Articles for

ORAL PRESENTATIONS

SESSION 2-5

Microbiology

Sym'Previus :
Probabilistic approach
to simulate bacterial growth in food

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Type of presentation. *ORAL*

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Abstract. *Sym'Previus is a decision making tool based on predictive microbiology.*

Sym'Previus is a collection of tools for food safety inspections designed for food sector businesses to help: Strengthen HACCP plans, Develop new products, Better understand and quantify microbial behaviour, Determine shelf lives and improve food safety.

Sym'Previus describes and simulates microbial growth/destruction in food. Sym'Previus models parameters with biological significance. Sym'Previus takes into account batch or intra-species variability in a given food.

Keywords. Predictive microbiology, microbial behavior in food, food product shelf-life, food product safety, decision making tool, microbial growth, new product development

High pressure treatment of dehydrated products

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Abstract. Decontamination of dried food products is still a challenge. Produced most of the time in Southern countries, spices are raw products highly exposed to contamination due to their manufacturing conditions. Most of the current decontamination processes go through a hydration phase followed by a drying phase, such as bleaching or steam treatment. These treatments are expensive and energy consuming. Ionization, another well known process, is efficient but raises concerns among consumers.

High hydrostatic pressure for mild treatment of liquid products is already used for fruit juices or other hydrated products and attributes high organoleptic properties. Gases under pressure such as carbon dioxide are widely studied, but require most of the time water, very long residence time and heating of the product to kill most resistant forms. Recently, it has been proven that dried microorganisms like yeasts could be inactivated by more than 6 log of destruction by high isostatic gas pressure. The major benefit of this process is the use of inert gases such as nitrogen, which, in theory, does not affect sensitive molecules. Latest experiments show that spores of bacteria and fungi can also be inactivated by this process reaching more than 2 log of inactivation. The efficiency of this treatment seems to be governed by different parameters: nature of the gas, level of pressure, holding time, release kinetic of the gas and level of hydration of the microorganisms. The lower the hydration is, the higher the destruction. The process is separated into three phases: first, the pressure is raised to a certain level (between 150 and 500 MPa), then a maintenance time permits the sorption of the gases, and finally, the gas is quickly released from the matrix (at about 75 $MPa.s^{-1}$). This last step is very important for the destruction; if the gas is released slowly, dried microorganisms can support very high levels of pressure without any damage. An avenue to explain the destruction of microorganisms could be in the sorption of the gases in different compartments of the cells followed by the rapid desorption of gases, causing an alteration of the membrane integrity. The above mentioned mechanisms are not yet well understood. The study of the effects of these treatments on macromolecules could offer additional explanations, and permit the development of new athermal decontamination technologies.

Keywords. Bacterial spore, high pressure, gas, inactivation, dried products

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Evaluation of the role of water content on bacterial spores heat inactivation

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Abstract. *Bacterial spores are formed when conditions for growth are unfavorable. Spores are metabolically dormant and resistant to a wide range of environmental conditions including wet and dry heat, UV and gamma radiation, extreme desiccation, and oxidizing agents. Multiple factors are involved in spore resistance properties, most related to the structure of the spore including the relative dehydration of spore core. The purpose of this study was to investigate the role of the water content of Bacillus subtilis spores in their heat inactivation.*
Bacillus subtilis spores were equilibrated at different water activities (a_w 0.1 to 0.5) and Differential Scanning Calorimetry (DSC) was used to simulate a heat treatment under controlled conditions of linearly increasing temperature (25 to 220°C). Aluminium pans with different pressure resistances (0.1 and 0.5 MPa) were used in order to allow water to evaporate at different temperatures. After the treatment, spore viability was assessed by plate count. The thermally induced transitions were determined and the relationship between the evaporation of water and cell death was evaluated.

It was observed that even spores with the lowest water activities presented at least one peak related to water evaporation in the thermogram. This result demonstrates that there is water present in the spore protoplast even when the a_w is 0.1. When they were submitted to a second heating the thermogram did not exhibit any peaks. The inactivation, after the first heating, was higher in the most resistant pan, which withstood a pressure build up of 0.5 MPa before the evaporation of water. For both pans, the spores with higher water activity were less resistant to inactivation. Therefore, this study indicates that it is possible to maximize inactivation if the heat treatment is applied before the evaporation of water.

Keywords. Bacterial spores, *Bacillus subtilis*, DSC, water content.

Biopreservation, a New Hurdle Technology to Improve Safety and Quality of Seafood Products

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Abstract. The biopreservation technology consists in preventing growth of unwanted microorganisms in food by using added selected protective microflora. Three lactic acid bacteria strains selected for their inhibition properties and their ability to grow at low storage temperatures have been added in raw salmon fillets and cooked peeled shrimp packed under modified atmosphere, in comparison with a commercial bacterial strain. For each product, microbiological analyses and sensory evaluations were performed during the chilled storage to determine the effect of the protective flora to prevent the spoilage activities. *Leuconostoc gelidum* EU2247 and *Lactococcus piscium* EU2229 allowed to improve the sensory shelf-life of raw salmon and cooked shrimp respectively by comparison to the non-inoculated control, without any adverse effects suggesting that biopreservation technology is promising for such applications.

