ANTIMICROBIAL ACTIVITY AND PRELIMINARY CHARACTERIZATION OF PEPTIDES PRODUCED BY LACTIC ACID BACTERIA ISOLATED FROM SOME VIETNAMESE FERMENTED FOODS

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ABSTRACT

In this study, 170 strains isolated from 22 samples of fermented foods were identified as lactic acid bacteria by biochemical and morphological methods. Of which, fifty two isolated strains had antibacterial activity when tested using the agar well-diffusion method. Two strains, CS3.7 and FME1.7, expressed high antimicrobial activity against all of the four indicator bacteria: *E. coli*, *Bacillus cereus*, *Listeria monocytogenes*, and *Salmonella* spp., and were selected for further studies. The results showed that both strains had antimicrobial activity against the indicator strains 2 hours before the bacteria reached the stationary phase. The antimicrobial activity of the cell free supernatants was completely lost when incubated with papain enzyme for 2 hours at room temperature. This result led us to conclude that antimicrobial substances of the free cell supernatants were peptides. Characterization of the peptides demonstrated that they were highly stable at 68^{o}C, in which residual activity of FME1.7 and CS3.7 was above 90% and 75% for 20 min, respectively. At 100^{o}C for 10 min, the antimicrobial activity of the two strains remained around 40%. The study also indicated that the peptides were stable at pH 5. However, antimicrobial activity was significantly reduced when incubated in other pHs. The results showed that peptides of CS3.7 and FME1.7 are quite promising to be used as biopreservatives because of their high range of antimicrobial activity and thermostability.

Keywords: Antimicrobial activity, indicator bacteria, lactic acid bacteria, peptide.

Xác định khả năng kháng khuẩn và đặc tính của peptide được sinh ra bởi các chủng vi khuẩn lactic phân lập từ một số thực phẩm lên men của Việt Nam

TÓM TẮT

Trong nghiên cứu này, 170 chủng phân lập từ 22 mẫu thực phẩm lên men được xác định là vi khuẩn lactic dựa vào phương pháp hóa sinh và đặc điểm hình thái. Trong đó, 52 chủng vi khuẩn này được xác định là có khả năng kháng khuẩn thông qua phương pháp khuếch tán bằng đĩa thạch. Hai chủng CS3.7 và FME1.7 có khả năng kháng khuẩn cao nhất với cả 4 chủng kiểm định *E. coli*, *B. cereus*, *L. monocytogenes*, *Salmonella* spp. được chọn cho các nghiên cứu tiếp theo. Kết quả chỉ ra rằng cả hai chủng đều có hoạt tính kháng khuẩn với cả 4 chủng kiểm định và hoạt tính cao nhất trước thời điểm pha cân bằng 2 giờ nuôi cấy. Dịch nuôi cấy mất hoàn toàn khả năng kháng khuẩn bởi enzyme papain sau 2 giờ ủ ở nhiệt độ thường, điều đó có thể sơ bộ rằng nguyên nhân kháng khuẩn của dịch nuôi cấy từ hai chủng này là peptide. Nghiên cứu xác định đặc tính của peptide cho thấy chúng bền ở nhiệt độ 68^{o}C, trong đó hoạt tính còn lại của FME1.7 và CS3.7 tương ứng là trên 90% và 75% trong vòng 20 phút. Ở 100°C trong 10 phút, hoạt tính kháng khuẩn của hai chủng còn lại khoảng 40%. Nghiên cứu cũng chỉ ra rằng peptide bền ở pH 5, nhưng hoạt tính kháng khuẩn giảm đáng kể khi tăng hoặc giảm pH. Với kết quả nghiên cứu này, peptide được sản xuất bởi hai chủng trên có tiềm năng sử dụng như một chất bảo quản sinh học bởi đặc tính kháng khuẩn rộng và bền nhiệt của chúng.

Từ khóa: Hoạt tính kháng khuẩn, peptide, vi khuẩn lactic, vi khuẩn kiểm định.

1. INTRODUCTION

Food is essential for human being's to live, and as a result, food safety has received increased attention. Consumption of food contaminated with pathogens may cause certain disease events even when it is contaminated with a very low infective dose. In addition, foods contaminated with antibiotic resistant bacteria could be a major threat to public health as the antibiotic resistance determinants can be transferred to other pathogenic bacteria that later on cause compromises in the treatment of severe infections.

