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Selection of Probiotic Lactic Acid Bacteria Isolated from Fermented Plant Beverages

[1]Y. Duangjitcharoen, [2]D. Kantachote, [2]M. Ongsakul, [3]N. Poosaran and [1]C. Chaiyasut
[1]Department of Pharmaceutical Science, Faculty of Pharmacy, Chaing Mai University, Muang District, Chiangmai, 50200, Thailand
[2]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat-Yai 90112, Thailand
[3]Department of Biotechnology, Faculty of Agro-Industry, Chaing Mai University, Muang District, Chiangmai, 50200, Thailand

Abstract: Screening for probiotic bacteria from non-human sources were performed in this study. Three hundred and twenty-seven strains of Lactic Acid Bacteria (LAB) were isolated from 90 samples of Fermented Plant Beverages (FPBs) and pickles collected from around Thailand. Potentially useful probiotic properties were investigated *in vitro* in parallel with a commercial probiotic strain of *Lactobacillus casei*, R obtained from a dairy probiotic product in Thailand. An isolate SS2, selected from a fermented star fruit beverage, survived in the human biological barriers (0.15 and 0.30% bile salt, pH values between 3-8, presence or absence of oxygen), resistance to some antibiotics in general use and showed other benefits to the host (antibacterial activity, utilizations of protein and starch). The isolate SS2 had a higher specific growth rate and better inhibitory properties against food borne pathogenic bacteria and spoilage organisms than the commercial probiotic R strain. It also grew well in MRS and the SPY2 medium that is free from animal-derived ingredients, ($r>0.8$). The isolate SS2 was therefore considered to be a potentially useful probiotic LAB for a non dairy product such as FPBs and was provisionally identified as a strain of *Lactobacillus plantarum*.

Key words: Probiotic lactic acid bacteria, biological barriers, antibiotic resistance, antibacterial activity, non-human origin, fermented plant beverages

INTRODUCTION

Probiotics are known as live microbial feed supplements that provide many benefits to the host by improving its microbial intestinal balance (Guarner and Schaafsma, 1998). In general, the best probiotic strains to give to humans are those that originated from the human intestine as they may be assumed to be the true probiotics (Surawicz 1998; Marteau and Rambaud, 1993). Most probiotic microbes are LAB such as *Lactobacillus casei*, *L. plantarum* and *Streptococcus lactis* (Sindhu and Khetarpaul, 2001). Probiotic LAB are normally used as starter cultures for fermented dairy products like yogurt. In recent years, consumers who are lactose intolerant and worry about the cholesterol content in milk as well as vegetarians increasingly demand for non-dairy-based probiotics. In Thailand, fermented plant beverages are considered to be non-alcoholic and people who consume these FPBs believe that plant beverages are healthy due to their high nutritional value and presence of bioactive compounds derived from the plant substrates used and also during the fermentation processes (Kantachote and Charernjiratrakul, 2007). However, scaling up the beverage production for commercial use can have a problem with a high contamination by yeasts. To restrict this, starter cultures have been advocated (Prachyakij *et al*., 2007). Consequently, the aims of this study were to isolate and select an appropriate probiotic LAB from fermented plant products to use as a starter culture and restrict yeast growth for the production of FPBs.

MATERIALS AND METHODS

Selection and isolation of probiotic LAB: LAB strains were isolated from 90 samples of fermented plant beverages and pickles collected from different areas in Thailand and they were used to investigate their probiotics during year 2005-2006. The selection was conducted to investigate probiotic properties of LAB in order steps as following provided while any strains which showed a good performance for each probiotic property were again selected for a next step. Possible biological barriers were investigated as described by Conway *et al*. (1987), such as survival in 0.15 and 0.30% (w/v) bile salt and also tolerance to pH values of 2, 3, 4, 5, 8 and 9. Briefly, LAB strains were grown in MRS (de Man Rogosa

