Session at SUT (Thailand) – Fourth year of food and biochemistry engineering at INSA (France) – 2007

Isolation, Identification and characterization of lactic acid bacterial strains and detection of anti-microbial activity against *Staphylococcus aereus*, in traditional Thai fermented fruits and vegetables.

Marcadet Virginie - vmarcade@etud.insa-toulouse.fr

Edited 4 September 2007

Abstract

Lactic acid bacteria were isolated from various Thai fermented vegetables and characterized morphologically. From five different food products, 51 bacterial strains were isolated and an agar well diffusion assay (AWDA) was performed on broth culture and supernatant for each isolated strain, for detection of antagonistic activity against *Staphylococcus aureus*. After characterization, 16 isolated bacterial strains were conserved as putative lactic acid bacteria. Six of these isolated stains were then tested with API gallery and identified as effective lactic acid bacteria.

Keywords ; Lactic acid bacteria ; *Staphylococcus aureus* ; Antimicrobial activity ; Fermented vegetables ; Fermented food

Introduction:

Thailand has a strong tradition for the production and consumption of fermented foods. Fermented vegetables such as Madan pickles (fruits), bamboo shoots, fermented cabbage, fermented lettuce or also fermented ginger are some of the products currently found. Indeed fermentation has been used for a long time ago in Thailand and many other countries, as a biopreservation technique to food products. Lactic acid bacteria (LAB) have been isolated yet from many fermented food products and were found to be responsible of their long biopreservation ability.

Lactic acid bacteria are gram-positive, catalase negative, anaerobic facultative and non-sporulating microaerophilic bacteria whose main fermentation product from carbohydrates is lactate.

In fact, in fermented food industry, lactic acid bacteria are used as starter cultures in fermentation process. Substances produced by these bacteria do not only contribute to flavor and aroma development but also have inhibitory activity against spoilage bacteria and food-borne pathogens. The antibacterial activity of lactic acid bacteria is due to the production of organic acids, hydrogen peroxide and bacteriocins.

Responsible Person: Asst. Prof. Piyasan Goodluck

Project coordinators : Asst. Prof. Jamthorn Kanchanatawee

The aim of the project conducted by INRA (Institut National de la Recherche Agronomique), France and SUT (Suranaree University of Technology), Thailand, is to isolate and identify lactic acid bacterial strains from traditional Thai fermented food and characterize them in terms of growth conditions, production of antimicrobial substances and inhibitory potential against *Staphylococcus aereus*. The finality of the project would be to find new lactic acid bacterial stains with potential interest for fermented food industries.

Materials and Methods

1. Isolation of the Bacterial strains and culture conditions

Bacterial strains were isolated from different fermented vegetables available on local markets. Thai fermented vegetables used in this study included madan pickles, fermented bamboo, cabbage (2 different varieties), and garlic. The food samples were collected randomly and cut into little bits. A sample of 25 grams was mixed with 225 ml of distilled water (dilution 10^{-1}) and put into a stomacher for 15 minutes. The liquid part of the mixture was then diluted successively at 10^{-2}, 10^{-3} and 10^{-4}. Dilutions 10^{-2} to 10^{-4} were propagated in MRS-agar medium (67,15g/L), and incubated anaerobically, at 30°C for 3 to 5 days. MRS-agar medium was supplemented with carbonate calcium (5g/L) to prevent pH from getting to low. As lactic acid bacteria are anaerobic facultative, anaerobic conditions were used to select them and avoid potential contamination.

About 5 colonies were picked up from each plate with a wire loop and transferred, by streaking method, onto new plates containing fresh MRS-agar medium so as to isolate the different bacterial strains. In anaerobic conditions, many bacterial strains grew very little and within a long time. As a consequence it resulted difficult to characterize or identify them. Furthermore the risk of contamination in petri dishes was high after five days of culture. For that reason new inoculated plates were incubated aerobically, at 30°C and for 24 hours only. In aerobic conditions bacterial strains grew much better and in only 24 hours. Aerobic conditions also allowed detection of anaerobic obligate bacterial strains.

