml) at pH 2.5 after 2 h of incubation (Table 3). The acid tolerance of LAB depends on the pH profile of H^+-ATPase and on the composition of the cytoplasmic membrane. This is largely influenced by the type of bacterium, the growth medium, and the incubation conditions (Hood and Zoitola, 1988).

Bacterial resistance to acid in the pH range of 2.5–3.0 is desirable for probiotic cultures. What should also be born in mind is that the combination of probiotic bacteria with other food ingredients present in food products may improve the viability of microorganisms during gastric transit. This is because of the protection exerted by certain food components leading to an enhanced gastric survival (Charteris et al., 1998; Wang et al., 1999; Zárate et al., 2000; Huang and Adams, 2004).

Bile salt tolerance is considered to be one of the essential properties required for LAB to survive in the small intestine (Park et al., 1998; Hosseini et al., 2009). The concentration of bile salt is the key factor which affects the viability of LAB. The concentration of bile intestinal contents of animals is dependent on the age and the foods or feeds taken in (Lin et al., 2007). Most *Lb. fermentum* isolated from swine and poultry were acid tolerant in the pH 2.6–3.2 gastric juice but less strains were bile intolerant (Lin et al., 2007). *Ent. faecium* SH328, *Ent. faecium* SH632, and *P. pentosaceous* SH740 isolated from GIT of broiler chickens were resistant to 0.5% bile salts and remained viable after 2 h at pH 3.0 (Shin et al., 2008).

In the present study, *Ent. faecium* CE5-1 showed good survival in the presence of 1, 3, 5, and 7% fresh chicken bile (Table 3). The probable reason for this was that the bacteria isolated from animal intestines have had more chance to be exposed to bile salts (Tanaka et al., 1999). Bile salts at high concentrations can rapidly dissolve membrane lipids and cause dissociation of integral membrane proteins resulting in the leakage of cell contents and cell death (Begley et al., 2005). It has been suggested that the major effect of bile acids would be the disaggregation of the lipid bilayer structure of the cell membrane. The tolerance to bile salts was initially associated with the presence of bile salt hydrolase activity (Moser and Savage, 2001; Taranto et al., 2006).

The two stresses resulting from stomach transit and small intestinal transit might interact and thereby affect the viability of the strains in a synergistic fashion. Therefore, it is important to evaluate all components (enzymes, low pH, bile salts, and food vehicle) in one system, rather than to evaluate the effect of each component in separate experiments. In this investigation the effect of exposure to gastric juice was combined with the following effects of exposure to bile salts on the viability of the probiotic strain. *Ent. faecium* CE5-1 could survive in the sequential study by showing a survival of 8.78 log CFU/ml (Table 3). These results indicate that *Ent. faecium* CE5-1 may resist the effects of pepsin during gastric transit and may also be intrinsically resistant to the action of pancreatin during the small intestinal transit. Overall, *Ent. faecium* CE5-1 showed high capacity of upper gastrointestinal transit tolerance and could provide an alternative source to enterococci for future probiotic development.

Antibiotic resistance of *Ent. faecium* CE5-1

Our enterococci were observed to be clinically resistant to chloramphenicol, streptomycin, and tetracycline (Table 4). On the other hand, they were susceptible to erythromycin, penicillin G, and vancomycin. *Ent. faecalis* VanB and *Ent. gallinarum* VanC were strongly resistant to almost all antibiotics tested in this study. In addition, *Ent. faecium* 139 was strongly susceptible to almost all tested antibiotics. However, *Ent. faecium* CE5-1 isolated from the GIT of Thai indigenous chicken was less resistant to tested antibiotics when compared with other strains. Robredo et al. (2000) established that vancomycin-resistant enterocci (VRE) were found in chicken products and were identified as *Ent. duran* (n = 11), *Ent. faecalis* (n = 10), *Ent. faecium* (n = 10), and *Ent. hirae* (n = 2). The increased use of antibiotics in poultry production to treat infection and as a growth promoter in feed has led to the problem of the development of antibiotic resistance in recent years (Apata, 2009).