Recently, food safety has not only been an intractable problem in developing countries like Vietnam, but also in many countries around the world. The risk of pathogenic microorganism contamination is increasing in agricultural products and food processing products. Undoubtedly the major threat to food safety is the emergence of pathogens such as *Escherichia coli, Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens*, or *Bacillus cereus*, which have been considered to be foodborne microorganisms (Castellano *et al.*, 2008). There are several methods used to prevent foods from pathogenic contamination, such as freezing and thawing or using chemical substances. However, food quality is decreased in terms of both nutrition and food safety when using those methods (Parada *et al.*, 2007). So, new approaches to controlling foodborne pathogens in food processing and food preservation have been prompted. For the past two decades, many studies have focused on the natural compounds produced by lactic acid bacteria (LAB) to apply in food preservation as LAB have been, so far, considered a food grade organism (Fricourt *et al.*, 1994; Ogunbanwo *et al.*, 2003; Parada *et al.*, 2007). Moreover, LAB produce antimicrobial substances, such as acids, peptides, and hydrogen peroxide, among others, during their growth and development, of which, peptides have been proven to be the main group to have antimicrobial activity and to safely be applied in food preservation (Deegan *et al.*, 2006; Settanni and Corsetti, 2008). A great deal of evidence has been reported that peptides produced by LAB have broad range capabilities against pathogenic bacteria activity (Nomoto, 2005). In addition, peptides are safe and stable in food processing and preservation, and are not deleterious to food. Therefore, up to date, many studies on antimicrobial peptides from isolated lactic acid bacteria with expectations for food preservation have been published.

However, peptides from these studies have narrow range antimicrobial activity, and almost all of them against only gram-positive bacteria (Ivanova *et al.*, 1998). Meanwhile, many bacteria contaminating food are gram-negative bacteria, such as *E.coli* and *Salmonella* spp. That is why this study aims to isolate lactic acid bacteria from a selection of Vietnamese fermented foods, including fermented vegetables, fermented milks, and fermented meats, to explore new peptides with high ranges of antimicrobial activity and characterize the peptides for further applications.

2. MATERIALS AND METHODS

2.1. Sample collection

Twenty-two samples of 6 different fermented foods described in Table 1 were used to isolate LAB.

2.2. Indicator strains

E. coli, *B. cereus*, *L. monocytogenes*, and *Salmonella* spp. supplied by the Faculty of Veterinary Medicine, Vietnam National University of Agriculture were chosen as pathogen indicators for antimicrobial activity testing.

2.3. Isolation of lactic acid bacteria

Isolation of LAB was done as described by Chen (2010). After crushing, samples were diluted to a 10^{-1} - 10^{-6} concentration by mixing with sterilized water. A 100 ml sample of diluted solution was spread directly onto the surface of MRS agar plates with an added 1% $CaCO_3$.

Table 1. Vietnamese fermented foods that were collected to isolate lactic acid bacteria

Sample	Location	Sample Symbol	Number of isolated strains	Number of isolated strains that have antimicrobial activity
Fermented milk	Hanoi	FMI1, FMI2, FMI3, FMI4	26	5
	Son La	FMI5, FMI6	19	4
Fermented eggplant	Hanoi	FE1, FE2, FE3	60	29
Fermented meat	Phu Tho	FME1, FME2, FME3	30	2
Chilli sauce	Lao Cai	CS1, CS2, CS3, CS4, CS5, CS6	40	3
Fermented cassava leaf	Hoa Binh	FCL1, FCL2	16	8
Fermented bamboo shoots	Phu Tho	FBS1, FBS2	6	1
Total		22	197	52

Samples were incubated under anaerobic conditions at 37°C for 24 hours. After incubation, colonies creating a clear zone on the agar plates were selected for further studies.

2.4. Identification of colonies

Identification of colonies as LAB was performed using biochemical and morphological tests as described by Barnali Ashe (2010).

2.5. Antimicrobial activity test of isolates

Cell free supernatant of 16 hour cultivation medium was used to determine antimicrobial activity by using the well-diffusion method described by Al-Allaf (2009). After adjusting to pH 6.5, 100 µl of the supernatant was filled in 5-mm diameter wells of an agar plate previously spread with pathogenic bacteria. The plate then was incubated at 37°C for 24 h. After incubation, the diameter of inhibition zone was measured with calipers. The bacterial isolate showing the widest inhibition zone against the pathogen bacteria was selected for further studies.