2. Preservation of isolated bacterial strains and test strain

a. Preservation for use routine

One colony from each plate (pure aerobic cultures) was picked up and transferred onto MRS-agar slants by streaking method. Slants were incubated aerobically, 24 hours at 30°C and then stored at 4°C in fridge. An example of agar slants with bacterial colonies can be observed on figure 1.

A similar method was used to preserve *Staphylococcus au*reus TISTR118 strain, used for antimicrobial activity assays (see further). The strain was grown in Nutrient broth medium at 37°C for 24 hours. Then, 20 µL of culture was spread on the surface of Nutrient-agar slants. Slants were incubated 24 hours at 37°C and then stored at 4°C in fridge.

b. Long preservation

One colony from each plate (pure aerobic cultures) was picked up and transferred into screw-capped tubes containing 10 mL of MRS-broth medium (52g/L) supplemented with 5g/L of carbonate calcium. The tubes were incubated aerobically at 30°C for 24 hours. For each bacterial strain 0,75mL of broth culture was transferred into an eppendorf tube and the same volume of sterile skim milk (10% solid) was added. Eppendorf tubes were stored at –20°C in freezer.

The same method was employed to store *Staphylococcus au*reus TISTR118 strain. The strain was grown in Nutrient broth medium at 37°C for 24 hours. Then, 0,75mL of culture was transferred into an Eppendorf tube with the same volume of sterile skim milk and stored at –20°C in freezer.

Examples of broth cultures in tubes as well as preserved culture in skim milk in Eppendorf tubes are presented respectively on figure 1 and 3.

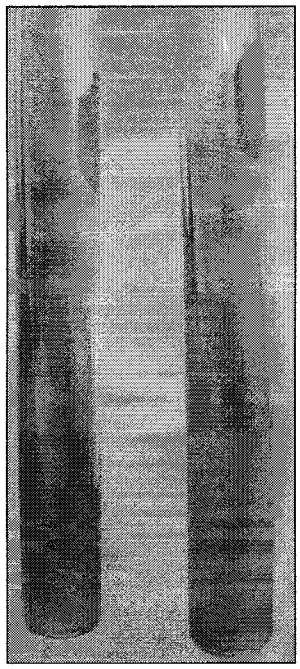

Figure 1: MRS-agar slants with bacterial colonies on surface after 24 hours of incubation at 30°C in aerobic conditions.

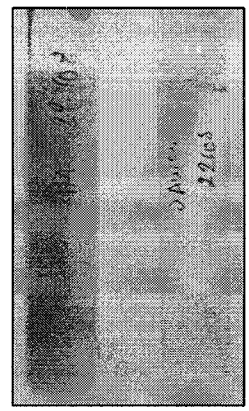

Figure 2: MRS-broth culture of strain D1 (left) and Nutrient-broth ulture of S.aureus (right).

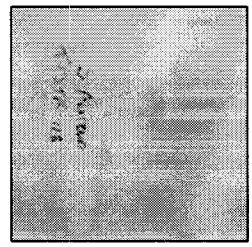

Figure 3: Preserved cultures in skim milk in Eppendorf tubes.

3. Morphological characterization and identification of the lactic acid bacteria

a. Twenty-four-hour aerobic cultures of isolated strains on MRS-agar were gram staining. One colony from each plate was picked off and diluted in a drop of distilled water on a glass slide. The smear were then heat-fixed by passing the slice through the Bunsen flame. The gram's staining method was then applied and slides were examinated with a light microscope using the oil-immersion objective. In the meantime the shape of cells was observed.

b. The isolated bacterial strains were also tested for the presence of catalase enzyme by placing a drop of hydrogen peroxide on a glass slide. Using a sterile wire loop, a small amount of growth was then picked up from a culture on MRS-agar slant and

added onto the drop of hydrogen peroxide. If bubbles appear, the organism is said to be *catalase-positive*; if not, the organism is *catalase-negative.*

c. Six Gram-positive isolated bacterial strains checked for catalase test (negative) were characterized by API CHL50 tests (Biomérieux, France) from MRS-broth cultures as described by the manufacturer. The 50 tubes of the strips were filled with the inoculated API 50 CHL Medium and all the tests were covered with mineral oil. The strips were then incubated aerobically at 30°C for 48 hours.