Growth curve and BLS production from *Ent. faecium* CE5-1

The highest antimicrobial activity of *Ent. faecium* CE5-1 (320 AU/ml) was reached at 15 h during

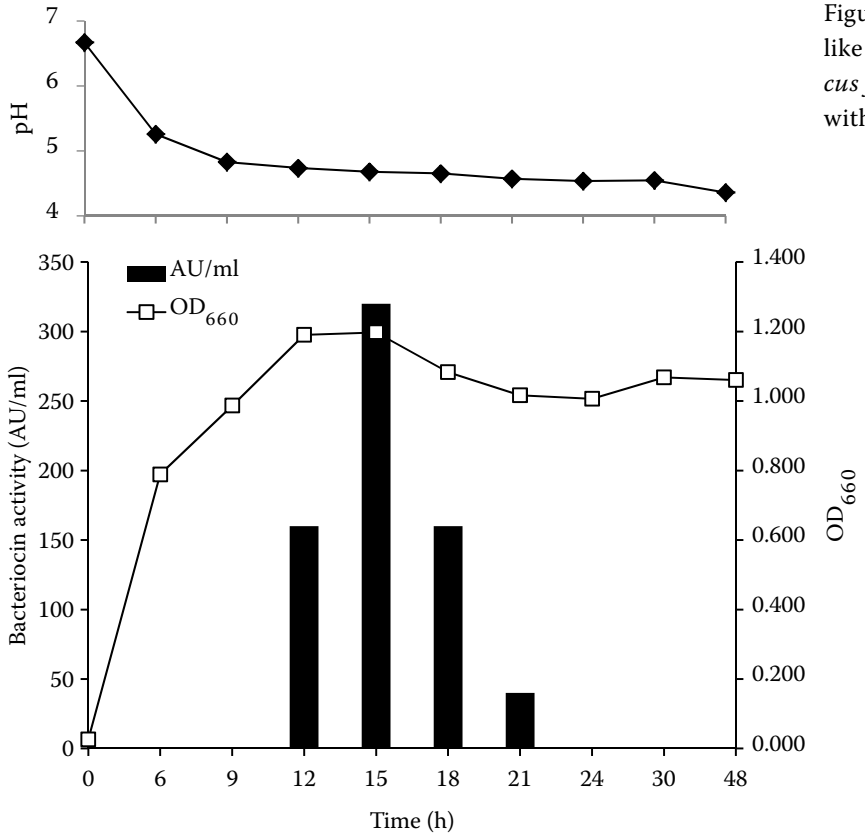

Figure 1. Growth curve and bacteriocin-like substance production of *Enterococcus faecium* CE5-1 in 1 l of MRS broth with agitation (100 rpm) at 37°C

the early stationary phase (Figure 1). However, it sharply decreased after 15 h of cultivation. This may be because of the digestion of the antagonistic compound by proteolytic enzyme released from the cells (He et al., 2006) or the binding of bacteriocin to producer cells (Todorov and Dicks, 2009). A decrease in the bacteriocin activity has also been observed for bacteriocins produced by *Ent. faecium* MMRA (Rehaiem et al., 2009), *Leuconostoc mesenteroides* E131 (Xiraphi et al., 2008), and *Lb. acidophilus* (Karthikeyan and Santhosh, 2009). On the other hand, bacteriocin production of *Ent. faecium* MMT21 was detected from the early logarithmic phase of growth; this reached a maximum at the end of exponential phase and remained constant until 34 h of incubation (Ghrairi et al., 2008).

In the same way, the pH strongly decreased from the initial pH 6.7 to around 4.8 in 9 h during the logarithmic growth and slightly decreased towards the end of incubation (pH 4.3 at 28 h). The decreasing of pH during the growth rate was usually found in the fermentation of LAB, the most common being lactic acid, acetic acid, propionic acid, and carbon dioxide (Salminen and Wright, 1998). Similar results have been reported for bacteriocin ST311LD production by *Ent. faecium* ST311LD, where the pH decreased from 6.2 to 4.4 (Todorov and Dicks, 2005).