Keywords. Lactic acid bacteria, salmon, shrimp, inhibition, spoilage, sensory analysis, modified atmosphere packaging.

Introduction

Lightly preserved seafood products like fresh fish fillets packed under modified atmosphere, cold smoked fish, carpaccios or cooked shrimp are very sensitive to the growth of pathogenic and spoiling bacteria, due to their physico-chemical characteristics. To improve their shelf-life, chemical preservatives and modified atmosphere packaging (MAP) or a combination of these techniques are generally applied to seafood products. However the growth of undesirable microflora is not always totally prevented and the interest for alternative mild technology such as biopreservation has increased in the last years.

This technology consists in adding in food a selected protective microflora for which the inhibition properties have been demonstrated towards pathogenic and/or spoilage bacteria without changing the organoleptic characteristics of the products. Lactic acid bacteria (LAB) have been widely studied for these applications in fermented products. Those last decades, presence of LAB in lightly preserved seafood has been highlighted and selection of protective cultures has intensified. However, an application in seafood still remains a challenge as the LAB must have the capacity to grow in fish matrix poor in carbohydrate, at low temperature and should not change the delicate flavour of marine products.

In a previous study, the use of *Carnobacterium divergens* V41, a bacteriocin-producing strain, allowed keeping the level of *Listeria monocytogenes* below 50 CFU/g in cold-smoked salmon during 28 days of vacuum storage at 4 and 8°C (Brillet, Pilet, Prévost, Bouttefroy, & Leroi, 2004). Others studies showed that in the presence of the bacteriocinogenic strains *C. maltaromaticum* CS526 (Yamazaki, Suzuky, Kawai, Inoue, & Montville, 2003) or *C. divergens* M35 (Tahiri, Desbiens, Kheadr, Lacroix, & Fliss, 2009), the population of *L. monocytogenes* in cold-smoked salmon decreased after 1 to 3 weeks at 4°C. Non producing-bacteriocin strains can also be used as protective culture against pathogenic bacteria (Nilsson, Hansen, Garrido, Buchrieser, Glaser, Knochel, *et al.*, 2005; Matamoros *et al.*, 2009a). In this case, the inhibition mechanisms are not always known but could be explained by a nutrient competition or a pH decrease due to the production of lactic acid.

The use of the biopreservation technology to prevent spoilage is more complex since many bacterial species, both Gram negative and positive, are involved in the off-odors production (Gram & Dalgaard, 2002). Matamoros, Leroi, Cardinal, Gigout, Kasbi Chadli, Cornet *et al.* (2009a) have shown the capacities of psychrotrophic strains of *Lactococcus piscium* and *Leuconostoc gelidum* to significantly improve the sensory quality of cold-smoked salmon and cooked peeled shrimp however the effect was very variable depending on strains and products. For that reason, it is interesting to select the best strain adapted to each product taking into account the variability of the raw material and to extend the application to other marine products such as raw salmon fillets stored under MAP. In this work, two strains of psychrotrophic *Ln. gelidum*, one strain of *Lc. piscium* and one commercial starter LLO have been tested on MAP cooked peeled shrimp and fresh salmon fillets, two increasing products on the European market.

Materials and Methods

Bacterial Strains and Cultures Conditions

Ln. gelidum strains (EU2247 and EU2262) and *Lc. piscium* (EU2229) were isolated from commercial seafood products by Matamoros, Pilet, Gigout, Prévost & Leroi (2009b). The starter LLO is a commercial strain used as a bioprotective agent in seafood products (Biocéane,

Nantes, France). The strains were stored at -80°C in their growth medium with 10% (v/v) sterile glycerol. All strains were subcultured for 48h at 15°C and cultured for 18h at 20°C in Elliker broth (Biokar Diagnostics, Beauvais, France).

Antibacterial Activity of **Leuconostoc gelidum** *and* **Lactococcus piscium** *in seafood products (challenge tests)*

The following products were provided by industrial partners : two batches of commercial fresh salmon fillets (*Salmo salar* farmed in Norway, portions 140 g) which differ with the processing plant (factories A and B) and the quality of fillets (Red Label and Norway Superior) and two batches of commercial cooked peeled shrimp (*Penaeus vannamei*, 71/90 size) which differ with the origin of farming place (Colombia and Thailand) and the presence/absence of sulphites additives. Products were freshly processed and inoculated within the following hours.

