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Research Article

Characterization of lactic acid bacteria and coagulase negative staphylococci isolated from raw meat and cured meat products

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Abstract

The aims of this study were to screen lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) from 50 samples of raw beef, raw pork, bacon, Nham and Saigrork Esan (Thai fermented pork sausages) to select the most suitable strains for use as meat starter cultures. The 403 LAB and 250 Micrococcaceae isolates were characterized. The 184 LAB isolates inhibited growth of at least one of 11 indicator organisms by agar spot test. Twenty-five LAB isolates were nitrate reductase positive and amino acid decarboxylase negative which may not accumulate biogenic amine in fermented food. After further characterization, three LAB isolates (the isolate S0602 isolated from Nham, the isolates T0903 and T0904 isolated from Saigrork Esan) with ability to produce bacteriocins and highest resistance to lowest pH (pH 1.5) of hydrochloric and lactic acids and highest concentration of sodium chloride (11% w/v) and bile salts (4-5% w/v) were identified as *Lactobacillus plantarum* S0602, *Lactobacillus brevis* T0903 and *L. brevis* T0904. The only one of 19 selected CNS isolates with catalase, nitrate and nitrite reductases positive was the isolate SC0903 isolated from raw beef. This bacterium was amino acid decarboxylase negative and was identified as *Staphylococcus xylosus*. Following this, *L. plantarum* S0602, *L. brevis* T0904 and *S. xylosus* SC0903 were selected to study the ability to deplete sodium nitrite in culture media. Among all bacterial strains tested, *L. plantarum* S0602 had highest ability to reduce the residual nitrite in the culture medium added with 1,000 mg/l during fermentation at 37°C.

Keywords: fermentation, LAB, nham, Micrococcaceae, starter culture, *Lactobacillus*, Thailand.

Introduction

Lactic acid bacteria (*Lactobacillus*, *Pediococcus*) and Micrococcaceae (*Staphylococcus*, *Micrococcus*) are often used as meat starter cultures. Lactic acid bacteria (LAB) produce inhibitory substances and improve sensory quality in final products [1]. Coagulase-negative *Staphylococcus* (CNS) are members of the family Micrococcaceae. They are gram-positive, catalase-positive cocci (GCC) [2]. CNS is a group of the most important microorganisms used as starter cultures. They play an important role in development of colour, flavour and aroma in cured

meat products [3]. Some *Staphylococcus* species are found as normal microflora in animals [2]. *S. saprophyticus and S. xylosus* have been found as dominating CNS in a southern Italian fermented sausage [4]. They are commonly used as commercial starters. Selection of suitable strains are based on some important criteria to be used as meat starter cultures such as ability to produce bacteriocins, nitrate reductase, nitrite reductase, inability to produce amino acid decarboxylase and some probiotic properties [5]. Therefore, the aim of this study was to screen, characterize and identify new suitable LAB and CNS strains from raw meat and cured meat products to be used as functional starter cultures.

Material and Methods

Physicochemical and microbiological analysis of raw meat and cured meat products

Samples of raw beef, raw pork, bacon, Nham and Saigrork Esan (Thai fermented pork sausages) (10 samples each) were purchased from local markets in Bangkok and Samut Prakarn, Thailand. Water activity (a_w) at 25°C and pH values of all samples were measured by using water activity meter (Aqualab Series 3TE, USA) and pH meter (Testo 205, Germany), respectively. Residual nitrite content was measured using the method of Kirk and Sawyer [6]. Total microbial and Micrococcaceae counts in all samples were determined by spiral plating onto Plate Count Agar (PCA) and Mannitol Salt Agar (MSA, Difco Laboratories, USA), respectively. Total LAB counts were determined by spread plating onto deMan Rogosa Sharpe agar (MRS, Difco Laboratories, USA) containing 0.5% $CaCO_3$. Typical colonies of Micrococcaceae on MSA (opaque white or cream colonies) and LAB with clear zone on MRS agar were randomly picked and purified.