4. Test of antimicrobial activity

An agar well diffusion assay (AWDA) was used for detection of antagonistic activity.

Tests of antimicrobial activity were performed with *Staphylococcus au*reus TISTR118, used as an indicator strain. The bacterial strain was coagulase positive and produced DNase which are typical characteristics of pathogens strains. The strain was obtained from the culture collection of Department of Food Technology, Suranaree University of Technology (Thailand).

The strain stored at 4°C in Nutrient-agar slants, was propagated in Nutrient broth tubes and incubated aerobically at 37°C for 24 hours before use.

Composition of Nutrient medium: Peptone from meat, 5,0g/L; beef extract 3,0g/L; agar-agar, 12g/L (not present in Nutrient broth).

1mL of *Staphylococcus aureus* broth culture was inoculated into screw-capped tubes containing 10 mL of Nutrient broth medium and incubated aerobically at 37°C for 24 hours. 100 µL of prepared culture was then transferred to Mueller-Hinton-agar plates and spread to surface with a sterile swab. Plates were allowed to dry for about 15 minutes.

Four wells were formed on each plate by pressing the largest top of 200 µL sterile pipette tips on the surface of the medium. The tops of the pipette tips were then carefully removed to leave wells of 6 mm in diameter.

60 µL of culture of the putative inhibitor strains (incubated 24 hours at 30°C) were placed into the wells. Alternatively, the wells were filled with 60 µL of cell free culture supernatant obtained by centrifuging at 10 000 xg for 15 minutes at 4°C.

The plates were left at room temperature for about one hour and then incubated aerobically at 37°C. After 24 and 48 hours of incubation, the plates were subsequently examined for zones of inhibition.

Results

1. Morphological characterization

After isolation of the various bacterial strains, colonies grown on agar plates in aerobic conditions were characterized morphologically. There was not a large diversity of the morphology of colonies. Most of the colonies appeared white and fairly circular in shape. Besides it could be noticed that several bacterial strains developed a strong smelt of French mature cheese!

The photos below show a few examples of the isolated bacterial strains when grown aerobically on MRS-agar. The pictures were taken after several days of growth.

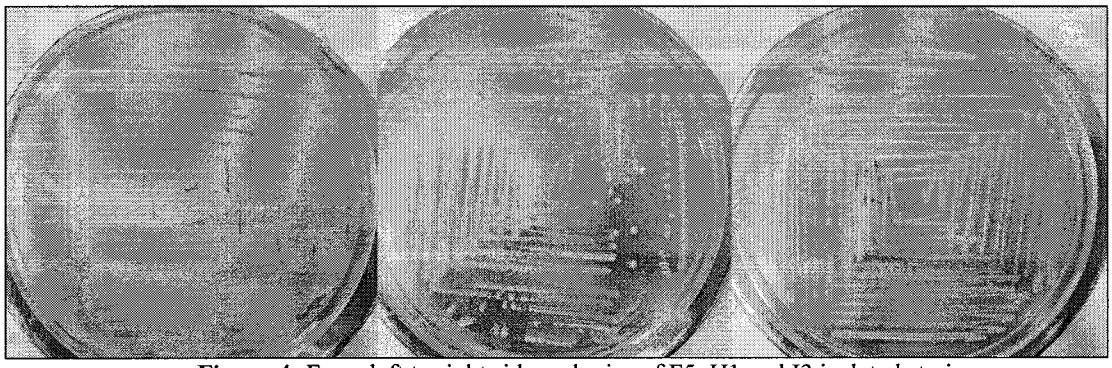

Figure 4: From left to right side: colonies of F5, H1 and I3 isolated strains.

All the bacterial strains isolated from fermented vegetables were gram-positive, except one that was thus supposed to be a contamination.

The morphological shape of cells was also observed with a light microscope while examinating slides resulting from gram staining test. Rod and coccal shaped cells could be observed on the various isolated bacterial strains.

The catalase test revealed the presence of many catalase positive isolated strains while lactic acid bacteria should be catalase negative. As a consequence, these strains must be considered as non lactic acid bacteria.

