Inhibitory Activity of Fermented Milk with *Lactobacillus Casei* Strain Shirota against Common Multidrug-Resistant Bacteria Causing Hospital-Acquired Infections

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Objective: *To determine inhibitory activity of fermented milk with Lactobacillus casei strain Shirota (FMLC) against common multi-drug-resistant (MDR) bacteria causing hospital-acquired infections.*
Material and Method: *Time-kill methods of FMLC and cell-free filtered fluid of FMLC (CF-FMLC) against Acinetobacter baumannii, Pseudomonas aeruginosa, ESBL-producing Escherichia coli & Klebsiella pneumoniae and methicillin-resistant Staphylococcus aureus were conducted. The control solutions were Mueller Hinton broth (MHB) and distilled water. The mixtures of FMLC, CF-FMLC, MHB and DW with 10^5 to 10^6 CFU/ml of each bacterium were prepared and incubated at 35°C. Each mixture was quantified for viable bacteria at 0, 1, 3, 6 and 24 hr after incubation onto brain heart infusion agar plates. The inoculated agar plates were incubated at 35°C for 24-48 hr. Bacterial colonies on agar plates were counted and compared among the mixtures.*
Results: *Log CFUs of each organism in MHB and distilled water after incubation were increased from 5.1-6.3 at 0 h to 6.4->11 at 24 hr. Log CFUs of each organism in FMLC and CF-FMLC after incubation with study bacteria for 0, 1, 3, 6 and 24 hr were decreased to undetectable amounts at 24 hr.*
Conclusion: *FMLC and CF-FMLC exerted slow inhibitory activity against MDR bacteria resulting in eradication of all study bacteria at 24 hr. Such inhibitory effects were probably due to the products of the milk fermented by Lactobacillus casei strain Shirota. Clinical study is needed to determine if consumption of FMLC can prevent and treat colonization and infection with MDR bacteria in hospitalized patients.*

Keywords: *Inhibitory activity, Probiotics, Fermented milk, Lactobacillus casei strain Shirota, Multidrug-resistant bacteria, Hospital-acquired infections*

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Hospital-acquired infection (HAI) is a serious and growing problem at every level of the healthcare system. HAI is associated with an increased attributable mortality, length of hospital stay and healthcare costs incurred by patients, insurers and healthcare facilities. The burden of HAI in developing countries is much more than that in developed countries[1,2]. The prevalence of HAI in Thailand was 6.5% with highest prevalence in university hospitals (7.6%)[3,4]. The common sites of HAI in Thailand were lower respiratory tract (36%) and urinary tract (26%). The common bacteria causing HAIs were usually resistant to antibiotics including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, ESBL-producing *Klebsiella pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA). Colonization with resistant bacteria are common in hospitalized patients especially those who received antibiotics resulting in developing HAI due to such resistant bacteria[5-8]. Therefore, non-antibiotic measures to prevent or eradicate colonization of resistant bacteria in hospitalized patients are welcome.

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host[9]. Lactic acid bacteria and bifidobacteria are the most common types of microbes

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used as probiotics. A recent meta-analysis of randomized controlled trials comparing probiotics and control in patients undergoing mechanical ventilation revealed that administration of probiotics was associated with lower incidence of ventilator-associated pneumonia than control[10]. However, there were many types of probiotics with different dosing regimens in the aforementioned review and only some of them showed benefits. Siriraj Hospital is conducting a randomized controlled study to determine an effectiveness of fermented milk with *Lactobacillus casei* strain Shirota (FMLC) for prevention of ventilator-associated pneumonia (VAP) in hospitalized patients. Since the common causative bacteria causing VAP at Siriraj Hospital are *A. baumannii*, *P. aeruginosa*, ESBL-producing Gram negative bacteria and MRSA, the objective of the study was to determine inhibitory activity of fermented milk with *Lactobacillus casei* strain Shirota against such common MDR bacteria causing HAIs.