Screening of lactic acid bacteria

Inhibitory activities of all selected LAB isolates were assessed by agar spot test against 11 indicator organisms including *Bacillus cereus* DMST 5040*, Escherichia coli* DMST 42112, *Listeria monocytogenes* DMST 11256, *Pseudomonas fluorescens* DMST 20076, *Salmonella* Typhimurium DMST 0562, *Staphylococcus aureus* TISTR 118, *Vibrio parahaemolyticus* OYI, *Yersinia enterocolitica* DMST 9380, *Enterococcus faecium* TISTR 1283, *Lactobacillus plantarum* TISTR 050 and *Pediococcus acidilactici* TISTR 051 [7]. The isolates inhibiting at least one indicator bacterium were selected for characterization such as tolerance of hydrochloric acid, lactic acid, sodium chloride and bile salts [8], production of catalase [9], nitrate reductase [10], nitrite reductase [11], amino acid decarboxylase [12] and bacteriocin production by agar well diffusion [7].

Screening of coagulase negative Staphylococcus

Differentiation of staphylococci and micrococci were performed by testing acid production from glucose, acid production from glycerol, susceptibility to furazolidone and lysostaphin resistance to bacitracin and oxidase production [13]. The isolates which were considered as *Staphylococcus* could produce acid from glucose and glycerol. They were oxidase negative and susceptible to furazolidone and lysostaphin, but resistant to bacitracin. All *Staphylococcus* isolates were selected for coagulase test [9], production of catalase [9], nitrate reductase [10], nitrite reductase [11] and amino acid decarboxylase [12].

Identification of lactic acid bacteria and coagulase negative Staphylococcus

All isolates were tested for the characteristics of gram reaction and cell morphology. The selected LAB isolates were tested for carbon dioxide production from glucose and growth at different conditions such as growth at 10°C and 45°C, growth in the presence of 6.5% and 18% NaCl and growth at pH 4.4 and 9.6 to identify at genus level [14]. The selected LAB isolates were then characterized by their carbohydrate fermentation patterns using the API-50 CH system

(bioMérieux, France), while the CNS isolates were identified at species level by the API Staph identification system (bioMérieux, France).

Sodium nitrite depletion by selected lactic acid bacteria and coagulase negative* Staphylococcus *isolates

This experiment was performed according to the method of Yan, *et al* [15]. Briefly, the selected LAB and CNS isolates ($1x10^7$cells/ml) were inoculated into 10 ml MRS and TSB broth containing 1000 mg/l of sodium nitrite, respectively. The MRS broth was overlaid with paraffin oil. All samples were incubated at 37°C for 4 days. The turbidity (OD_{620} nm), pH values and residual nitrite content [6] of all culture media were measured at days 0, 1, 2, 3 and 4 of incubation.

Results and Discussion

Physiochemical and microbiological characteristics of raw meat and cured meat products

Raw meat samples had slightly acidic pH (5.30-5.98), while bacon, Nham and Saigrork Esan samples had lower pH values (4.32-5.56). The pH values of raw pork samples (5.30-5.52) were lower than those of raw beef samples (5.50-5.98). Among all samples, raw beef and pork had the highest a_w values (0.991-0.997). Residual nitrite content in raw meat and cured meat products was detected in acceptable range (0.71-23.26 mg/kg) (Table 1). Total microbial counts and total Micrococcaceae counts in raw beef and pork were found in higher number, compared to other samples. However, these raw meat samples contained slightly lower LAB counts than those found in Nham and bacon (Table 2). The LAB isolates (403 isolates) and Micrococcaceae isolates (250 isolates) were purified and stored at 4°C for further characterization.

Table 1. Physiochemical characteristics of raw meat and cured meat products.