The different results and observations for each isolated putative lactic acid bacterial strain are reported in Table 1. Some further details are given next.

Table 1: General observations and characteristics of isolated bacterial strains.
In the first two columns, the different letters refer to samples of different origins. The time of culture refers to the number of day required for the apparition of colonies on MRS-agar plates, in anaerobic conditions.

Fermented food	**Bacterial isolate**	**Culture delay (days)**	**Morphological observations of colonies**	**Morphological observations of cells**	**Catalase**
Madan pickles (A)	A1	5	All the colonies isolated from Madan appeared small, white and circular in shape. They did not develop any strong odor.	Cocci	+
	A2	5		Cocci	+
Madan pickles (B)	B1	5		Cocci	+
	B2	5		Cocci	+
	B3	5		Cocci	+
	B4	5		Cocci	+
	B5	5		Cocci	+
	B6	5		Cocci	+
	B7	5		Cocci	+
	B8	5		Cocci	+

	B9	5		Cocci	+
	B10	5		Cocci	+
	B11	5		Cocci	+
	B13	5		Cocci	+
Madan pickles (C)	C1	5		Rod and cocci	+
	C2	5		Cocci	+
	C3	5		Cocci	+
Fermented garlic (D)	D1	3 ½	Cream color, circular shape, flat	Large rods	-
	D2	3 ½	Cream color, small circular shape	Rods	-
	D4	3 ½	Yellow, punctiform, hard consistency	Small cocci	-
Fermented garlic (E)	E2	3 ½	Cream color, small circular shape	Large rods	-
	E3	3 ½	Cream color, circular shape	Large rods	-
Fermented bamboo (F)	F1	3 ½	White, circular shape	Rods	-
	F2	3 ½	White, circular shape	Rods	-
	F3	3 ½	White, circular shape	Small cocci	+
	F4	3 ½	White, circular shape	Small cocci	+
	F5	3 ½	White, circular shape	Large rods	-
	F6	3 ½	Almost transparent, circular shape	Large rods	-
	F7	3 ½	Almost transparent, circular shape	Large rods	-
Fermented bamboo (G)	G1	3 ½	White, circular and convex shape	Thin rod	-
	G2	3 ½	White, circular and convex shape	Long thin rods	-
	G3	3 ½	Almost transparent, circular shape	Rods	-
Fermented baby bok choy (H)	H1	3 ½	White, circular shape	Cocci	-
	H4	3 ½	White, circular shape	Cocci	-
	H5	3 ½	White, circular shape	Cocci	-
	H6	3 ½	Almost transparent, circular shape	Long thin rods	-
	H7	3 ½	White, convex and circular shape	Rods	-
	H8	3 ½	White, circular shape	Cocci	-
	H9	3 ½	White, circular shape	Rods	-
Fermented baby bok choy (I)	I1	3 ½	Almost transparent, irregular shape	Large rods	-
	I2	3 ½	White, irregular shape, hard consistency	Rod and cocci	-
	I3	3 ½	White, circular shape	Small rods	-
	I5	4 ½	White, circular shape	Cocci	+
Fermented cabbage	J1	3 ½	Cream color, irregular shape	Large rods	-
	J2	3 ½	Cream color, irregular shape	Large rods	-
	J3	4 ½	White, circular shape	Cocci	+
	J4	4 ½	White, circular shape	Cocci	+
Fermented cabbage	K1	3 ½	Cream color, irregular shape	Large rods	-
	K2	3 ½	Cream color, irregular shape	Large rods	-
	K3	3 ½	Cream color, irregular shape	Large rods	-
	K4	4 ½	White, circular shape	cocci	-

Five strains isolated from fermented vegetables did not grow aerobically and thus, were not conserved as lactic acid bacteria. These strains are not mentioned in the table.

Strain C1 isolated from madan pickles had alternatively coccal or rod shaped cells. At first it was thought that the culture was not pure. Thus five colonies were picked off randomly on different places of the plate, transferred on fresh plates, incubated at 30°C for 24 hours and then gram staining again. It resulted that there were still both coccal and rod shaped cell on all five plates. Then one colony of the previous plate was picked off, transferred into MRS broth tubes, incubated at 30°C for 24 hours and gram staining. Surprisingly all cells were coccal shaped. As a consequence, it was supposed that strain C1 could have both coccal and rod shaped cells depending on the growing conditions. The same characteristic was observed for strain I2 isolated from fermented "baby bok choy".

