Biotechnol Lett (2009) 31:571–576
DOI 10.1007/s10529-008-9902-3

ORIGINAL RESEARCH PAPER

Probiotic potential of lactic acid bacteria isolated from fermented dairy milks on antiproliferation of colon cancer cells

Mongkol Thirabunyanon · Pongphun Boonprasom · Piyanuch Niamsup

Received: 1 September 2008 / Revised: 1 December 2008 / Accepted: 4 December 2008 / Published online: 31 December 2008

Abstract Fifty-four strains of lactic acid bacteria obtained from fermented dairy milks were investigated for possible use as probiotics and for colon cancer biological products. Five of these strains inhibited growth of eight food-borne pathogens including *Helicobacter pylori*, *Escherichia coli*, and *Salmonella typhimurium*. Three of these strains survived at pH 2.5 and in 0.3% bile salts. Additionally they produced no haemolysis, were resistant to kanamycin and adhered to Caco-2 cells. 16S rRNA gene sequences of probiotic strains indicated that RM11 and RM28 were *Enterococcus faecium* and *Lactobacillus fermentum*, respectively. Both the cultured medium and live whole cells from probiotic strains were tested for antiproliferation of colon cancer cells through MTT and Trypan Blue exclusion assays. The probiotic strains of *E. faecium* RM11 and *L. fermentum* RM28 also triggered antiproliferation of colon cancer cells at the rates of 21–29%, and 22–29%, respectively. This suggested that both strains could be used as potential probiotics in functional food or for colon cancer biological products.

Keywords Caco-2 cells · Colon cancer · Fermented milk · Lactic acid bacteria · Probiotic

Introduction

Colon cancer is one of the leading causes of death through cancer. Dietary behavior such as a high-fat, low-fiber diet is an important contribution to a major risk of colon cancer (Commane et al. 2005). The diverse complex of microflora in the human intestines may play a critical role for diseases or human health. Metabolic capabilities from these microflora-produced bacterial enzymes—such as β-glucuronidase, nitroreductase, azoreductase, 7-α-dehydroxylase, and cholesterol dehydrogenase—can mediate the formation of the carcinogenic process, releasing carcinogens in the intestinal tract (Ewaschuk et al. 2006; Rafter 2003). Pathogenic *Escherichia coli* is a normal inhabitant in the human intestine. It has also been implicated in the initiation of colon carcinoma by producing a harmful toxin, cytotoxic necrotizing factor I (Travaglione et al. 2008). Conversely, a probiotic is "a live microbial food ingredient that is beneficial to health" (Salminen et al. 1998). Some probiotic bacterial strains have been suggested as having the potential to protect against colon cancer, through several mechanisms such as: alteration of the metabolic activities of the intestinal microflora; alteration of the physicochemical conditions in the colon; binding and degradation of potential carcinogens; quantitative or qualitative alterations in

Electronic supplementary material The online version of this article (doi:10.1007/s10529-008-9902-3) contains supplementary material, which is available to authorized users.

M. Thirabunyanon (✉) · P. Boonprasom · P. Niamsup
Biotechnology Section, Faculty of Science,
Maejo University, Chiang Mai 50290, Thailand
e-mail: mthirabun@yahoo.co.th

the intestinal microflora; production of anti-tumorigenic or antimutagenic compounds; enhancement of the host's immune response; and effects on host physiology (Commane et al. 2005; Ewaschuk et al. 2006; Rafter 2003; Saikali et al. 2004). The role of using a probiotic as a functional food in many food products has recently been applied, and its significance for human health is continually increasing each year. The probiotic bacteria which are applied most as a functional food are the group of lactic acid bacteria (LAB). The consumption of fermented milk using probiotic LAB as starter cultures could play a role in colon cancer risk reduction (Moreno et al. 2007; Saikali et al. 2004). Therefore, the development of probiotic selections and the application of these in functional foods—for food fermentation, food value, and human health (e.g., prevention of colon cancer)—are very important focuses in recent days.

In this study, the characteristics of LAB strains which were screened from fermented dairy milks for use as potential probiotics, and their efficiency in antiproliferation of colon cancer cells, were investigated.

Materials and methods

Isolation of LAB strains and their antagonistic activity

Several samples of dairy milk were collected from markets in Chiang Mai province, Thailand. The samples were fermented in anaerobic conditions at 37°C for 48 h. Then the fermented milks were diluted with serial dilutions of 0.85% (v/v) NaCl in sterile distilled water. Suitable dilutions of 100 µl were spread plated onto MRS agar, and all morphologically different colonies were collected. Well diffusion assay was used to evaluate against eight food-borne pathogens (Table 1).