Corresponding Author: Duangporn Kantachote, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat-Yai 90112, Thailand Tel: +6674288310 Fax: +6674446661

and Sharp) broth at 37°C for 24 h and were inoculated by streak on bile salt agar plates, whereas acid-base tolerance was tested in MRS broth with various pH. All plates and tubes were incubated at the same condition as above to observe their growth. Utilizations of protein, starch and lipid were examined using agar plates of milk, starch and tributyrin, respectively. Utilization ability was observed as a clear zone around bacterial colonies in each agar medium. Growth as facultative anaerobe was investigated in MRS broth and then the culture tubes were separated to incubate in an anaerobic jar while another set was separated to incubate in an aerobic incubator at 37°C for 24 h. Bacterial growth was measured using spectrophotometer ($OD_{660\ nm}$). Requirement for vitamin B12 was tested in vitamin B12 assay medium and the growth was compared with *Lactobacillus delbrueckii* subsp. *lactis* growth. Antibiotic susceptibility tests to 15 antibiotics in regular use (Table 1) and antagonistic activity against 13 strains of food borne potentially pathogenic bacteria and spoilage organisms (Table 2) were carried out according to standard methods as described by Seeley *et al.* (1991) and Spelhaug and Halander (1989). Bacterial indicators used in this study were provided by Department of Microbiology, Faculty of Science, Prince of Songkla University, Thailand. In addition, an experiment examined the possibility that the isolates could grow as well in a medium without animal derived ingredients (SPY2) as they did in MRS. A commercial probiotic dairy product in Thailand, *Lactobacillus casei* namely R was used as a reference strain (Lee *et al.*, 2004).

RESULTS AND DISCUSSION

One probiotic lactic acid bacterium, isolate SS2, was selected from 327 strains of LAB isolated from samples of FPBs and pickles. The isolate SS2 was provisionally identified as *Lactobacillus plantarum* (data not shown) and it was isolated from a fermented star fruit (*Averrhoa carambola*) beverage from Si-Sa-Ket province in the Northeast part of Thailand. As shown in Table 1, the selected strain SS2 and a commercial strain R were able to grow with both 0.15 and 0.30% bile salt in a modified MRS medium. Both strains had survival rates of more than 90% at pH 3 and 4 after a 3 h incubation time. It was surprising that more than 50% of the isolate SS2 survived at pH 8, but the R strain died. However, neither grew at pH 2 or 9. These tests for screening probiotic bacteria are appropriate because normally they must transit through the acidic condition of the stomach and bile in the intestine (Lee and Salminen, 1995). In addition, oxygen, one of the human physiological barriers, has a strong negative effect on the survival of possible probiotics bacteria in commercial products (Shah *et al.*, 1995). Fortunately, isolate SS2 and the commercial strain showed no significant differences in their growth in the presence

Table 1: Comparisons of properties of the isolate SS2 and the probiotic commercial strain, R

	Probiotic properties							
Strain	0.15 and 0.30% bile salt	Acid-base tolerance	Nutrient[a] digested	Oxygen effect	Cobalamin free medium	Antibiotic[c] susceptibility test	SPY2/Animal ingredient free medium and MRS	Generation time
SS2	Growth	Growth at pH 3, 4, 5, 8	Protein, starch	NS[b]	Growth	Resistance to VC, BC, GT, KC, ST, NF, PB	Growth r >0.8, p<0.05	51 min
R	Growth	Growth at pH 3, 4, 5	Protein	NS[b]	Growth	Resistance to VC, BC, GT, KC, ST, NF, PB	Growth r >0.8, p<0.05	71 min

[a]: Protein and starch utilizations were determined in 3% milk agar plates and 0.2% starch agar plates, respectively, [b]: No significant difference (p>0.01) between growth with or without oxygen present, [c]: VC: Vancomycin, BC: Bacitracin, GT: Gentamycin, KC: Kanamycin, ST: Streptomycin, NF: Norfloxacin, PB: Polymyxin B; other tested antibiotics were ampicillin (10 mcg), cephalothin (30 mcg), ceftazidime (30 mcg), chloramphenical (30 mcg), erythromycin (15 mcg), pencillin G (10 U), ceferperazone (75 mcg), tetracycline (30 mcg)