All strains isolated from madan pickles appeared to be catalase positive. To interpret this result it can be said that no lactic acid bacteria could be found in madan pickles.

For almost all isolated strains grown on MRS-agar at 30°C and in aerobic conditions, colonies appeared within 24 hours. However strains G1, G2 and D4 needed 48 hours. On the opposite strains H1, H4, H5, H6, H7, H8 and H9 could grow within 18 hours.

2. Antimicrobial activity

The plates resulting from the agar well diffusion assay were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. Each inhibition assay was performed in duplicate and on different plates for each isolated strain. The antibacterial activity was recorded in terms of the average surface of the inhibition zone, in millimeter-square. The absence of inhibition zone was interpreted as the absence of antimicrobial activity and a value of 0 mm^2 was attributed to the inhibition zone. The culture supernatant from isolated bacterial strains were tested to know if the antimicrobial metabolites produced by LAB were extracellular and released into the growth medium.

The main results observed for each isolated strain are presented in table 2 below. Results of catalase test were also reported in last column.

Table 2: Inhibition of *Staphylococcus aureus* with culture and supernatant of isolated bacteria.

Fermented food	Bacterial isolate	Culture inhibition (mm^2)	Supernatant inhibition (mm^2)	Catalase test
Madan pickles (A)	A1	0	0	+
	A2	0	0	+
Madan pickles (B)	B1	0	25	+
	B2	0	25	+
	B3	0	0	+
	B4	0	0	+
	B5	25	0	+
	B6	0	0	+
	B7	25	0	+
	B8	63	0	+

	B9	86	0	+
	B10	111	0	+
	B11	138	0	+
	B13	0	0	+
Madan pickles (C)	C1	25	0	+
	C2	0	11	+
	C3	0	25	+
Fermented garlic (D)	D1	0	0	-
	D2	0	0	-
	D4	0	0	-
Fermented garlic (E)	E2	0	25	-
	E3	25	68	-
Fermented bamboo (F)	F1	0	0	-
	F2	0	0	-
	F3	11	0	+
	F4	50	0	+
	F5	105	42	-
	F6	25	0	-
	F7	0	0	-
Fermented bamboo (G)	G1	0	0	-
	G2	25	0	-
	G3	25	0	-
Fermented baby bok choy (H)	H1	50	25	-
	H4	25	11	-
	H5	50	11	-
	H6	0	0	-
	H7	25	0	-
	H8	50	11	-
	H9	0	0	-
Fermented baby bok choy (I)	I1	0	0	-
	I2	25	0	-
	I3	0	0	-
	I5	0	25	+
Fermented cabbage	J1	68	0	-
	J2	68	0	-
	J3	0	0	+
	J4	25	0	+
Fermented cabbage	K1	50	0	-
	K2	50	68	-
	K3	0	0	-
	K4	0	0	-

Of 51 bacterial strains isolated from fermented vegetables, 31 (60,8%) strains seemed to have antibacterial activity against *Staphylococcus aureus* indicator strain and only 16 of them (31,4%) were also catalase negative.

The antimicrobial properties of the bacterial strains tested were very variable. Many of the strains showed weak or no inhibition of the pathogenic strain. Furthermore, the inhibition zones were really unclear and for all assays it was hard to say if what was observed could be interpreted as an inhibition or not. Consequently these results should be considered as putative.

Besides it seems nonsense that for several strains, antimicrobial activity could be detected on the supernatant while the culture was found inactive. It would also be expected to find residues of antimicrobial activity of the supernatant when the culture is active. However this result could be interpreted as a competitive growth being responsible of the antimicrobial activity.

In tests of supernatant, positive antimicrobial activities suggest that the inhibitory metabolites produced by the isolates were extracellular and diffusible because inhibition took place by diffusing through a layer of agar.

A few examples of inhibition assays are presented below.