Effect of antibacterial activity of BLS against bacterial indicators

The CS of *Ent. faecium* CE5-1 adjusted to pH 6.5–7.0 were observed to show a narrow spectrum of activity towards the strains tested (Table 1). It could inhibit almost all LAB strains and some Gram-positive bacteria such as *Enterococcus* strains, *P. pentosaceus* 3CE27, *Lb. sakei* subsp *sakei* JCM1157, and *L. monocytogenes* DMST17303. However, no activity was observed against *B. cereus* DMST5040, *Lb. plantarum* D6SM3, *P. pentosaceus* DMST18752, *Stap. aureus* DMST8840, and all tested Gram-negative bacteria. That no activity was shown against Gram-negative bacteria may be due to the relative impermeability of their outer membranes (Jack et al., 1995). Rehaiem et al. (2009) reported that CS of *Ent. faecium* MMRA could inhibit *Lactobacillus*, *Leuconostoc*, *Enterococcus*, and *Listeria* species, but there was no inhibition of Gram-negative bacteria, *Hafnia*, *Serratia*, and *E. coli.* Moreover, the largest inhibition zones were observed against *Lb.* subsp *sakei* JCM1157. Thus, *Lb. sakei* subsp *sakei* JCM1157 was used as an indicator for the determination of antimicrobial activity for our study.

Furthermore, CS of *Ent. faecium* CE5-1 showed inhibition against antibiotic-resistant enterococci

Table 4. Antibiotic resistance patterns of enterococci

Bacterial species	Antibiotics (μg/ml)									
	Chloramphenicol		Erythromycin		Penicillin G		Streptomycin		Tetracycline		Vancomycin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Ent. faecium 139	10	320	1.25	40	1.25	5	80	160	< 0.625	40	1.25	40
Ent. faecium 174	80	1.282	5	40	20	20	> 1.282	> 1.282	160	> 1.282	2.5	80
Ent. faecium 348	80	> 1.282	2.5	80	10	40	> 1.282	> 1.282	> 1.282	> 1.282	1.25	40
Ent. faecalis VanB	80	80	320	> 1.282	10	80	1.282	> 1.282	40	> 1.282	160	> 1.282
Ent. gallinarum VanC	160	> 1.282	160	> 1.282	10	40	> 1.282	> 1.282	80	> 1.282	10	80
Ent. faecium CE5-1	10	320	2.5	40	1.25	2.5	320	640	< 0.625	40	1.25	40

MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration

strains including *Ent. faecium* 174, *Ent. faecium* 348, *Ent. gallinarum* VanC, and *Ent. faecalis* VanB except for *Ent. faecium* 139 and *Ent. faecium* CE5-1 itself. However, it was normally observed that bacteriocin from the producer's organism had no inhibitory effect on the organism producing it. This was due to the presence of structural enterocin A gene (*ent*A gene) and the immunity enterocin A gene (im*ent*A gene) located on the genomic DNA of *Ent. faecium* CE5-1 (data not shown). These results suggest that a BLS from *Ent. faecium* CE5-1 could possibly be used to control antibiotic-resistant enterococci infections.

PCR detection of enterocin structural gene

The results indicate that only the enterocin A gene was successfully detected in *Ent. faecium* CE5-1 (Figure 2). The PCR product was approximately 138 bp. After sequencing of the 138 bp PCR product, it exhibited 100% homology with the enterocin A gene of *Ent. faecium* E9 in the GenBank sequence database. Similar results showing the presence of structural *ent*A gene in *Enterococcus* strains isolated from animal, food, and feed were observed by Strompfová et al. (2008). Enterocin A was first characteried by Aymerich et al. (1996). It was grouped in the class IIa subgroup of class II bacteriocin, otherwise known as pediocin-like bacteriocin, and had high anti-listerial activity (O'Keeffe et al., 1999). However, the presence of structural *ent*A gene in genomic DNA of *Ent. faecium* CE5-1 does not necessarily mean the production of the corresponding enterocin A. In order to confirm the production of enterocin A from *Ent. faecium* CE5-1 it would be necessary to purify antimicrobial peptide in the future.