Acid and bile salt tolerance assay

For the acid tolerance assay, tested bacteria were incubated in MRS broth at 37°C for 18 h, and 1 ml of culture was transferred into 9 ml PBS adjusted to pH 2.5 with 5 M HCl and then incubated at 37°C. The numbers of viable bacteria were determined at 0 h and 3 h of incubation, on an MRS agar plate. Triplicates of each sample were done. For the bile salt tolerance assay, tested bacteria were incubated in MRS broth at 37°C for 18 h, and 1 ml of culture was transferred into 9 ml MRS broth containing 0.3% (w/v) bile salt, and then incubated at 37°C. The numbers of viable bacteria were determined at 0 h and 24 h of incubation on an MRS agar plate. Triplicates of each sample were performed.

Haemolytic activity and antibiotic resistance

Haemolysis was evaluated using Columbia blood agar (Sigma) plates containing 5% (v/v) human blood, and incubated at 37°C for 48 h. Recorded

Table 1 Antagonistic activity of LAB strains against food-borne pathogens (Mean ± SD)

LAB strains	Inhibition zone (mm)							
	Helicobacter pylori DMST 20165	*Escherichia coli* TISTR 780	*Salmonella enteritidis* DMST 15676	*Salmonella typhimurium* TISTR 292	*Staphylococcus aureus* TISTR 118	*Bacillus cereus* TISTR 687	*Listeria monocytogenes* DMST 1783	*Vibrio cholerae* DMST 2873
RM11	12 ± 1	11 ± 0	14 ± 1	16 ± 1	18 ± 1	12 ± 1	12 ± 1	13 ± 1
RM24	15 ± 1	15 ± 0	14 ± 1	14 ± 2	16 ± 1	12 ± 1	12 ± 1	13 ± 1
RM25	11 ± 1	11 ± 0	14 ± 1	17 ± 1	16 ± 1	12 ± 1	12 ± 1	13 ± 1
RM28	16 ± 1	15 ± 1	15 ± 1	19 ± 1	17 ± 1	15 ± 1	14 ± 1	14 ± 1
RM34	12 ± 1	11 ± 1	13 ± 1	14 ± 1	15 ± 1	11 ± 1	11 ± 1	11 ± 1

The well diffusion assay method was used. MRS agar plates were overlaid with 10 ml of MRS broth containing 0.7% (w/v) agar at 45°C and inoculated with pathogenic strains to obtain a final concentration of about 10^5 cfu/ml. Upon solidification of both agar layers, a sterile cork borer was applied to create wells 8 mm diam. Then the cell-free supernatant (100 µl) of tested bacteria was transferred into the wells and incubated at 37°C for 24 h. A clear (inhibition) zone around the well, showing no growth of indicator pathogens, was recorded. Triplicates of each sample were done

characteristics of haemolysis on blood agar were shown as β-, α-, and γ-hemolysis. The assay was performed in duplicate. Antibiotic resistance was examined by the disk diffusion method. Tested bacteria were grown in MRS broth at 37°C for 18 h. The suspensions were swabbed onto MRS agar plates. An antibiotic dish (Oxoid) including 30 μg chloramphenicol, 10 μg ampicillin, 15 μg erythromycin, 30 μg tetramycin, and 30 μg kanamycin was placed on MRS agar plates and incubated at 37°C for 48 h. The inhibition zone around the antibiotic disk was recorded. The assay was performed in triplicate.

Cell culture

Colon cancer cells (Caco-2 cells, CLS) were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, Sigma) supplemented with 10% (v/v) fetal calf serum (Hyclone) inactivated at 56°C for 30 min, with 1% (v/v) non-essential amino acid (Hyclone) and 1% (v/v) penicillin–streptomycin (10,000 IU/ml and 10,000 μg/ml; Gibco). Cells were incubated in a CO_2 incubator at 37°C in 5% CO_2 for 15 days to become fully differentiated.

Adhesion to Caco-2 cells

Caco-2 cells were seeded with 1 ml culture medium containing 10^6 cells/well in 24-well tissue culture plates. Tested bacteria from 18 h cultures in MRS broth were harvested and washed twice with PBS. These bacteria were resuspended in non-supplemented DMEM to give 10^8 cfu/ml. After washing the Caco-2 twice with PBS, 0.5 ml bacterial suspension was added to each well and incubated at 37°C for 1 h in 5% CO_2. Unattached bacteria were removed by washing with PBS three times. Caco-2 cells were lysed with 0.1% (v/v) Triton X-100 for 5 min. Adhering bacteria cells were enumerated by plate counting in triplicate with MRS agar, and then incubated at 37°C for 48 h. Adhesion assays were performed in five replicates.