Each batch of product was divided in five sub-batches inoculated with : (1) *Ln. gelidum* EU2247, (2) *Ln. gelidum* EU2262, (3) *Lc. piscium* EU2229, (4) the commercial starter LLO and (5) non-inoculated (control). For this, an appropriate dilution was aseptically sprayed on each side of raw salmon fillets (2% v/w) and cooked peeled shrimp (5% v/w) with a trigger sprayer ("Diamant 0.6L RCM", Nantes, France). The initial desired level of LAB in the flesh was 10^6 CFU g^{-1}. Immediately after inoculation, the salmon fillets and portions of shrimp (125 g) were packed in punnets under modified atmosphere (50% N_2 - 50% CO_2; Multivac T252, Wolfertschwenden, Germany) with a product/gas ratio of approximately 2/1. The salmon fillets and portions of shrimp were stored at 2°C (3 days and 7 days respectively) and then at 8°C according to the industrial recommendations until sensory rejection of the products.

Microbiological Analysis

Microbiological analyses were performed at day 0 and day of sensory spoilage. Salmon and shrimp samples (50 g taken from 3 punnets) were homogenized and diluted in 200 ml chilled physiological saline containing 0.85% (w/v) NaCl and 0.1% (w/v) tryptone (Biokar Diagnostics) for 2 min in a stomacher (Lab. Blender, London, UK). After 20 min at room temperature, the homogenate was 10-fold serially diluted in physiological saline, and 0.1 ml of each appropriate dilution was spread-plated in duplicate. For inoculated samples, total LAB were enumerated on Elliker or MRS medium. Plates were incubated at 12°C for 5-6 days in anaerobic conditions. For controls, total psychrotrophic counts were enumerated on Long & Hammer medium at 15°C, as described by Cardinal, Gunnlaugsdottir, Bjoernevik, Ouisse, Vallet, & Leroi (2004).

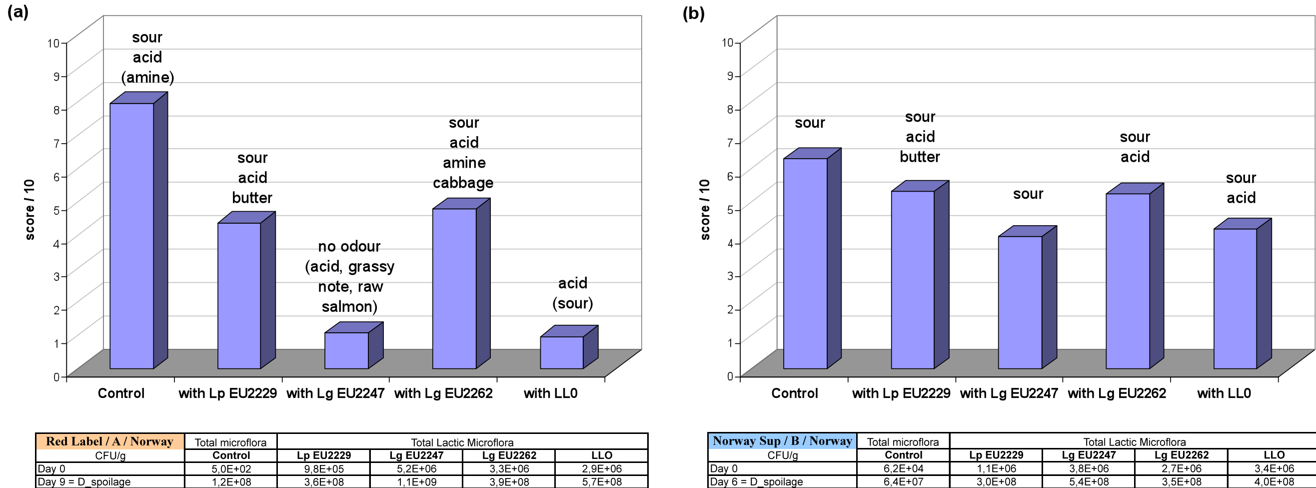
Sensory Analysis

After six days of storage, sensory analyses were performed daily on non-inoculated products (controls), the objective being to determine the sensory rejection time of controls. Nine regularly trained people from an internal panel (IFREMER) classified the controls into the following three classes defined by spoilage level on the basis of odor : not spoiled, lightly spoiled, strongly spoiled. Two major odor descriptors were also given by the trained panel. At the sensory rejection time, determined when 50% of the panelists classified controls in the "strong spoilage" level, all the samples of the respective batch inoculated with protective LAB were analyzed by the panel as described for the controls (spoilage level, two odor descriptors). Additionally the panelist scored the global off-odor perception on an unstructured line scale from zero (low intensity) to ten (high intensity).

Results and Discussion

Challenge tests in raw salmon fillets

Microbiological and sensory analyses were performed on two batches of raw salmon fillets inoculated with protective cultures during chilled storage (Figure 1). The spoilage of the Red Label control was noted after nine days of storage, and after six days for the Norway Superior control.