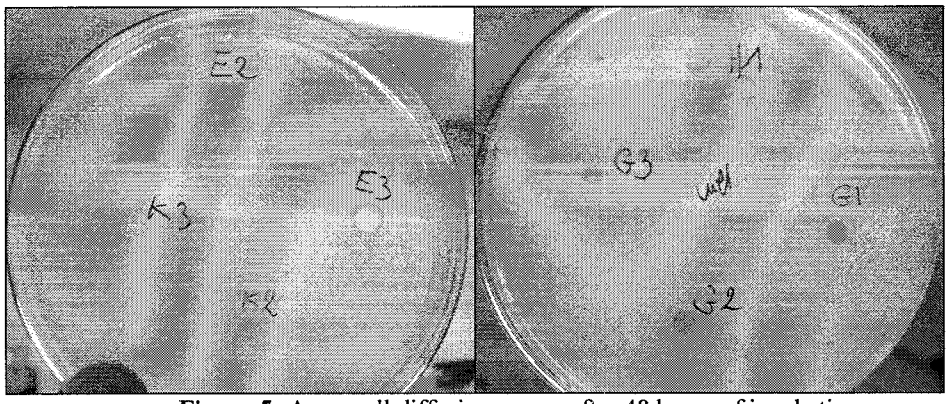

Figure 5: Agar well diffusion assays after 48 hours of incubation.

3. Identification of bacterial strains

The isolated bacterial strains tested with API CHL50 tests were strains I2, F5, K2, H1, J1 and H5. Results of strips were read and interpreted after 48 hours of incubation as described by the manufacturer.

A positive test corresponds to acidification revealed by the bromcresol purple indicator contained in the medium changing to yellow. For the esculin test (tube no. 25), a change in color from purple to black is observed.

Strain I2 was identified as *Lactobacillus fermentum 1.*

Strain F5 was identified as possible *Lactobacillus curvatus ssp curvatus.*

Strain H1 and H5 were both identified as Pediococcus pentosaceus 1.

Strain K2 was identified as *Lactobacillus plantarum.*

Strain J1 was identified as possible *Lactobacillus delbrueckii ssp bulgaricus.*

The following photo is an example of API CHL50 test after 48 hours of incubation.

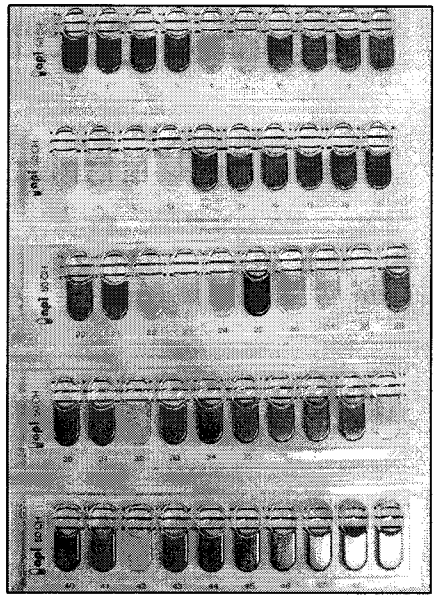

Figure 6: API CHL50 test with strain H5 after 48 hours of incubation.

Although only six strains were identified with API CHL50 tests, all the bacterial stains isolated from the different fermented vegetables were conserved for further investigation, including catalase positive strains.

Discussion

Despite very unclear results of the antimicrobial assays, it should be kept in mind that the antimicrobial activity of the isolated lactic acid bacterial strains was estimated only for one strain of *Staphylococcus aureus* and for one condition. Moreover there could be many other reasons to explain why no inhibition zone could be observed. However, many of the isolated strains may have antimicrobial activity even if it was not demonstrated. It might be supposed that the Mueller Hinton agar medium was not appropriate for the growth of test strains or for diffusion of antimicrobial substances. It may also be possible that test strains were not in log phase and had not produced antimicrobial substances yet. Furthermore, the strain of *Staphylococcus aureus* used for inhibition assays might be too strong. Consequently the results must not be taken as definitive and sure. Indeed for further investigation concerning the isolated bacterial strains, it would be necessary to check the antimicrobial activity on different strains of *Staphylococcus aureus* isolated on food-intoxicated patients and also other pathogens. It would also be useful to do the inhibition test using a filter paper disc diffusion method.