Type of samples (number of samples)	Physicochemical characteristics		
	a_w (at 25°C)	pH	Residual nitrite (mg/kg)
Raw beef (10)	0.991-0.995	5.50-5.98	0.71-1.41
Raw pork (10)	0.992-0.997	5.30-5.52	0.35-1.06
Bacon (10)	0.979-0.989	4.98-5.56	0.35-23.26
Nham (10)	0.964-0.972	4.45-5.20	1.40-4.56
Saigrork Esan (10)	0.958-0.975	4.32-4.85	1.80-4.93
Total (50)	-	-	-

Raw meat had slightly acidic pH. This is probably due to accumulation of lactic acid as a result of anaerobic glycolysis after slaughtering [16]. Final pH values of meat may correlate with glycogen contents. Blixt and Borch [17] found that 100g pork loin (pH 5.35) had higher glycogen content than 100g beef loin (pH 5.45). In this study, Nham and Saigrork Esan samples had lower pH values because they probably contained organic acids as fermentation products. Normally, carbohydrate added in fermented meat products at the concentration of 0.5-0.7% can reduce pH values to lower than 5.0 [18]. In addition, the low a_w value of Nham and Saigrork Esan may be the result of water releasing during fermentation. Vissanguan, *et al* [19] reported that Nham samples fermented for at least 36 h had pH values of 4.6 and their weight decreased due to release of water during fermentation. In this study, residual nitrite content in all raw and cured meat samples did not exceed the standard of the Ministry of Public Health, Thailand (125 mg/kg). However, the Department of Disease Control in Thailand reported toxicity of sodium nitrite in patients with excessive intake of 3,137 mg/kg nitrite in sausages. In this study, high microbial counts in raw

pork and beef indicated poor sanitary conditions during slaughtering and carcass handling of raw meat.

Table 2. Microbiological characteristics of raw meat and cured meat products.

Type of samples (number of samples)	Microbiological characteristics					
	Total microbial counts (CFU/g)	Total (LAB) counts (CFU/g)	Selected LAB (isolates)	LAB with antimicrobial activity (isolates)	Total Micrococcaceae counts (CFU/g)	Selected Micrococcaceae (isolates)
Raw beef (10)	$1.2x10^7$-$1.8x10^9$	$7.8x10^5$-$9.3x10^7$	100	21	$2.2x10^4$-$9.6x10^6$	80
Raw pork (10)	$3.7x10^6$-$1.7x10^9$	$7.0x10^5$-$4.0x10^7$	70	43	$1.8x10^4$-$1.0x10^7$	90
Bacon (10)	$2.7x10^6$-$5.4x10^7$	$1.1x10^6$-$1.2x10^7$	100	40	<2,500	0
Nham (10)	$3.2x10^7$-$1.1x10^8$	$2.5x10^6$-$6.7x10^8$	70	47	$5.0x10^3$-$2.9x10^4$	30
Saigoyisan (10)	$1.2x10^5$-$1.6x10^7$	$1.7x10^4$-$2.3x10^7$	50	33	$4.5x10^3$-$1.2x10^4$	50
Total (50)	-	-	403	184	-	250

Screening, biochemical characterization and identification of lactic acid bacteria

The 184 LAB isolates (45.66% of 403 isolates) exhibited antibacterial activity by inhibiting at least one of 11 indicator organisms (Table 2). The LAB isolates (25 of 184 isolates) with catalase and nitrate reductase positive, but amino acid decarboxylase negative were selected to test the resistance to acid, sodium chloride and bile salts (Table 3). Ten LAB isolates (40% of 25 isolates) had high ability to tolerate the lowest pH of HCl and lactic acid at pH 1.5 and the highest concentration of sodium chloride (11%) and bile salts (5%). They were selected to identify at genus level. These isolates were shown to be *Enterococcus* (the isolates B0407, B0410, T0701, T0704 and T0901), *Lactococcus* (the isolates B0708 and C0604) and *Lactobacillus* (the isolates S0602, T0903 and T0904).

The selected LAB isolates could inhibit growth of indicator bacteria as they may produce some inhibitory substances such as organic acids, bacteriocins, hydrogen peroxide and others. In addition, these isolates could tolerate hydrochloric and lactic acids at low pH level and a high concentration of bile salts. Probiotic bacteria are known to be beneficial to consumer health. They play an important role in balancing microflora in the gastrointestinal (GI) tract. These bacteria have been reported to inhibit the growth of undesirable bacteria, reduce disease caused by infection in the GI tract, stimulate the body's immune system, inhibit growth of cancer cells and produce enzymes that are essential to the body. To gain these health benefits, these bacteria need to survive passage through the GI tract. One of the important characteristics of probiotic bacteria is their resistance to all harmful acids and bile salts in the gut. In this study, the selected LAB which can tolerate acid, NaCl and bile salts may be suitable for use as starter culture [20, 21].