Red Label / A / Norway	Total microflora	Total Lactic Microflora			
CFU/g	**Control**	**Lp EU2229**	**Lg EU2247**	**Lg EU2262**	**LLO**
Day 0	5,0E+02	9,8E+05	5,2E+06	3,3E+06	2,9E+06
Day 9 = D_spoilage	1,2E+08	3,6E+08	1,1E+09	3,9E+08	5,7E+08

Norway Sup / B / Norway	Total microflora	Total Lactic Microflora			
CFU/g	**Control**	**Lp EU2229**	**Lg EU2247**	**Lg EU2262**	**LLO**
Day 0	6,2E+04	1,1E+06	3,8E+06	2,7E+06	3,4E+06
Day 6 = D_spoilage	6,4E+07	3,0E+08	5,4E+08	3,5E+08	4,0E+08

Figure 1. Sensory analysis of raw salmon fillets at the spoilage day and microbiological analysis: (a) Quality "Red Label", factory A, Norway (Day 9); (b) Quality "Norway Superior", factory B, Norway (Day 6). Note 0 : not spoiled, Note 10 : strongly spoiled.

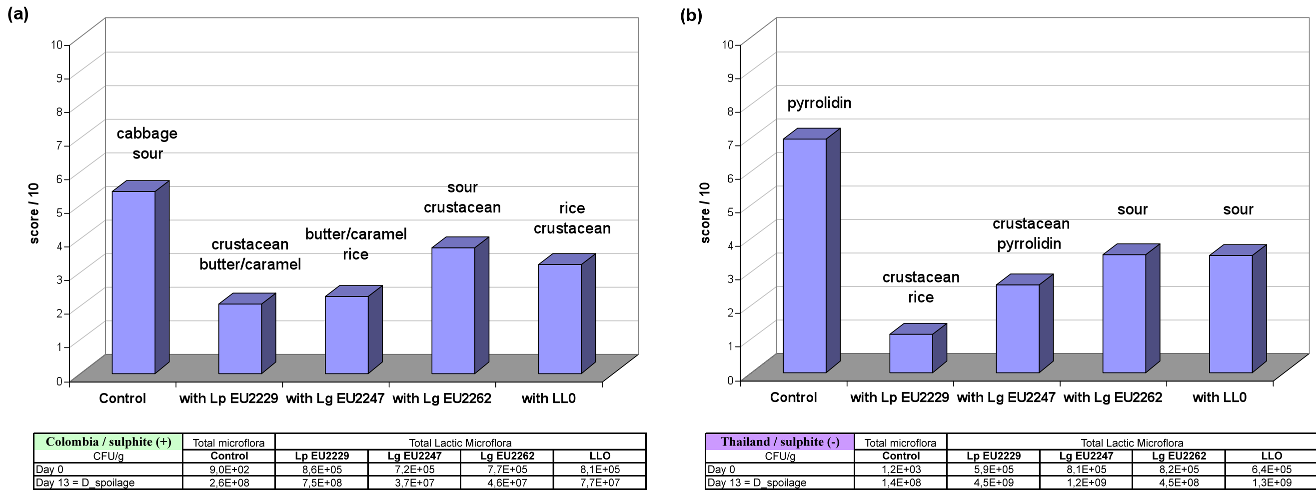
The results presented in Figure 1 show that all the protective cultures were inoculated at the desired level and grew easily in this product reaching 3.10^8 to 10^9 CFU g^{-1}. The addition of these protective cultures on raw salmon fillets led to a reduction of off-odors. The best results were obtained with the Red Label samples inoculated with *Ln. gelidum* EU2247 showing no off-odors (note <1) and characterized by freshness descriptors whereas the control was considered as strongly spoiled (score nearly 8/10) with strong sour and acid off-odors. The commercial starter LLO showed results as good as *Ln. gelidum* EU2247 with samples scored <1 on the spoilage scale (no significant difference with the Duncan test at the 0.05 level of probability). Additional sensory tests performed after the sensory rejection time of the control (data not shown) indicate that those strains could also increase the shelf-life but more analysis should be required to precisely determine the extension time.

In the Norway Superior samples, none of the protective cultures tested was able to prevent efficiently the spoilage although better results were again observed with *Ln. gelidum* EU2247 and starter LLO (score around 3-4 versus 6 for control). It is important to note that the initial total psychrotrophic microflora level of the control batch was clearly higher than the Red Label, leading to the rapid off-odors detection after processing. As previously described by Brillet, Pilet, Prévost, Cardinal & Leroi (2005), the biopreservation strategy can not improve quality nor safety in products of low hygienic quality but must be considered as an interesting extra hurdle technology in products of high hygienic quality. The better efficacy of *Ln. gelidum* EU2247 could be explained by the production of a bacteriocin-like component active against *Lactobacillus farciminis* which is sometime involved in MAP fish spoilage. On the other hand, this compound

was not active against other major spoiling microorganisms such as *Brochothrix thermosphacta, Shewanella putrefaciens, Pseudomonas* sp. and *Serratia liquefaciens* (Matamoros, Pilet, Gigout, Prévost & Leroi, 2009b) suggesting that other mechanisms may be involved.