2.6. Effect of cultivation time on antimicrobial activity

Isolated LAB were cultured in 1000 ml of MRS broth at 37°C. Every 2 hours, 100 ml of culture medium was taken out to centrifuge at 4°C, 6000 rpm for 15 min. Cell free supernatant was concentrated 3 times by rotary evaporator at 35°C, 70 rpm for 45 min before testing antimicrobial activity by the well diffusion method.

2.7. Characterization of antimicrobial activity

The effect of proteolytic enzymes on the concentrated cell free supernatant as described by Joshi *et al.,* (2006) was applied to show that the peptides of LAB are agents of antimicrobial activity. First, the concentrated cell free supernatant was adjusted to pH 6.5. Second, 5 ml of concentrated cell free supernatant was taken in test tubes and treated with papain at a final concentration of 1 mg/ml in pH 7. The test tubes with and without the enzymes (control) were incubated for 2 hours at 37°C and then heated for 3 min at 100°C to inactivate the enzymes. Both the control and the samples were assayed for antimicrobial activity using the well diffusion method.

2.8. Characterization of peptides

Heat stability: A volume of 5 ml of concentrated crude peptides in different test tubes were overlaid with paraffin oil to prevent evaporation and then heated at 68°C and 100°C for 10 and 20 min each, and at 121°C for 15 min under pressure. The heat-treated samples were then assayed for antimicrobial activity as described previously.

pH sensitivity: A volume of 5 ml of concentrated crude peptides was put in test tubes and adjusted to different pHs (2 - 9) using either sterile 1M NaOH or 1M HCl. Treated samples were incubated for 2 hours at room temperature and then adjusted to the original

pH of 6.5 before determination of antimicrobial activity as described previously.

3. RESULTS AND DISCUSSION

3.1. Identification of LAB isolated from fermented foods

In this study, a total of 192 colonies isolated from 22 samples of 6 different fermented foods produced clear zones in MRS agar plates with added $CaCO_3$. Of which, 170 colonies were identified as LAB by colony morphology and negative catalases were used to test antimicrobial activity.

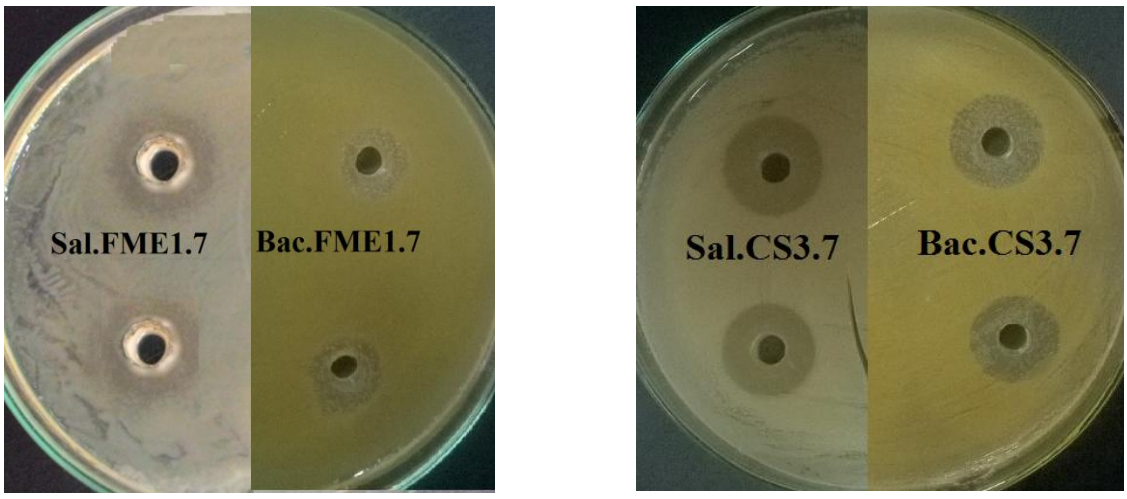
3.2. Screening for antimicrobial activity of isolated colonies

Antimicrobial activity of isolated LAB indicated that there were a total of 52 colonies that showed antimicrobial activity, of which, strains FME1.7 and CS3.7 had a wide range of antimicrobial activity against *E. coli, B. cereus, L. monocytogenes,* and *Salmonella* spp., as seen in Figure 1 and Table 2. The results showed that both FME1.7 and CS3.7 had broad range antimicrobial activity with a *Listeria, Salmonella, Bacillus,* and *E. coli*. So, FME1.7 and CS3.7 were used for the next studies.