***Screening, biochemical characterization and identification of coagulase negative* Staphylococcus**

The results of differentiation tests of 250 Micrococcaceae isolates showed that 19 isolates (7.6%) were *Staphylococcus* and 231 isolates (92.4%) were *Micrococcus*. All of the *Staphylococcus* isolates were coagulase negative (Table 4).

Table 3. Hydrochloric acid, lactic acid, sodium chloride and bile salt tolerance of lactic acid bacteria (LAB) isolated from raw meat and cured meat products.

Source of isolates	LAB isolates	pH[a] of HCl	pH[a] of lactic acid	NaCl (%w/v) [b]	Bile salts (%w/v)[b]
Raw beef	C0403, C0609	1.5	1.5	11.0	3.0
	C0604	1.5	1.5	11.0	5.0
	C0605	1.5	4.0	7.0	5.0
	C0608	1.5	1.5	11.0	1.5
	C0610	1.5	> 5.0	< 6.0	5.0
	C0708	1.5	3.0	7.0	5.0
Raw pork	P0509	2.0	2.0	10.0	3.0
	P0510, P0804, P0806	1.5	1.5	11.0	5.0
	P0706, P0708	2.5	2.0	10.0	2.0
Bacon	B0407, B0410	1.5	1.5	11.0	5.0
	B0708	1.5	1.5	10.0	2.0
	B0709	2.5	1.5	10.0	2.0
	B1002	2.0	1.5	10.0	2.0
	B1010	2.0	1.5	9.0	2.0
Nham	S0602	1.5	1.5	11.0	4.0
Saigrork Esan	T0701, T0901, T0904	1.5	1.5	11.0	5.0
	T0704, T0903	1.5	1.5	11.0	5.0

[a] The lowest pH of acid at which the isolates were able to grow.
[b]The highest concentration of sodium chloride and bile salts at which the isolates were able to grow.

Table 4. Differentiation of *Staphylococcus* and *Micrococcus* isolates in family Micrococcaceae.

Source of Isolates	Micrococcaceae (isolates)	Number of isolates	
		Staphylococcus [a]	*Micrococcus*[b]
Raw beef	80	8	72
Raw pork	90	4	86
Bacon	0	0	0
Nham	30	4	26
Saigrork Esan	50	3	47
Total	250	19	231

[a] Acid production from glucose and glycerol, furazolidone and lysostaphin susceptibility, bacitracin resistance and oxidase negative were found.
[b] Furazolidone and lysostaphin resistance, bacitracin susceptibility and oxidase positive were found, but acid production from glucose and glycerol was not found.

All 19 *Staphylococcus* isolates were coagulase negative, but catalase and nitrate reductase positive. 18 of these 19 isolates were amino acid decarboxylase positive, while the only *Staphylococcus* isolate which showed amino acid decarboxylase negative was the isolate SC0903 (Table 5). These isolates were identified as *S. xylosus* (8 of 19 selected CNS isolates) *S. saprophyticus* (2 isolates), *S. lentus* (2 isolates), *S. cohnii* ssp. *urealyticum* (3 isolates), *S. cohnii*

ssp. *cohnii* (2 isolates), *S. haemolyticus* (1 isolate), and *S. lugdunensis* (1 isolate). Only the isolate SC0903 (isolated from raw beef) with nitrite reductase positive was selected to identify using the API Staph kit. It was identified as *S. xylosus* (99.9% Identity).

All *Staphylococcus* isolates were catalase and nitrate reductase positive. Catalase is generally present in all aerobes and facultative anaerobes. This enzyme mediates degradation of hydrogen peroxide [22]. Nitrite reductase is also an essential enzyme for preservation, colour development and aroma formation. Nitrate in the medium will be reduced into nitrite by action of this enzyme. It also helps to recycle nitrite by converting this compound into nitrate in the sequential reaction with myoglobin [18]. In this study, *S. xylosus* SC0903 could produce both nitrate and nitrite reductases. Gøtterup, *et al* [3] reported that some strains of *Staphylococcus* isolated from cured meat products (*S. saprophyticus, S. simulans, S. sciuri* and *S. carnosus*) could produce both nitrate and nitrite reductases. The commercial API Staph system showed high accuracy in the identification of the isolate SC0903 which is in agreement with the study of Cunha, *et al* [23].