Material and Method

Probiotics products

They were fermented milk with *Lactobacillus casei* strain Shirota (FMLC) and cell-free filtered fluid of FMLC (CF-FMLC). CF-FMLC was prepared by filtering the FMLC with 0.2 µm membrane.

Control media

They were cation-adjusted Mueller-Hinton broth (MHB) and distilled water.

Study bacteria

They were MDR *A. baumannii*, MDR *P. aeruginosa*, ESBL-producing *E. coli*, ESBL-producing *K. pneumoniae* and MRSA.

Time-kill curve technique

The mixtures of the present study bacteria and the probiotics products or the control media were prepared as shown in Table 1. The mixtures were incubated at 35°C. The viable cells of bacteria were quantified at 0, 1, 3, 6 and 24 hr after incubation. The repetitive colony counts were performed by 10-fold serial dilution in sterile normal saline from 10^{-1} to 10^{-10}. An aliquot of 100 µl from each diluted tube was inoculated on brain-heart infusion (BHI) agar plates, and the inoculated plates were incubated at 35°C for 24-48 hr. In order to detect minimal amounts of viable bacteria, 100 µl of undiluted samples at 6 hr were inoculated on BHI plates and 1.0 ml of undiluted samples at 24 hr were inoculated on BHI plates and 5 ml of MHB. The inoculated BHI plates and MHBs were incubated at 35°C for 24-48 hr. Then subcultures of inoculated MHBs were performed to determine colony counts. The colony forming units (CFUs) of bacteria were calculated from the colony count and were expressed as CFUs/ml.

Results

The amounts of each type of study bacteria after mixing them with MHB, distilled water, FMLC and CF-FMLC and incubated at 35°C for 0, 1, 3, 6 and 24 hr are shown in Fig. 1 to 5. Log CFUs of each organism in MHB and distilled water after incubation were increased from 5.1-6.3 at 0 hr to 6.4-> 11 at 24 hr. The amounts of all types of bacteria incubated in MHB were more than those in distilled water. Log CFUs of each bacterium in FMLC and CF-FMLC after incubation for 0, 1, 3, 6 and 24 hr were decreased until reaching an undetectable amount at 24 hr after incubation.

Table 1. The mixtures of study bacteria and probiotics products or control media

Reagent	Tube Number			
	1	2	3	4
FMLC	5 ml	-	-	-
CF-FMLC	-	5 ml	-	-
MHB	-	-	5 ml	-
Distilled water	-	-	-	5 ml
Study organism 10^7 CFUs/ml	100 µl	100 µl	100 µl	100 µl

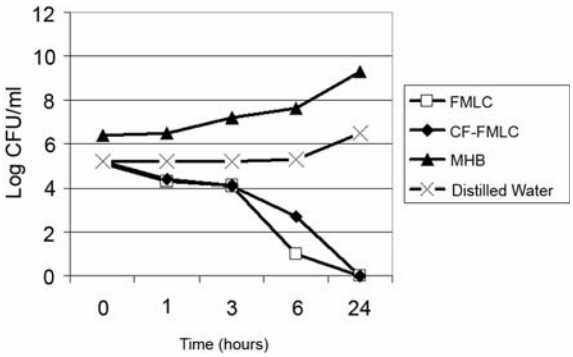

Fig. 1 Time-kill curve of fermented milk with *Lactobacillus casei* strain Shirota (FMLC), cell-free filtered fluid of FMLC (CF-FMLC), Mueller-Hinton broth (MHB) and distilled water against MDR *A. baumannii*