3.3. Effect of cultivation time on antimicrobial activity

To determine the relationship between the growth curve and antimicrobial activity of isolated LAB, FME1.7 and CS3.7 were cultured in MRS broth at 37°C. At 2 h intervals, the culture medium were taken out to measure the OD at 600 nm for the growth curve and centrifuged to get the cell free supernatant for antimicrobial activity tests as described previously. Results are indicated in Figure 2.

Figure 2 (a, b) shows that cell free supernatants of the two isolated strains started their antimicrobial activity after 4 hours of cultivation and inhibited the growth of all of test bacteria from 6 to 12 hours of cultivation. Interestingly, the total antimicrobial activity was highest 2 hours before the stationary phase (10 hours and 12 hours of cultivation with CS3.7 and FME1.7, respectively). The antimicrobial activity completely disappeared when the stationary phase was reached in both strains. So, 2 hours before the stationary phase, the free cell supernatants of both strains were used for further characterization of antimicrobial activity and peptides.

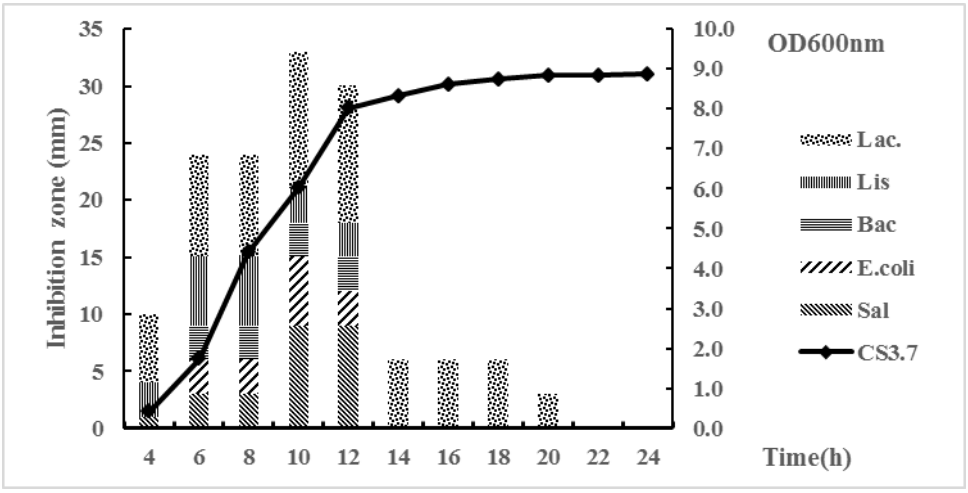


(a) (b)

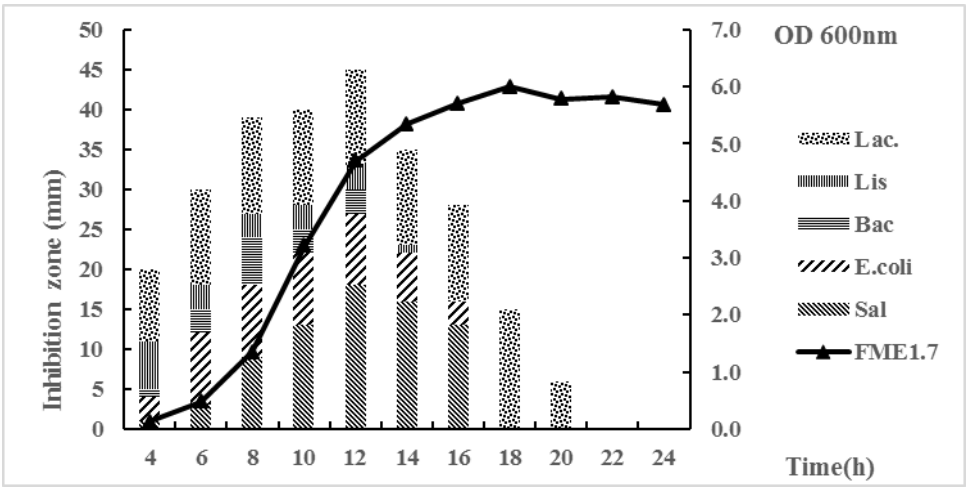
Figure 1. Anti- Bacillus and Salmonella activity of concentrated cell free supernatant of FME1.7 (a) and CS3.7 (b)