Table 5. Catalase, nitrate reductase, nitrite reductase and amino acid decarboxylase activity from selected *Staphylococcus* isolates.

Original sources of *Staphylococcus* isolates	*Staphylococcus* isolates	Number of isolates			
		Catalase	Nitrate reductase	Nitrite reductase	Amino acid decarboxylase activity
Raw beef	SC0204, SC0206 SC0209, SC0608 SC0609, SC0903 SC0904, SC0907	8	8	1[a]	7
Raw pork	SP0202, SP0204 SP0205, SC0207	4	4	0	4
Bacon	0	0	0	0	0
Nham	SS0301, SS0802 SS0804, SS0902	4	4	0	4
Saigrork Esan	ST0901, ST0704 ST1001	3	3	0	3
Total	-	19	19	1	18[b]

[a] The only one isolate with nitrite reductase positive was the isolate SC0903.
[b] The only one isolate with amino acid decarboxylase negative was the isolate SC0903.

Sodium nitrite depletion by selected lactic acid bacteria and coagulase negative* Staphylococcus *isolates

In this study, three bacterial isolates, *Lactobacillus plantarum* S0602, *Lactobacillus brevis* T0904 and *Staphylococcus xylosus* SC0903 were selected to study the depletion of sodium nitrite in culture media added with 1,000 mg/l sodium nitrite during fermentation at 37°C. The results showed that all media containing 1,000 mg/l sodium nitrite and inoculated with these three bacteria as starter cultures had residual nitrite concentration in the range of 130.20-134.48 mg/l at the beginning of fermentation. After incubation for 24 h, nitrite concentration in MRS broth inoculated with *L. plantarum* S0602 (19.03 mg/l) was significantly lower than those of other treatments ($P<0.05$). The culture medium with *L. brevis* T0904 had decreased nitrite concentration of 37.28 mg/l, while the medium with *S. xylosus* SC0903 contained 132.34 mg/l residual nitrite

after 24-hour fermentation. The amount of nitrite in culture medium of all treatments then slightly decreased until day 4 of fermentation (Figure 1a).