It would also be interesting to isolate, identify and characterize the antimicrobial substances produced by the most interesting strains such as bacteriocins, exopolysaccharides... Further studies may be focused on the characterization of amino acid and nucleotide sequences of these antibacterial compounds.

Moreover the experiments were conducted in-vitro, on synthetic media. In the first time, investigations should be carried on so as to determine the optimal conditions of growth and antimicrobial substances production. Indeed, according to previous

publications the inhibitory property of lactic acid bacteria was already found different when grown on different types of broths indicating that the media composition affects the production of antimicrobial metabolites. In a second time, isolated bacterial strains of putative industrial interest should be grown in a media more representative of food products such as cheese matrix to evaluate their growth, their antimicrobial activity and thus their capacity to be used in industrial food processes.

These researches are all vital in the sense that functional properties in lactic acid bacteria improve preservative effect and add flavor and taste. Lactic acid bacteria have an essential role in most food and beverage fermentation processes, one of the earliest known food preservation of fermented foods and beverage. These isolated strains can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving their hygiene and safety.

Acknowledgements

I am grateful to Dr Sunthorn Kanchanatawee for financial support obtained through the SUT (Suranaree University of Technology). I also thank a lot Dr Piyawan Kasaluk as well as all the graduate students of Food Technology department, for their help, their support and their friendship. I would also like to give a special thank to Natida, Paeng, Por and Som for always being attentive, for valuable discussions and for taking care of me. Finally I acknowledge Mr. Pascal Loubière for giving me the opportunity to do this session and thus to travel to Thailand.

References

Pongsak Rattanachaikunsopon, Parichat Phumkhachorn (2000). "A Bacteriocin Produced by Lactobacillus lactis subsp. Lactis Isolated from Thai Fermented Foods." ScienceAsia 26: 195-200.

T. Smitinont, C. Tansakul, S. Tanasupawat, S. Keeratipibul, L. Navarini, M. Bosco, P. Cescutti (1999). "Exopolysaccharide-producing lactic acid bacteria strains from thai traditional fermented foods: isolation, identification and exopolysaccharide characterization". International Journal of Food Mirobiology 51: 105-111.

Chatinan Ratanapibulsawat, Pumrussiri Kroujkaew, Ohomo Sadahiro, Sunee Nitisinprasert (2004). "Screening and Characterisation of Lactic Acid Bacteria Producing Antimicrobial Substances against Staphylococcus aureus."

Renata Bromberg, Izildinha Moreno, Cíntia Lopes Zaganini, Roberta Regina Delboni, Josiane de Oliveira (2004). "Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity." Brazilian Journal of Microbiology 35: 137-144.

Girum Tadesse, Eden Ephraim, Mogessie Ashenafi. "Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented beverages, on some foodborne pathogens and effect of growth medium on the inhibitory activity." Internet Journal of Food Safety V (5) 13-20.

Aly Savadogo, Cheik A.T. Ouattara, Imael. H.N. Bassole, Alfred S. Traore (2004). "Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Burkina Faso Fermented Milk." Pakistan Journal of Nutrition 3: 174-179.

Hicham LABIOUI, Laaroussi ELMOUALDI , Mohammed EL YACHIOUI, Mohammed OUHSSINE (2005). "Selection de souches de bacteries lactiques antibacteriennes." Bull. Soc. Pharm. Bordeaux 144: 237-250.

Cinta RACHMAN, Angélique FOURRIER, Abdoulaye SY, Marie France de la COCHETIERE, Hervé PREVOST, Xavier DOUSSET (2004). "Monitoring of bacterial evolution and molecular identification of lactic acid bacteria in smoked salmon during storage." Lait 84: 145-154.

Salim Ammor, Gregoire Tauveron, Eric Dufour, Isabelle Chevallier (2006). "Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility. 1-Screening and characterization of the antibacterial compounds." Food Control 17: 454-461.

WEB sources:

http://www.emdchemicals.com