Challenge tests in cooked peeled shrimp

Results of microbiological and sensory analyses for the two batches of cooked peeled shrimp inoculated with protective cultures are presented in Figure 2. The first batch (a in figure 2) was farmed in Colombia and sulphites were added after slaughtering. The second one (b) was farmed in Thailand and contained no sulphite. The spoilage was recorded at day 13 for the two batches.



Colombia / sulphite (+)	Total microflora	Total Lactic Microflora			
CFU/g	**Control**	**Lp EU2229**	**Lg EU2247**	**Lg EU2262**	**LLO**
Day 0	9,0E+02	8,6E+05	7,2E+05	7,7E+05	8,1E+05
Day 13 = D_spoilage	2,6E+08	7,5E+08	3,7E+07	4,6E+07	7,7E+07

Thailand / sulphite (-)	Total microflora	Total Lactic Microflora			
CFU/g	**Control**	**Lp EU2229**	**Lg EU2247**	**Lg EU2262**	**LLO**
Day 0	1,2E+03	5,9E+05	8,1E+05	8,2E+05	6,4E+05
Day 13 = D_spoilage	1,4E+08	4,5E+09	1,2E+09	4,5E+08	1,3E+09

Figure 2. Sensory analysis of cooked peeled shrimp at the spoilage day (day 13) and microbiological analysis: (a) Colombia, presence of sulphites; (b) Thailand, absence of sulphites. Note 0 : not spoiled, Note 10 : strongly spoiled.

All the LAB strains developed well in shrimp although higher counts (approx. 1-2 log CFU g^{-1}) were observed in the batch without sulphites (Figure 2). The four protective cultures significantly improved the sensory quality of the products but in all cases the best results were obtained with *Lc. piscium* EU2229 : at day 13, the score of the samples inoculated with this strain was below 2 and 1 in batches (a) and (b) respectively and the main odors descriptor were closed to those of fresh products (crustacean, rice). The others strains were less effective to limit the emergence of off-odors. It is noteworthy that *Lc. piscium* EU2229 grew better in shrimp that the other strains, and this is particularly the case for product with sulphites. Fall, Leroi, Cardinal, Chevalier & Pilet (2010) showed that the improvement of the sensory quality of cooked peeled shrimp by another *Lc. piscium* strain could be linked to the inhibition of the specific spoiling bacteria *B. thermosphacta.*

Conclusions

The results obtained in this study have demonstrated the efficiency of biopreservation to improve the sensory quality of seafood products during their storage. *Ln. gelidum* EU2247 and *Lc. piscium* EU2229 are promising agents for a biopreservation strategy in raw salmon fillets and cooked peeled shrimp respectively and could be proposed, as the LLO starter, as new protective culture for seafood products.

Acknowledgements

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Construction of RR Gene Mutants of *Lactobacillus paracasei* HD 1.7 and the Impact on the Production of Paracin1.7

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Abstract. [**Objective**] A suicide plasmid pUC18-prcR-tet was constructed to knock out *prcR* gene in *Lactobacillus paracasei* HD1.7, which was imported to *Lactobacillus paracasei* HD1.7. The knockout mutant strains of *prcR* gene were obtained and their situations of antimicrobial were observed. [**Methods**] On the basis of plasmid pUC18, two components were integrated, which were *prcR* gene from *Lactobacillus paracasei* HD1.7 and tetracycline resistance gene as tetracycline resistant marker. After enzyme digestion and ligation, the suicide plasmid pUC18-prcR-tet was constructed. In this plasmid, both upstream and downstream of tetracycline resistance gene have fragments (about 400 bp) of *prcR* as a way of homologous recombination. The pUC18-prcR-tet was imported to the *L.paracasei* HD1.7 by electrotransformation and the situation of the knockout mutant strains of *prcR* gene was observed by antimicrobial test. **[Results]** The mutant strain of *prcR* was accessed by the gene knockout technology. By antimicrobial test, that degree of antimicrobial of mutant strains was weaker than that of the original strains was discovered, which indicated that genes that affected antimicrobial peptides were changed, which affected production of antimicrobial peptides. [**Conclusions**] The suicide plasmid pUC18-prcR-tet was successfully constructed, which was used to knock out *prcR* gene in *Lactobacillus paracasei* HD1.7. By electrotransformation, the mechanism of the regulation gene *prcR* was explored, which affects the Quorum-sensing system. The research can be a potential basis for the exploration of lactic acid bacteria quorum-sensing system and the mechanism of related regulation genes of antimicrobial peptide and so on.