Table 2: The antagonistic activity of the isolate SS2 and the commercial probiotic strain R after 18 h of incubation time

		Food borne pathogenic bacteria and spoilage microorganisms												
Condition	Strain	*S. aureus* ATCC 25923	*B. cereus* ATCC 11778	*S. sonnei* PSSCMI 0032	*S. flexneri* PSSCMI 0035	*P. vulgaris* PSSCMI 0041	*P. rettgeri* PSSCMI 0044	*E. cloacae* PSSCMI 0040	*E. aerogenes* PSSCMI 0039	*E. coli* ATCC 25922	*E. coli* O157:H7	*S. typhi-murium* PSSCMI 0035	*S. typhi* PSSCMI 0034	*V. para-hemolyticus* VP4
1	SS2	+++	+++	++	+++	++	++	++	+++	+++	++	+++	+++	++
	R	+	+	++	+	++	+	-	+	+	+	+	++	++
2	SS2	+++	++	++	+	+++	++	++	++	++	++	++	++	+++
	R	++	+	+	++	+++	+	+	+	+	+	+	+	+
3	SS2	+	+	+	+	+	+	+	+	+	+	+	+	+
	R	-	+	-	+	+	+	-	+	-	-	-	+	+

1: Productions of organic acids and H_2O_2, 2: No control of organic acid production but a limitation of H_2O_2, 3: Control of organic acids and H_2O_2 production, +++: ≥10 mm inhibition zone, ++ : 7-9 mm inhibition zone, +: <7 mm inhibition zone and -: No inhibition zone

or absence of oxygen. In addition, both strains achieved an $OD_{660\ nm}$>2.00 in a cobalamin deficient medium so neither had a growth requirement for vitamin B_{12}. Hence, the isolate SS2 should not need to compete with the human body for cobalamin. This is especially important for vegetarians whose vitamin B_{12} levels are normally low because of its absence in their foods (Herbert, 1973). Utilization of protein and starch was observed in the milk and starch agar plates by the isolate SS2 but the R strain utilized only protein. This meant that the selected strain may provide more benefit to human digestion than the commercial strain.

The isolate SS2 had a higher antibacterial activity against 13 strains of food borne pathogenic bacteria and spoilage organisms than that found with the R strain in all conditions tested (Table 2). The antagonist activity of probiotics has long been used to preserve foods. The antimicrobial substances produced by probiotics bacteria act to improve the composition and the activity of the normal microbiota in the intestine (Ouwehand and Vesterlund, 2004). In addition, the antibiotic susceptibility tests showed that the SS2 and R strains were resistant to 7 of the antibiotics used, vancomycin (30 mcg), bacitracin (10 mcg), gentamicin (10 mcg), kanamycin (30 mcg), streptomycin (10 mcg), norfloxacin (10 mcg) and polymyxin (300 mcg). Results of a clinical trial indicated the advantages of administering both antibiotics and a probiotic strain to patients with recurrent *Clostridium difficile* infections (McFarland *et al*., 1994). Besides, co-administration may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of various antimicrobial agents (Salminen *et al*., 1998). Both SS2 and R strains grew well in the MRS and SPY2 broths with a Pearson's correlation level r >0.8. That means both strains could grow well in media containing either animal or plant ingredients. The isolate SS2 had a generation time of 51 min in SPY2 medium, whereas the R strain took 71 min for one generation. This could indicate that SS2 could respond more quickly than R to any potential antagonist activity.

We conclude that the selected strain, *L. plantarum* SS2, could be developed as an effective starter culture for use as a probiotic in a fermented plant beverage produced especially for vegetarians or others preferring to use a fermented plant product free from animal products.

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