Antiproliferation of colon cancer cells

MTT assay

Colon cancer cells were added at 100 μl (10^6 cells/well) in 96 well plates, incubated at 37°C for 24 h then, 100 μl cultured medium (CM) was added into each well and incubated further for 24 h. Cells were then washed twice with PBS; 10 μl MTT (0.5 mg/ml in DMSO) was added into each well and incubated further for 4 h. The formazan precipitates were solubilized by the addition of 100 μl DMSO, and incubated for 5 min. The absorbance was measured at 595 nm using a microplate reader. The absorbance of the control group using MRS broth was set as 100% of cell viability. Analysis was performed in three independent experiments, each conducted in triplicate [% cell viability = (sample O.D/control O.D) × 100].

Trypan Blue exclusion assay

Colon cancer cells were seeded at of 10^6 cells/well with 1 ml cell suspension being added into 24 well plates. The suspensions were incubated at 37°C for 24 h. Then, 1 ml of CM or live whole cells was added into each well and incubated further for 24 h. The cell survival was examined using Trypan Blue exclusion staining with a Neubauer haemocytometer. The analysis was evaluated in three independent studies, each conducted in triplicate [% cell viability = (live cell count/total cell count) × 100].

Statistical analysis

A one-way analysis of variance (ANOVA) was employed, using SPSS (version 15.0) to evaluate the experimental data. The significant differences were accepted at $P < 0.05$ by Duncan's multiple range test.

Results

Characteristics of probiotic LAB strains

Fifty-four of the LAB strains obtained from fermented dairy milks were Gram-positive, with morphological forms of rods or cocci. Five of these strains (RM11, RM24, RM25, RM28, and RM34) inhibited growth of all eight food-borne pathogens (Table 1). Of these strains, RM11 and RM28 induced more antimicrobial substances against harmful strains as compared to the others. Survival of the five LAB strains in a gastrointestinal tract model is shown in Figs. 1 and 2. A high survival rate of the RM11,

RM24, and RM28 strains was observed after their exposure to pH 2.5 (Fig. 1). These three strains also exhibited a high tolerance rate when tested in 0.3% bile salt conditions (Fig. 2).

Five of the tested strains displayed no haemolysis (γ-hemolysis) when challenged with human blood. They were resistant only to kanamycin, however, and exhibited sensitivity to chloramphenicol, ampicillin, erythromycin and tetramycin. The efficiency of the LAB strains in adhering to Caco-2 cells is shown in Fig. 3. There was a significant difference ($P < 0.05$) in the abilities of the RM11, RM28, and RM24 strains to adhere to Caco-2 cells, with rates of 13, 7, and 4%, respectively. Comparison of the 16S rRNA gene sequences (see Supplementary data) indicated that RM11 was *Enterococcus faecium* with 100% identity (accession no: AB362603.1) and RM28 was

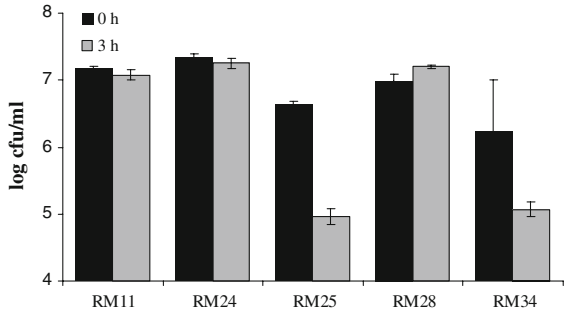

Fig. 1 Acid tolerance activity of LAB strains (Mean ± SD). Survival of LAB strains was compared by plate counting after exposure to low pH 2.5 in PBS for 0 and 3 h

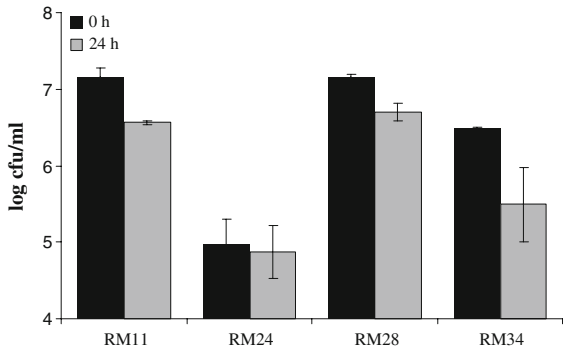

Fig. 2 Bile salt tolerance activity of LAB strains (Mean ± SD). Survival of LAB strains was compared by plate counting after exposure with bile salt 0.3% in MRS broth for 0 and 24 h