Effect of co-culturing of *Ent. faecium* CE5-1 with antibiotic-resistant enterococci in MRS broth

Competition between *Ent. faecium* CE5-1 and the antibiotic-resistant *Ent. faecalis* VanB and *Ent. gallinarum* VanC was brought during their co-culture in 200 ml of MRS broth at 37°C. The results showed that the viability of both *Ent. faecalis* VanB and *Ent. gallinarum* VanC strongly declined from an initial 6.68 to 4.29 log CFU/ml (Figure 3a) and 6.76 to 4.31 log CFU/ml (Figure 3b), respectively. This was after

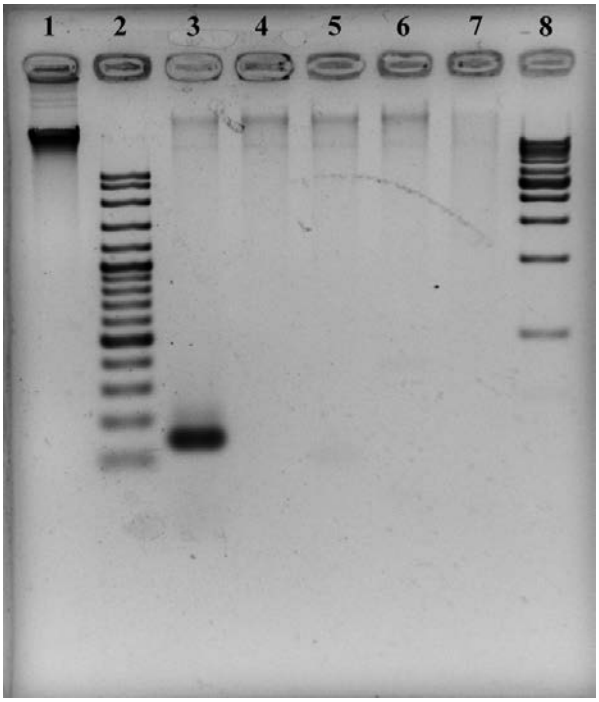

Figure 2. Agarose gel electrophoresis of polymerase chain reaction products of the bacteriocin encoding gene from *Enterococcus faecium* CE5-1 obtained using enterocins specific primer

1 = chromosomal DNA of *Enterococcus faecium* CE5-1, 2 = 100 bp DNA ladder, 3 = enterocin A, 4 = enterocin B, 5 = enterocin P, 6 = enterocin L50B, 7 = enterocin IIa, 8 = 1 kb DNA ladder