Keywords. Quorum-sensing, *Lactobacillus paracasei* HD1.7, suicide plasmid, the gene knockout technology, *prcR*, electrotransformation

Introduction

Lactobacillus paracasei HD1.7 was isolated from Chinese sauerkraut juice in 2003. *Lactobacillus paracasei* HD1.7 was the Gram-positive bacteria and belonged in the low GC group (De Vos, Kleerebezem, & Kuipers, 1997)); the fermentation broth of *Lactobacillus paracasei* HD1.7 contained a kind of peptide Paracin1.7 that could inhibit the growth of several Gram-positive bacteria, Gram-negative bacteria and yeast (Ge, et al., 2009). The process of production of Paracin1.7 had the characteristics of Quorum-sensing. NAKAYAMA AKKERMANS & De Vos (2003) got a series of genes (including histidine protein kinase gene *prcK* and response regulator *prcR*) from *L.paracasei* E93490, which were defined the putitive quorum-sensing components, and predicted that the signaling molecule of *L.paracasei* E93490 might have antibacterial activity, but they did not do further research about functions of these genes in Quorum Sensing and whether or not the output of antimicrobial peptides related to Quorum Sensing. Accordingly, the similar condition might be existed in *L.paracasei* HD1.7. Therefore, it is of great significance for us to study fuctions of these related genes in Quorum Sensing and the process of producing antimicrobial peptides by molecular methods.

In this study, to construct the suicide plasmid pUC18-prcR-tet applied to the insertional inactivation-based gene knockout technology, plasmid pUC18 was used as the backbone plasmid, and two components were integrated into the plasmid pUC18, which were *prcR* gene used for homologous recombination and tetracycline resistance gene as tetracycline resistant marker. The pUC18-prcR-tet was imported to the *L.paracasei* HD1.7 by electrotransformation and knockout mutant strains of *prcR* gene were obtained. The growth of colonies on the tetracycline resistant plate indicated that homologous recombination between the suicide plasmid pUC18-prcR-tet and the host cell had accomplished. Antimicrobial test was used to show the effect of deletion of *prcR* on the output of antimicrobial peptides. That provided the basis for further exploring substantial functions of these related genes in Quorum Sensing of *L.paracasei*.

Materials and Methods

Bacterial strains, growth media, and culture conditions

Bacterial strains used were *Lactobacillus paracasei* HD1.7, *Escherichia coli* DH5α, *Bacillus subtilis*, and *Staphyloccocus aureus*. *Lactobacillus paracasei* HD1.7 strains were grown in MRS broth and were incubated at 30°C. Strains of *Escherichia coli* DH5α, *Bacillus subtilis*, and *Staphyloccocus aureus* were grown in Luria-Bertani broth and were incubated at 37°C.

Primers

Primers were synthesized by Invitrogen. The sequences of the primers were shown in Table. 1.

Table 1 Primer sequences, templates and their restriction endonuclease sites

Gene	Primer sequences (5'→3')	oriteiation	templates	restriction endonuclease sites
tet	CCGCAATTGTCTCATGTTTGACAGCTT	+	HD1.7 genome	*Mun* I
	GTCCAATTG*TAATAG*ATATGTTCTGCCAAGGGT	—	HD1.7 genome	*Mun* I
prcR-1	TTAGATTACACATCCACACCG	+	HD1.7 genome	
	CTGCCAGGTTATGGGAAT	—	HD1.7 genome	

prcR-2	ATGACNAAYCAYCARAC	+	*prcR*-1	
	TGCCAGGTTATGGGAAT	—	*prcR*-1	

Notes: In the orientation of primers, "+" represents the upstream primer and "-" represents the downstream primer; the underlined part is the sequence of restriction endonuclease site.

Construction of the recombinant plasmid pUC18-prcR-tet

Plasmid pUC18-prcR was constructed by inserting a 907 bp *Eco*R I fragment containing *prcR* from pGM-T-R into the *Eco*R I gap of plasmid pUC18. Plasmid pUC18-prcR-tet was constructed by inserting a 1.4 kbp *Mun* I fragment containing *tet* from pMD18-T-tet into the *Mun* I gap of pUC18-prcR. The recombinant plasmid pUC18-prcR-tet was then transformed into *E. coli* competent cells. The plasmid pUC18-prcR-tet was isolated from *E. coli* cells and restriction enzymes analysis and PCR identification were used to investigate whether the recombinant plasmid pUC18-prcR-tet met the need of the experimental design.

Importing the recombinant plasmid pUC18-prcR-tet into Lactobacillus paracasei HD1.7 by electrotransformation

2 μl of the recombinant plasmid pUC18-prcR-tet and 40 μl of *Lactobacillus paracasei* HD1.7 competent cells were mixed together well on the ice. The mixture was transferred into precooling 0.2 cm biorad and then the biorad was put on the ice for 5 min. Then the electroporation generator discharged the electric pulse in the 1.8 kV/cm of field strength. After electric shock, 400 μl of MRS broth was added into the biorad immediately and then the mixture was slightly mixed well. The mixture was transferred into 1.5 ml centrifuge tube and was incubated at 30°C under static condition for 5 h. The culture solution was diluted with 100 times. Then 100 μl of the diluent was coated on MRS screening plates with tetracycline and these plates were incubated at 30°C for 7 h.