Table 2. Antimicrobial activity of concentrated cell free supernatant of FME1.7 and CS3.7

	Inhibition zone diameter (mm)			
	E. coli	*B.cereus*	*L. monocytogenes*	*Salmonella* spp.
FME1.7	4	8	8	8
CS3.7	8	12	6	12



(a)



(b)

Figure 2. Effect of cultivation time on antimicrobial activity of FME1.7 and CS3.7

Note: (a) Growth curve and antimicrobial activity of CS3.7; (b) Growth curve and antimicrobial activity of FME1.7

3.4. Characterization of concentrated cell free supernatant

Effect of enzymes

Antimicrobial activity of concentrated cell free supernatants was completely lost when the samples were treated with papain. The results indicated that the major antimicrobial factors were sensitive to proteolytic enzymes, which is in agreement with an earlier report that bacteriocins are short peptides and sensitive to proteolytic enzymes (Joshi et al., 2006). In addition, the bacteriocin pediocin ACH from *Pedicoccus acidilacti* was sensitive to proteolytic enzymes and was completely inactivated by several proteolytic enzymes (Bhunia et al., 1988;

Bonade et al., 2001). In comparing our results to those of previous studies, it can be concluded that the major antimicrobial factors in the cell free supernatant of this study are peptides.

3.5. Characterization of peptides

3.5.1. Heat stability of peptides

The effects of temperature on the antimicrobial activity of FME1.7 and CS3.7 are described in Table 3. The results indicated that crude peptides were highly stable at 68°C for up to 20 min, at which time the residual antimicrobial activity of FME1.7 and CS3.7 remained higher than 90% and 75%, respectively. At 100°C after 10 min of incubation, antimicrobial activity of FME1.7 and CS3.7 was reduced approximately 40% of that of the original. During the same treatment but for 20 min, the highest remained antimicrobial activity and lowest remained activity were 50% of CS3.7 with *E.coli* and 20% of FME1.7 with *E.coli* and *Salmonella* spp., respectively. At 121°C for 15 min of incubation, antimicrobial activity was completely lost in both CS3.7 and FME1.7. This study was compatible to the study of Joshi *et al.* (2006), which indicated that bacteriocins produced by *Lactobacillus* sp. were stable at 68°C for 20 min, and at 100°C for 10 min, 55 % of antimicrobial activity was retained. Moreover, these peptides are comparable with the OR-7 bacteriocin purified from *Lactobacillus salivarius* strain NRRL B-30514, which was stable at 90°C for 15 min (Stern *et al.,* 2006). The heat stability of peptides in this study indicated that they are highly promising for use as bio-preservatives in combination with thermal processing to preserve food products.