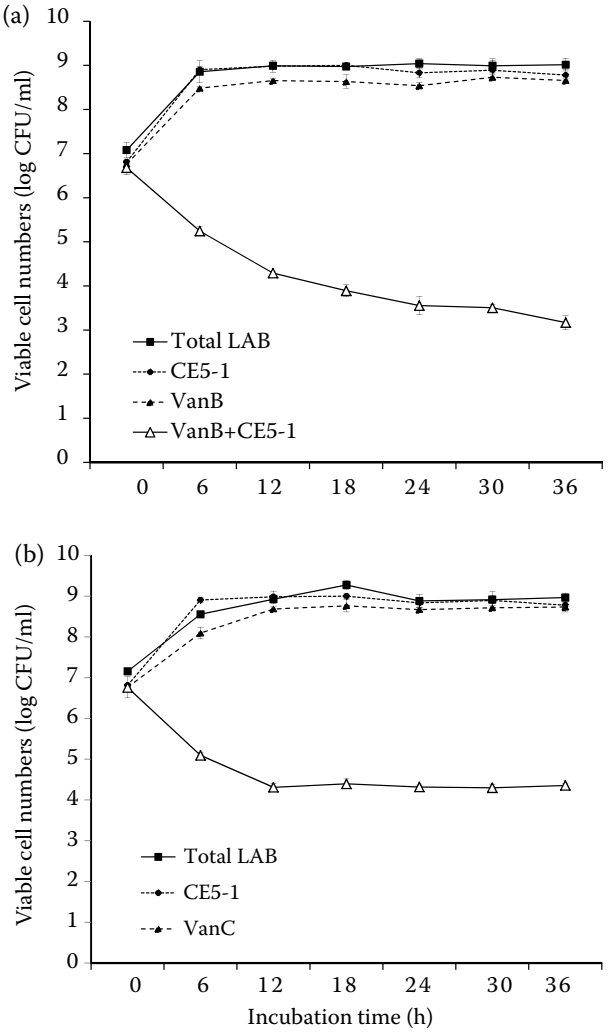

Figure 3. Growth of the inoculated indicators in *Enterococcus faecium* CE5-1 culture

(a) growth of the *Ent. faecalis* VanB in MRS broth with *Ent. faecium* CE5-1, (b) growth of the *Ent. gallinarum* VanC in MRS broth with *Ent. faecium* CE5-1

12 h of cultivation and the count decreased slightly after 12 h of cultivation in a co-culture.

This is the first report on using *Ent. faecium* as a probiotic strain to control antibiotic-resistant enterococci. Almost all researchers have been interested in using *Ent. faecium* or enterocin to control foodborne pathogens such as *L. monocytogenes*. *Ent. faecium* MMRA supernatant reduced *L. ivanovii* BUG 496 at 60 AU/ml (Rehaiem et al., 2009). In the same way, the number of cells of *L. innocua* LMG 13568 decreased from 4.8×10^3 CFU/ml to 2.0×10^2 CFU/ml for 32 h in a co-culture of *Ent. mundtii* ST4SA and *L. innocua* LMG 13568 (Todorov and Dicks, 2009).

CONCLUSION

This study suggests that BLS-producing *Ent. faecium* CE5-1 showed better tolerance to simulate gastric juice in pH 2.5 and to simulate intestinal juice which contained 7% of fresh chicken bile at pH 8.0. It was sensitive to tetracycline, erythromycin, penicillin G, and vancomycin. The culture supernatant showed activity against *L. monocytogenes* DMST17303, *P. pentosaceus* 3CE27, *Lb. sakei* subsp. *sakei* JCM1157, and antibiotic-resistant enterococci strains. It may be concluded that *Ent. faecium* CE5-1 can be used as a probiotic strain for antibiotic-resistant enterococci control in chickens in the future. However, biochemical and genetic characteristics of the BLS produced from *Ent. faecium* CE5-1 should be studied, too.

REFERENCES

Abriouel H., Lucas R., Omar N.B., Valdivia E., Maqueda M., Martínez-Cañamero M., Gálvez A. (2005): Enterocin AS-48RJ: A variant of enterocin AS-48 chromosomally encoded by *Enterococcus faecium* RJ16 isolated from food. Systematic and Applied Microbiology, 28, 387–397.

Almeida L.S.B., Murata R.M., Yatsuda R., Dos Santos M.H., Nagem T.J., Alencar S.M., Koo H., Rosalen P.L. (2008): Antimicrobial activity of *Rheedia brasiliensis* and 7-epiclusianone against *Streptococcus mutans*. Phytomedicine, 15, 886–891.

Apata D.F. (2009): Antibiotic resistance in poultry. International Journal of Poultry Science, 8, 404–408.

Aymerich T., Holo H., Havarstein L.S., Hugas M., Garriga M., Nes I. (1996): Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Applied and Environmental Microbiology, 62, 1676–1682.

Begley M., Gahan C.M.G., Hill C. (2005): The interaction between bacteria and bile. FEMS Microbiology Reviews, 29, 625–651.

Casaus P., Nilsen T., Cintas L.M., Nes I.F., Hernandez P.E., Holo H. (1997): Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology, 143, 2287–2294.

Charteris W.P., Kelly P.M., Morelli L., Collins J.K. (1998): Development and application of an *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. Journal of Applied Microbiology, 84, 759–768.

Cintas L.M., Casaus P., Havarstain L.V., Hernandez P.E., Nes I.F. (1997): Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with abroad antimicrobial spectrum. Applied and Environmental Microbiology, 63, 4321–4330.