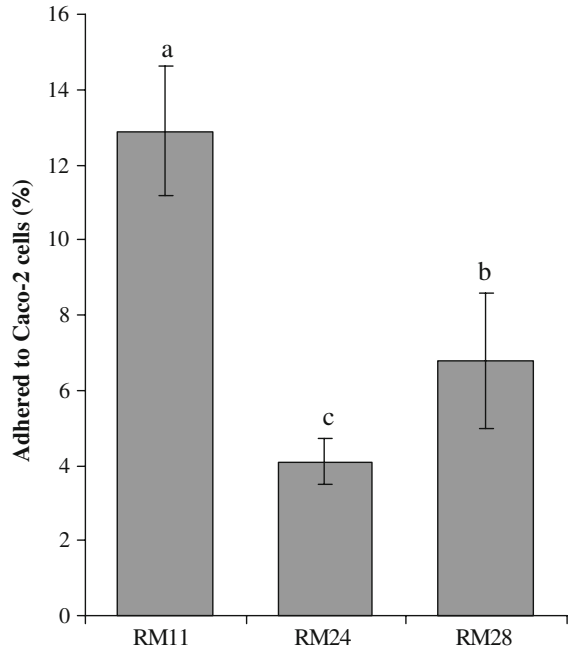

Fig. 3 Adhesion ability of LAB strains to Caco-2 cells (Mean ± SD). [a,b,c]Mean values with different superscript letters were significantly different at $P < 0.05$. The adherence percentage of LAB strains to Caco-2 cells was compared by plate counting between initial and adhered bacteria

Lactobacillus fermentum with 99% identity (accession no: AP008937.1).

Potential of probiotic LAB strains on antiproliferation of colon cancer cells

The activity of culture medium (CM) or live whole cells correlates with the effect of growth inhibition of colon cancer cells (Table 2). CM from *E. faecium* RM11 and *L. fermentum* RM28 strains inhibited proliferation of colon cancer cells at 21 and 23%, respectively, by using MTT. The use of CM and live whole cells from these probiotic strains with a Trypan Blue exclusion exhibited significantly higher ($P < 0.01$) anti-proliferation of colon cancer cells as compared to the control group.

Discussion

The application of probiotics as a functional food is of continually increasing significance to human health each year. One purpose of using probiotics as a

Table 2 Potential of probiotic LAB on antiproliferation of colon cancer cells (Mean ± SD)

Probiotic strains	Antiproliferation (%)		
	MTT Cultured medium	Trypan Blue exclusion	
		Live whole cells	Cultured medium
Control	–	1 ± 0^{a}	4 ± 2^{a}
Enterococcus faecium RM11	21 ± 9	29 ± 3^{b}	26 ± 10^{b}
Lactobacillus fermentum RM28	23 ± 5	29 ± 4^{b}	22 ± 8^{b}

Two bacteria forms were applied, as CM and live whole cells. Bacteria of each tested strain were grown in MRS broth at 37°C for 18 h. The CM was prepared by the removal of live whole cells using centrifugation at 5,000*g* for 10 min followed by filtration through a sterilized filter of 0.2 μm

[a,b] Mean values with different superscript letters in the same column were significantly different at $P < 0.01$

biological product is for prevention of human cancers such as colon cancer (Commane et al. 2005; Rafter 2003). Of the 54 strains of LAB, obtained after fermenting dairy milks, five produced antimicrobial substances which inhibited the growth of eight food-borne pathogens. Maragkoudakis et al. (2006) also reported that potential probiotic LAB strains isolated from dairy products could inhibit the growth of *H. pylori*, *E. coli*, and *S. typhimurium*. Some of these pathogenic strains are associated with causing cancers, such as *H. pylori* with gastric and colon cancer (Shmuely et al. 2001) or *E. coli* with colon cancer (Travaglione et al. 2008). Three of the LAB strains—RM11, RM24, and RM28—survived in a gastrointestinal tract model of pH 2.5 with 0.3% bile salt. Similarly, probiotic *Lactobacillus* can survive at pH 2.5 in 0.3% bile salts (Pennacchia et al. 2004; Zoumpopoulou et al. 2008). No blood haemolysis was observed in our LAB strains, indicating that they were non-pathogenic (Maragkoudakis et al. 2006) they were, however, kanamycin resistant, in this investigation. Resistance to kanamycin was also reported for *Lactobacillus* strains isolated from infant faeces (Arici et al. 2004). Good adherence activity of RM11 and RM28 to Caco-2 cells was observed. Maragkoudakis et al. (2006) reported that lactobacilli isolated from dairy products had different rates of Caco-2 cell adherence, ranging from 0.2% to 25.5%. Most had a low adhesion rate (<4%). Also, a probiotic strain of *L. fermentum* isolated from food adhered to Caco-2 cells (Baccigalupi et al. 2005). Our result suggests that probiotic LAB strains can adhere to epithelial cells of the intestine which is where the probiotic action or colon cancer originates. Two probiotic strains in our finding, *E. faecium* and *L. fermentum*, are normally found as original strains in fermented dairy products, and are recommended as beneficial probiotics (Leroy et al. 2003; Zoumpopoulou et al. 2008).