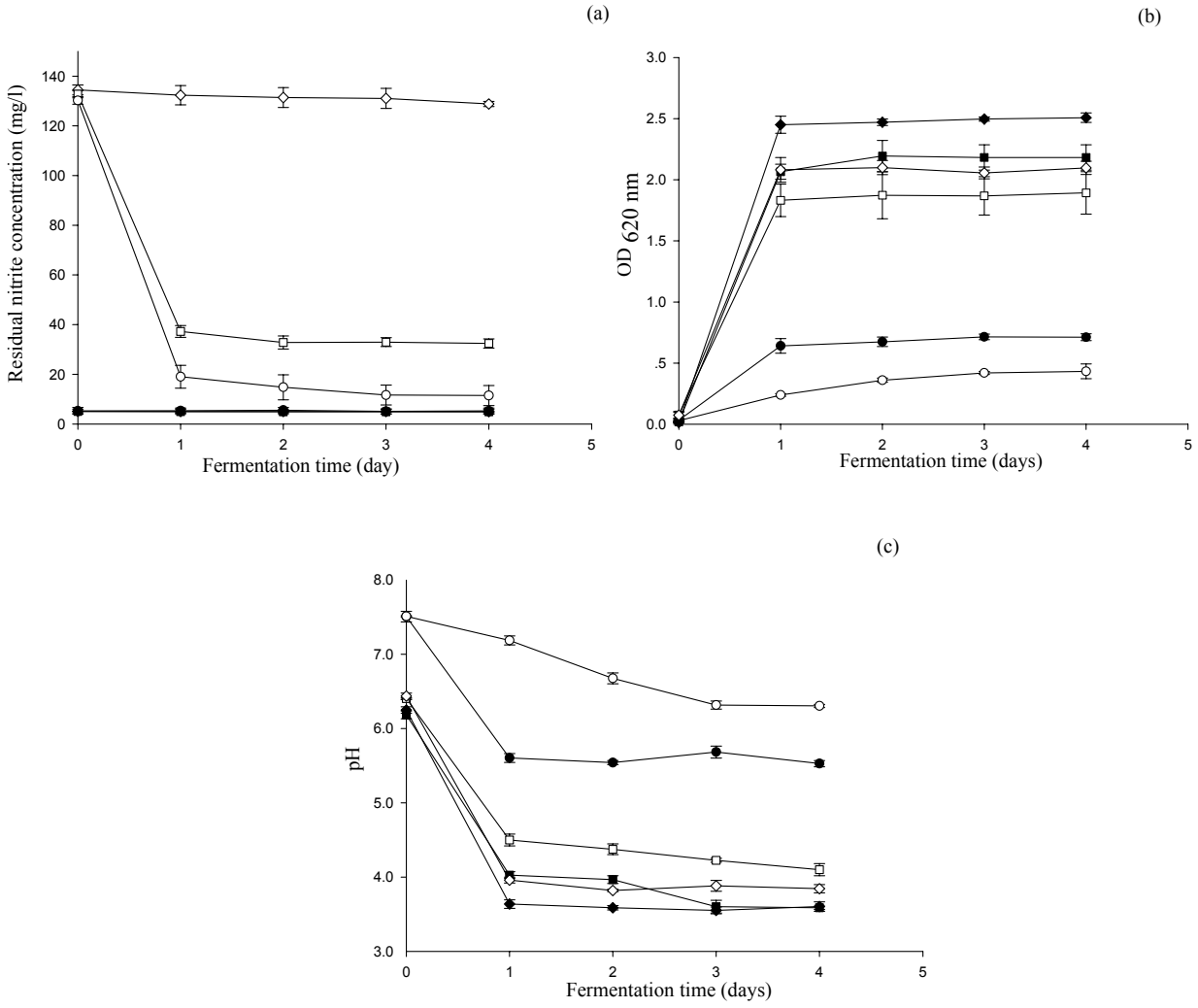

Figure 1. Change of residual nitrite content (a), optical density (b), and pH values (c) in culture media with 1,000 mg/l sodium nitrite (TSBN or MRSN) or without sodium nitrite (TSB or MRS broth).

TSB inoculated with *Staphylococcus xylosus* SC0903 (●), TSBN inoculated with *S. xylosus* SC0903 (○), MRS broth inoculated with *Lactobacillus brevis* T0904 (■), MRSN broth inoculated with *L. brevis* T0904 (□), MRS broth inoculated with *Lactobacillus plantarum* S0602 (♦), MRSN inoculated with *L. plantarum* S0602 (◊).

Considering the optical density at 620 nm after incubation for 24 hours, *L. plantarum* S0602 in the culture medium grew rapidly with the highest $OD_{620\ nm}$ (2.08) followed by *L. brevis* T0904 (1.82) and *S. xylosus* SC0903 (0.24). $OD_{620\ nm}$ then slightly changed when the fermentation time increased until 4 days of fermentation (Figure 1b). The depletion of sodium nitrite was concomitant with the increasing of $OD_{620\ nm}$ and the decreasing of pH value of culture medium.

As can be seen in Figure 1c, the pH values of the culture medium inoculated with *L. plantarum* S0602 and that with *L. brevis* T0904 decreased rapidly from 6.20-7.50 at the beginning of fermentation to 3.64-4.50 after 24 hours of fermentation, while the pH values of the culture medium inoculated *S. xylosus* SC0903 had higher pH values (5.60-7.19). The pH values of all treatment media then changed slightly until the end of fermentation (Figure 1c).

Conclusion

Results of this research revealed that two bacteriocin-producing LAB strains, *L. plantarum* S0602 and *L. brevis* T0904 with probiotic properties could potentially be used as functional starter cultures in fermented meat products. In addition, *S. xylosus* SC0903 could also be used in these products as a starter bacterium. These bacterial strains could provide significant health benefits, enhance safety of the products and decrease the residual nitrite concentration in products.

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