Cintas L.M., Casaus P., Holo H., Hernandez P.E., Nes I.F., Havarstain L.S. (1998): Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50 are related to staphylococcal haemolysins. Journal of Bacteriology, 180, 1988–1994.

Cleveland J., Montville T.J., Nes I.F., Chikinda M.L. (2001): Bacteriocins: safe, natural antimicrobials for food preservation. International Journal of Food Microbiology, 71, 1–20.

D'Aimmo M.R., Modesto M., Biavati B. (2007): Antibiotic resistance of lactic acid bacteria and *Bifidobacterium* spp. isolated from dairy and pharmaceutical products. International Journal of Food Microbiology, 115, 35–42.

De Vuyst L., Degeest B. (1999): Heteropolysaccharides from lactic acid bacteria. FEMS Microbiology Reviews, 23, 153–177.

Franz C.M.A.P., Holzapfel W.H., Stiles M.E. (1999): Enterococci at the crossroads of food safety. International Journal of Food Microbiology, 47, 1–24.

Franz C.M.A.P., van Belkum M.J., Holzapfel W.H., Abriouel H., Gálvez A. (2007): Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiology Reviews, 31, 293–310.

Fuller R. (1989): Probiotics in man and animals. Journal of Applied Bacteriology, 66, 365–378.

Ghrairi T., Frere J., Berjeaud J.M., Manai M. (2008): Purification and characterisation of bacteriocins produced by *Enterococcus faecium* from Tunisian rigouta cheese. Food Control, 19, 162–169.

He L., Chen W., Liu Y. (2006): Production and partial characterization of bacteriocin-like peptides by *Bacillus licheniformis* ZJU12. Microbiology Research, 161, 321–326.

Hood S.K., Zoitola E.A. (1988): Effect of low pH on the ability of *Lactobacillus acidophilus* to survive and adhere to human intestinal cells. Journal of Food Science, 53, 1514–1516.

Hosseini S.V., Arlindo S., Böhme K., Fernández-No C., Calo-Mata P., Barros-Velázquez J. (2009): Molecular and probiotic characterization of bacteriocin producing *Enterococcus faecium* strains isolated from nonfermented animal foods. Journal of Applied Microbiology, 107, 1392–1403.

Huang Y., Adams M.C. (2004): *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. International Journal of Food Microbiology, 91, 253–260.

Jack R.W., Tagg J.R., Ray B. (1995): Bacteriocins of Gram-positive bacteria. Microbiology Reviews, 59, 171–200.

Karthikeyan V., Santhosh S.W. (2009): Study of bacteriocin as a food preservative and the *L. acidophilus* strain as probiotic. Pakistan Journal of Nutrition, 8, 335–340.

Klare I., Konstabel C., Badstübner D., Werner G., Witte W. (2003): Occurrence and spread of antibiotic resistances in *Enteroccoccus faecium*. International Journal of Food Microbiology, 88, 269–290.

Lauková A., Strompfová V., Simonová M., Štyriak I., Swiecicka I. (2008): Species of *Enterococcus faecalis* associated with free-living rodents. Folia Microbiologica, 53, 173–178.

Lin W.H., Hwang C.F., Chen L.W., Tsen H.Y. (2006): Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microbiology, 23, 74–81.

Lin W.H., Yu B., Jang S.H., Tsen H.Y. (2007): Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. Anaerobe, 13, 107–113.

Madureira A.R., Pereira C.I., Truszkowska K., Gomes A.M., Pintado M.E., Malcata F.X. (2005): Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. International Dairy Journal, 15, 921–927.

Moser S.A., Savage D.C. (2001): Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. Applied and Environmental Microbiology, 67, 3476–3480.

Musikasang H., Tani A., H-kittikun A., Maneerat S. (2009): Probiotic potential of lactic acid bacteria isolated from chicken gastrointestinal digestive tract. World Journal of Microbiology and Biotechnology, 25, 1337–1345.

Ogier J.C., Serror P. (2008): Safety assessment of dairy microorganisms: The *Enterococcus* genus. International Journal of Food Microbiology, 126, 291–301.