Both CM and live whole cells from *E. faecium* RM11 and *L. fermentum* RM28 strains trigger antiproliferation of colon cancer cells. Ewaschuk et al. (2006) concluded that, the conditioned medium of probiotic LAB (VSL3) reduced the viability and induced apoptosis of colon cancer cells (Caco-2 and HT-29). Gonet-Surowka et al. (2007) suggested that only some species of lactobacilli were probiotic and that both live and heat-killed forms had strongly activated pan-caspases, resulting in colon cancer cell apoptosis. The action mode of both probiotic strains in our finding might trigger a mechanism in colon cancer cells, resulting in cell apoptosis.

Acknowledgements Financial support for this research was provided by the National Research Council of Thailand.

References

Arici M, Bilgin B, Sagdic O, Ozdemir C (2004) Some characteristics of *Lactobacillus* isolates from infant faeces. Food Microbiol 21:19–24

Baccigalupi L, Donato AD, Parlato M, Luongo D, Carbone V, Rossi M, Ricca E, Felice MD (2005) Small surface-associated factors mediate adhesion of a food-isolate strain of *Lactobacillus fermentum* to Caco-2 cells. Res Microbiol 156:830–836

Commane D, Hughes R, Shortt C, Rowland I (2005) The potential mechanisms involved in the anti-carcinogenic action of probiotics. Mutat Res 591:276–289

Ewaschuk JB, Walker JW, Diaz H, Madsen KL (2006) Bioproduction of conjugated linoleic acid by probiotic bacteria occurs in vitro and in vivo in mice. J Nutr 136:1483–1487

Gonet-Surowka AK, Strus M, Heczko PB (2007) Influence of Lactobacilli probiotic strains on apoptosis of colon cancer cells lines. Int J Antimicrob Agents 29:S343–S344

Leroy F, Moreno MRF, Vuyst LD (2003) *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. Int J Food Microbiol 88:235–240

Maragkoudakis PA, Zoumpopoulor G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006) Probiotic potential of

Lactobacillus strains isolated from dairy products. Int Dairy J 16:189–199

Moreno D, LeBlanc DA, Matar C, Perdigón G (2007) The application of probiotics in cancer. Br J Nutr 98 (Suppl 1):S105–S110

Pennacchia C, Ercolini D, Blaiotta G, Pepe O, Mauriello G, Villani F (2004) Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. Meat Sci 67:309–317

Rafter J (2003) Probiotics and colon cancer. Best Pract Res Clin Gastroenterol 17:849–859

Saikali J, Picard C, Freitas M, Holt P (2004) Fermented milks, probiotic cultures, and colon cancer. Nutr Cancer 49:14–24

Salminen S, Wright A, Morelli L, Marteuau P, Brassart D, Vos WM, Fonden R, Saxelin M, Collins K, Mogensen G, Birkeland SE, Mattila-Sandholm T (1998) Demonstration of safety of probiotics—a review. Int J Food Microbiol 44:93–106

Shmuely H, Passaro D, Figer A, Niv Y, Pitlik S, Samra Z, Koren R, Yahav J (2001) Relationship between *Helicobacter pylori* CagA status and colorectal cancer. Am J Gastroenterol 96:3406–3410

Travaglione S, Fabbri A, Fiorentini C (2008) The Rho-activating CNF1 toxin from pathogenic *E. coli*: A risk factor for human cancer development? Infect Agent Cancer 3:4

Zoumpopoulou G, Foligne B, Christodoulou K, Grangette C, Pot B, Tsakalidou E (2008) *Lactobacillus fermentum* ACA-DC 179 displays probiotic potential in vitro and protects against trinitrobenzene sulfonic acid (TNBS)-induced colitis and *Salmonella* infection in murine models. Int J Food Microbiol 121:18–26