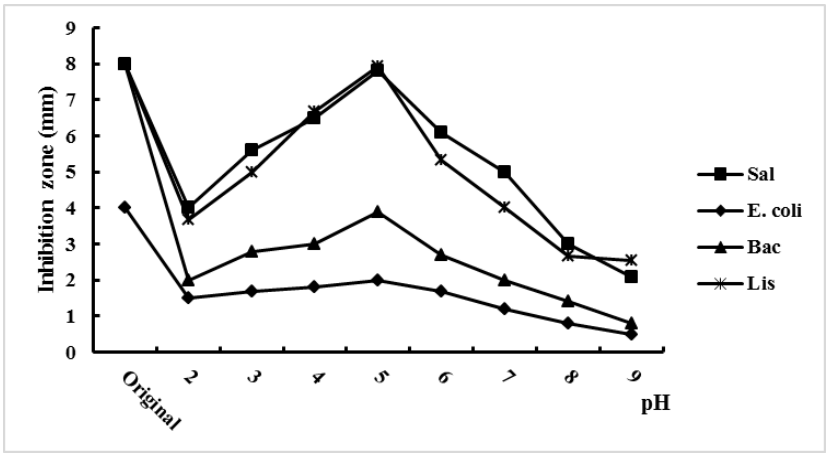
3.5.2. pH sensibility of crude peptides

pH sensibility of crude peptides is described in Figure 3 (a, b). Results showed that the peptides were very sensitive to strong acids and strong bases. At pHs of 2 to 5, the antimicrobial activity gradually increased and reached maximal activity at pH 5. When the pH was increased over 5, the antimicrobial activity was drastically reduced and nearly lost at pH 9 for *E. coli* with CS3.7 and *Listeria* with FME.1.7. Results of this study were comparable with the peptides of *Lactococcus lactis* D53 and 23, which showed that the optimal pH for peptide stability was 5 (Schillinger *et al.,* 1989). In one previous study, the bacteriocins produced by an isolated *Bacillus* sp. strain 8A was active in a pH range of 5-8 but was inactivated when incubated outside this range (Bizani *et al.,* 2002).

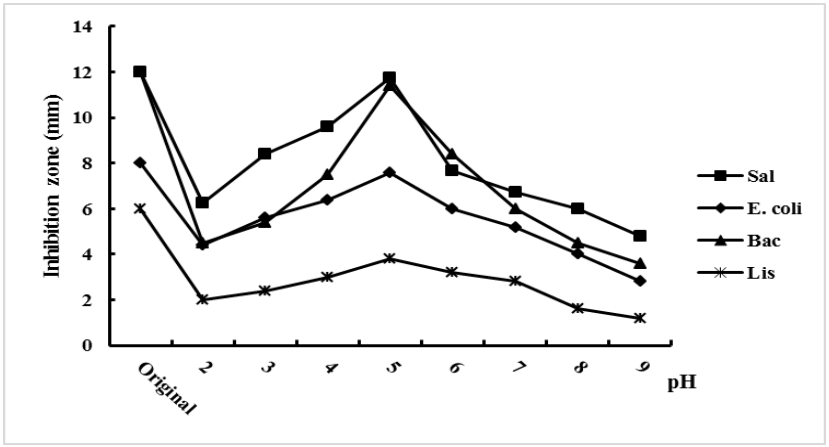
Table 3. Heat stability of peptides at different incubation times

Treatment temperature	Sample	Inhibition zone diameter (mm)			
		E. coli	*B.cereus*	*L. monocytogenes*	*Salmonella* spp.
68°C /10min	FME1.7	3.6 (90)	8.0 (100)	8.0 (100)	7.5 (93.8)
	CS3.7	8.0 (100)	10.5 (87.5)	6.0 (100)	12.0 (100)
68°C/20min	FME1.7	3.6 (90)	7.6 (95)	8.0 (100)	7.5 (93.8)
	CS3.7	7.0 (87.5)	9.0 (75)	5.1 (85)	10.8 (90)
100°C/10min	FME1.7	2.4 (60)	5.0 (62.5)	5.3 (66.6)	3.0 (37.5)
	CS3.7	5.0 (62.5)	6.0 (50)	3.6 (60)	7.2 (60)
100°C/20min	FME1.7	0.8 (20)	3.0 (37.5)	3.3 (41.6)	1.6 (20)
	CS3.7	4.0 (50)	4.5 (37.5)	1.5 (25)	3.0 (25)
121°C/15min	FME1.7	0.0	0.0	0.0	0.0
	CS3.7	0.0	0.0	0.0	0.0
Concentrated peptides	FME1.7	4.0	8.0	8.0	8.0
	CS3.7	8.0	12.0	6.0	12.0

Note: Values in parentheses represent retention of antimicrobial activity (in %)



(a)



(b)

Fig 3. Antimicrobial activity of concentrated crude bacteriocin of FME1.7 (a) and CS3.7 (b) to pathogenic bacteria with a pH range of 2-9 (Original: crude peptides)

4. CONCLUSIONS

The results of this study indicate that peptides produced by isolated lactic acid bacteria strains FME1.7 and CS.3.7 had a wide range of antimicrobial activity against indicator bacteria, including *E. coli*, *B. cereus*, *L. monocytogenes*, and *Salmonella* spp. As such, these strains are very promising to produce peptides and then safely apply them in food preservation

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