O'Keeffe T., Hill C., Ross R.P. (1999): Characterization and heterologous expression of the genes encoding enterocin A production, immunity, and regulation in *Enterococcus faecium* DPC1146. Applied and Environmental Microbiology, 65, 1506–1515.

Parente E., Brienza C., Moles M., Ricciardi A. (1995): A comparison of methods for the measurement of bacteriocin activity. Journal of Microbiological Methods, 22, 95–108.

Park H.S., Lee S.H., Uhm T.B. (1998): Selection of microorganisms for probiotics and their characterization. Korean Journal of Food Science and Nutrition, 27, 433–440.

Rehaiem A., Martínez B., Manai M., Rodríguez A. (2009): Production of enterocin A by *Enterococcus faecium* MMRA isolated from 'Rayeb', a traditional Tunisian dairy beverage. Journal of Applied Microbiology, 108, 1685–1993.

Robredo B., Singh K., Baquero F., Murray B., Torres C. (2000): Vancomycin-resistant enterococci isolated from animal and food. International Journal of Food Microbiology, 54, 197–204.

Salminen S., Wright A. (1998): Lactic Acid Bacteria: Microbiology and Functional Aspects. 2nd Ed. Marcel Dekker, Inc., New York, USA.

Schillinger U., Lücke F.K. (1989): Antibacterial activity of *Lactobacillus sake* isolated from meat**.** Applied and Environmental Microbiology, 55, 1901–1906.

Shin M.S., Han S.K., Ji A.R., Kim K.S., Lee W.K. (2008): Isolation and characterization of bacteriocin-producing bacteria from the gastrointestinal tract of broiler chickens for probiotic use. Journal of Applied Microbiology, 105, 2203–2212.

Souza M.R., Moreira J.L., Barbosa F.H.F., Cerqueira M.M.O.P., Nunes A.C., Nicoli J.R. (2007): Influence of intensive and extensive breeding on lactic acid bacteria isolated from *Gallus gallus domesticus* ceca. Veterinary Microbiology, 120, 142–150.

Strompfová V., Lauková A. (2007): *In vitro* study on bacteriocin production of *Enterococci* associated with chickens. Anaerobe, 13, 228–237.

Strompfová V., Lauková A., Simonová M., Marciňáková M. (2008): Occurrence of the structural enterocin A, P, B, L50B genes in enterococci of different origin. Veterinary Microbiology, 132, 293–301.

Tanaka H., Doesburg K., Iwasaki T., Mierau I. (1999): Screening of lactic acid bacteria for bile salt hydrolase activity. Journal of Dairy Science, 82, 2530–2535.

Taranto M.P., Perez-Martinez G., de Valdez G.F. (2006): Effect of bile acid on the cell membrane functionality of lactic acid bacteria for oral administration. Research in Microbiology, 157, 720–725.

Todorov S.D., Dicks L.M.T. (2005): Optimization of bacteriocin ST311LD production by *Enterococcus faecium* ST311LD, isolated from spoiled black olives. Journal of Microbiology, 43, 370–374.

Todorov S.D., Dicks L.M.T. (2009): Effect of modified MRS medium on production and purification of antimicrobial peptide ST4SA produced by *Enterococcus mundtii*. Anaerobe, 15, 65–73.

Wang X., Brown I.L., Evans A.J., Conway P.L. (1999): The protective effects of high amylose maize (amylomaize) starch granules on the survival of *Bifidobacterium* spp. in the mouse intestinal tract. Journal of Applied Microbiology, 87, 631–639.

Xiraphi N., Georgalaki M., Rantsiou K., Cocolin L., Tsakalidou E., Drosinos E.H. (2008): Purification and characterization of a bacteriocin produced by *Leuconostoc mesenteroides* E131. Meat Science, 80, 194–203.

Yi H., Zhang L., Tuo Y., Han X., Du M. (2010): A novel method for rapid detection of class IIa bacteriocin-producing lactic acid bacteria. Food Control, 21, 426–430.

Zárate G., Chaia A.P., González S., Oliver G. (2000): Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. Journal of Food Protection, 63, 1214–1221.

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