Screening and PCR identification of the knockout mutant strains of prcR gene

The inoculating loops were used to pick up single colonies on the MRS resistant plates with 5 μg/ml (final concentration) of tetracycline. And these single colonies were continuously passage cultured on the MRS resistant plates of tetracycline for 3~4 times. Then the stability of the knockout mutant strains was observed. At the same time, genomic DNA was isolated from the knockout mutant strains with high stability. PCR was used to identify whether homologous recombination had accomplished, which was based on the theoretical design.

Antimicrobial condition of fermentation broth of knockout mutant strains

Cylinder plate method (Shen, Fan & Li, 1999) was applied to antimicrobial tests, in which indicator bacteria were *Escherichia coli*, *Bacillus subtilis*, and *Staphyloccocus aureus*, respectively.

Results

PCR amplification of three components of constructing the recombinant plasmid pUC18-prcR-tet

The results of the sequencing of *tet*, *prcR*-1 and *prcR*-2 were analyzed. The result showed that *tet*, *prcR*-1 and *prcR*-2 were successfully cloned (Fig. 1).

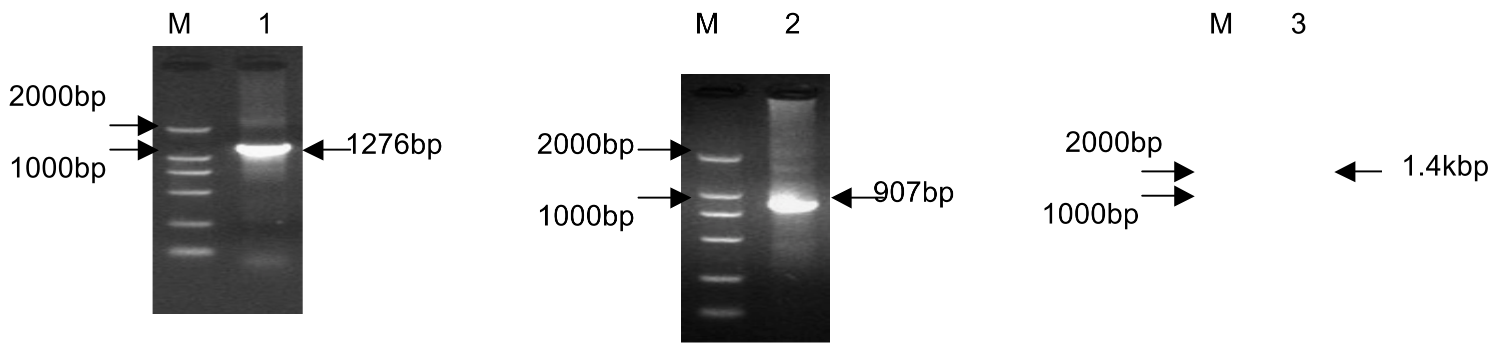

Fig. 1 PCR results of *tet*, *prcR*-1, *prcR*-2

M, DNA Marker; Lane 1, PCR product of *prcR*-1 (1276bp); Lane 2, PCR product of *prcR*-2 (907bp); Lane 3, PCR product of *tet* (1.4kbp)

PCR identification and restriction enzymes analysis of the recombinant plasmid pUC18-prcR-tet

The pUC18-prcR-tet was isolated from three *Escherichia coli* DH5α transformants. For PCR identification of pUC18-prcR-tet, pUC18-prcR-tet and the primers PrcR-2-up and PrcR-2-down were used as template and the primers, respectively. It is the same with pUC18-prcR-tet and the primers Tet-up and Tet-down. The results of agarose gel electrophoresis were shown in Fig. 2. For the first PCR, the result showed that an about 2.3 kbp DNA fragment was amplified. The length of this fragment accorded with the total length of *prcR* (907 bp) and *tet* (1.4 kbp) both cloned successfully before. For the second PCR, the result showed that an about 1.4 kbp DNA fragment is amplified. The length of this fragment accorded with the length of *tet*.

For restriction enzymes analysis of pUC18-prcR-tet, pUC18-prcR-tet was digested with *Bam*H I and *Cla* I respectively to judge the relative direction between *prcR* and *tet* in the plasmid. The need of the experimental design was that the reading directions of *prcR* and *tet* were opposite in order to prevent the protein produced was not the target protein due to continuously reading of *prcR* and *tet,*. According to these results and analysis, the recombinant plasmid pUC18-prcR-tet was constructed successfully.