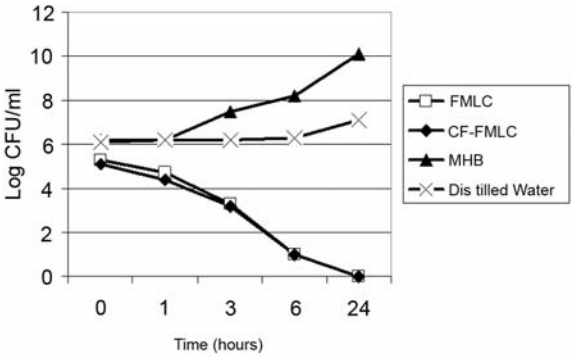

Fig. 2 Time-kill curve of fermented milk with *Lactobacillus casei* strain Shirota (FMLC), cell-free filtered fluid of FMLC (CF-FMLC), Mueller-Hinton broth (MHB) and distilled water against MDR *P. aeruginosa*

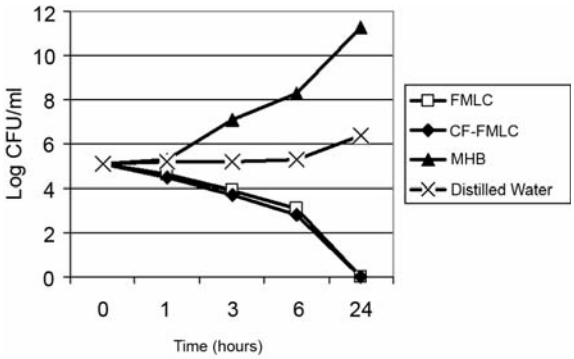

Fig. 3 Time-kill curve of fermented milk with *Lactobacillus casei* strain Shirota (FMLC), cell-free filtered fluid of FMLC (CF-FMLC), Mueller-Hinton broth (MHB) and distilled water against ESBL-producing *E. coli*

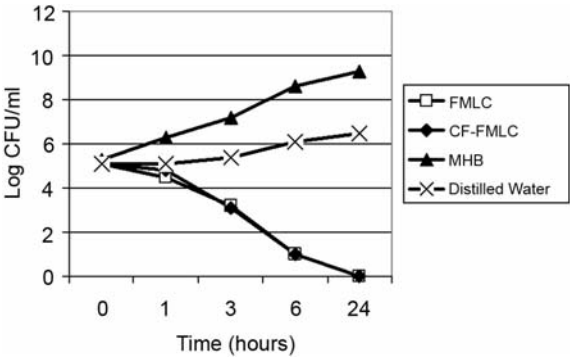

Fig. 4 Time-kill curve of fermented milk with *Lactobacillus casei* strain Shirota (FMLC), cell-free filtered fluid of FMLC (CF-FMLC), Mueller-Hinton broth (MHB) and distilled water against ESBL-producing *K. pneumoniae*

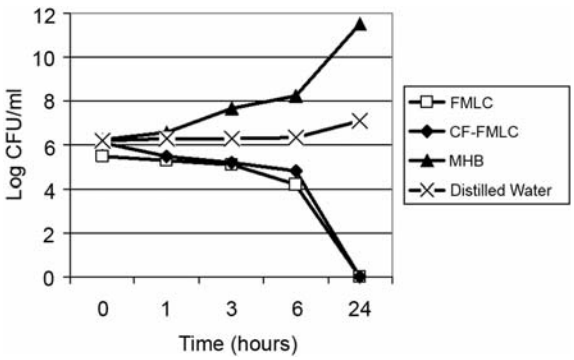

Fig. 5 Time-kill curve of fermented milk with *Lactobacillus casei* strain Shirota (FMLC), cell-free filtered fluid of FMLC (CF-FMLC), Mueller-Hinton broth (MHB) and distilled water against MRSA