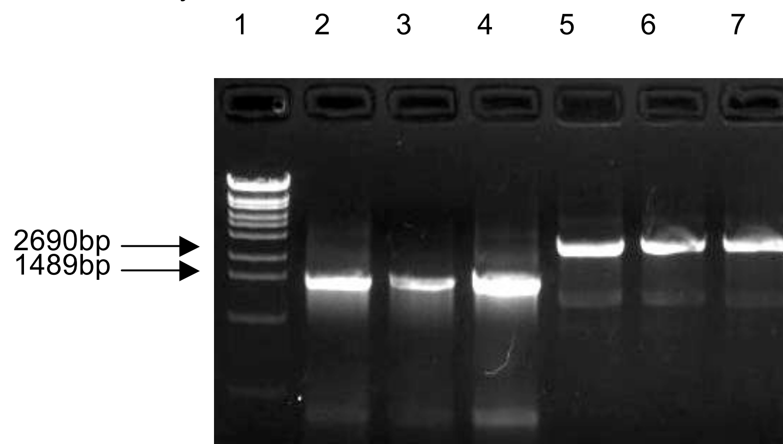

Fig. 2 PCR identification results of the recombinant plasmid pUC18-prcR-tet

1: DNA Marker λ-EcoT14 I digest; 2-4: PCR product of No.1-3 strains with Tet-up and Tet-down

primers; 5-7: PCR product of No.1-3 strains with prcR-2-up and prcR-2-down primers

Screening and PCR identification of the knockout mutant strains of prcR gene

Two single colonies growing well on the MRS resistant plates with 5 μg/ml (final concentration) of tetracycline were selected as knockout mutant strains of *prcR* that are called GXJ-M-1 and GXJ-M-2, respectively. The original strain *Lactobacillus paracasei* HD1.7 was used in the negative control experiment. The results of agarose gel electrophoresis were shown in Fig. 3. The results showed that there was no DNA fragment that was amplified in the negative control experiment, but an about 500 bp DNA fragment definitely that was amplified in both GXJ-M-1 and GXJ-M-2. However, the concentration of this amplified products were low. This might be due to that *tet* of plasmid pUC18-prcR-tet was partly integrated into genomic DNA of *L.paracasei* HD1.7.

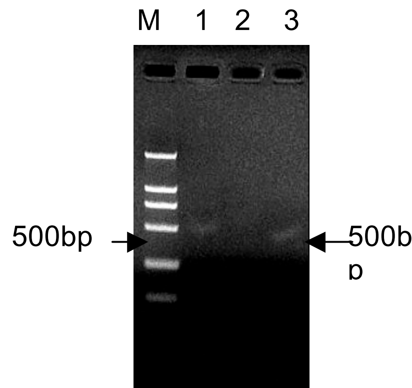

Fig. 3 The dectection of knockout mutant strains by PCR

M, DNA Marker; Lane 1, PCR result of *tet* using GXJ-M-1 gDNA as template; Lane 2, PCR result of *tet* using the original strain gDNA as template; Lane 3, PCR result of *tet* using GXJ-M-2 gDNA as template

Antimicrobial condition of fermentation broth of knockout mutant strains

Antimicrobial results of fermentation broth of knockout mutant strains were shown in Table 2. Table 2 showed that the inhibition degree of mutant strains to *Bacillus subtilis* was generally the same with that of original strains to *Bacillus subtilis*; the inhibition degree of mutant strains to *Escherichia coli* was weaker than that of original strains to *Escherichia coli*, which showed that the output of antimicrobial peptides produced by mutant strains was less than that of antimicrobial peptides produced by original strains; by comparing the inhibition degree of mutant strains to *Staphyloccocus aureus* with that of original strains to *Staphyloccocus aureus*, we could infer that the output of antimicrobial peptides produced by mutant strains was also less than that of antimicrobial peptides produced by original strains. *Bacillus subtilis* was not sensitive to the variation of the concentration of antimicrobial peptides, because it had the spore and stronger tolerance to inhibitors than *Escherichia coli* and *Staphyloccocus aureus*.

Table 2 Titer of antimicrobial peptide

No.	1	2	3
Titer (AU/ml) (*B.sub*)	2013.17	1527.50	1876.55
Titer (AU/ml) (*E.coli*)	268.97	191.19	153.30

Titer (AU/ml) (*S.aureus*)	181.83	43.71	131.87

Conclusions

Gene knockout vector was the tool of the gene knockout technology. So the effect of its construction was greatly significant. This study chose the plasmid pUC18 as the backbone plasmid, which was the narrow host range plasmid and had one single copy and even no copy in G^+ bacteria. Because pUC18 could not autonomously replicate in G^+ bacteria, the recombinant plasmid pUC18-prcR-tet was integrated into genomic DNA of *L.paracasei* HD1.7 and replicated with the genomic DNA of *L.paracasei* HD1.7, only according to the fact that homologous recombination between *prcR* in the plasmid pUC18-prcR-tet and genome of *L.paracasei* HD1.7 had accomplished. After homologous recombination, *tet* in pUC18-prcR-tet was brought in genomic DNA of *L.paracasei* HD1.7, which made recombinant strains resistant to tetracycline, and the recombinant strains showed the expected pattern for a double cross-over.

The successful construction of the recombinant plasmid pUC18-prcR-tet had some influence on the output of antimicrobial peptides of *L.paracasei* HD1.7, which provided the basis for further exploring substantial functions of these related genes in Quorum Sensing of *L.paracasei* HD1.7.

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