Discussion

The present study results showed that FMLC and CF-FMLC exerted slow inhibitory activity against MDR bacteria resulting in eradication of all study organisms at 24 hr. Since both FMLC and CF-FMLC had similar effects on inhibiting bacterial growth, such inhibitory effects were probably due to the products of the milk fermented by *Lactobacillus casei* strain Shirota, such as lactic acid. However, the aforementioned observations are only *in vitro* effects of fermented milk with *Lactobacillus casei* strain Shirota against MDR bacteria. A recent study reported a protective effect of *Lactobacillus casei* strain Shirota against lethal infection with multi-drug resistant Salmonella enterica serovar Typhimurium DT104 in mice[11]. Many clinical studies of various types of probiotics with different dosing regimens revealed that many probiotics products were beneficial for several health conditions whereas some of them were not[10,12-16]. Therefore, clinical study is needed to determine if consumption of specific type and dose of probiotics is effective for a particular health condition. The evidence of inhibitory effect on MDR bacteria from fermented milk with *Lactobacillus casei* strain Shirota supports the rationale of ongoing clinical study on effectiveness of fermented milk with *Lactobacillus casei* strain Shirota for prevention of ventilator-associated pneumonia in hospitalized patients.

Potential conflicts of interest

None.

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ฤทธิ์ของนมเปรี้ยวที่มี *Lactobacillus casei* สายพันธุ์ Shirota ในการยับยั้งแบคทีเรียดื้อยาที่ก่อการติดเชื้อในโรงพยาบาล

สุรภี เทียนกริม, วิษณุ ธรรมลิขิตกุล

วัตถุประสงค์: *เพื่อทราบฤทธิ์ของนมเปรี้ยวที่มี Lactobacillus casei สายพันธุ์ Shirota ในการยับยั้งแบคทีเรียดื้อยาที่ก่อการติดเชื้อในโรงพยาบาล*

วัสดุและวิธีการ: *นำนมเปรี้ยวที่มี Lactobacillus casei สายพันธุ์ Shirota และนมเปรี้ยวที่กรอง Lactobacillus casei สายพันธุ์ Shirota ออกแล้วมาผสมกับเชื้อ Acinetobacter baumannii, Pseudomonas aeruginosa, ESBL-producing Escherichia coli & Klebsiella pneumoniae และ methicillin-resistant Staphylococcus aureus ในปริมาณ 10^5-10^6 CFUs ต่อ มล. โดยมี Mueller Hinton broth (MHB) และน้ำกลั่นเป็นสารเปรียบเทียบ นำส่วนผสมของสารดังกล่าวและแบคทีเรียดังกล่าวไปอบที่อุณหภูมิ 35 องศาเซลเซียส แล้วนำส่วนผสมดังกล่าวมาเพาะเชื้อเป็นระยะที่ 0, 1, 3, 6 และ 24 ชั่วโมงในจานวุ้น brain heart infusion แล้วนับจำนวนแบคทีเรียที่เจริญเติบโตในจานวุ้น*

ผลการศึกษา: *แบคทีเรียใน MHB และน้ำกลั่นมีจำนวนเพิ่มขึ้นอย่างต่อเนื่องจาก 5.1 ถึง 6.3 Log CFUs ที่ 0 ชั่วโมงเป็น 6.4 ถึงมากกว่า 11 Log CFUs ที่ 24 ชั่วโมง ส่วนแบคทีเรียในนมเปรี้ยวที่มี Lactobacillus casei สายพันธุ์ Shirota และนมเปรี้ยวที่กรอง Lactobacillus casei สายพันธุ์ Shirota ออกแล้วมีจำนวนลดลงอย่างต่อเนื่องจนไม่พบเชื้อที่ 24 ชั่วโมง*

สรุป: *นมเปรี้ยวที่มี Lactobacillus casei สายพันธุ์ Shirota และนมเปรี้ยวที่กรอง Lactobacillus casei สายพันธุ์ Shirota ออกแล้วมีฤทธิ์ยับยั้งแบคทีเรียดื้อยาได้อย่างช้าๆ และสามารถกำจัดเชื้อได้หมดที่ 24 ชั่วโมง ฤทธิ์ดังกล่าวน่าจะเป็นผลของสารที่มีอยู่ในนมเปรี้ยว ควรมีการศึกษาทางคลินิกว่านมเปรี้ยวที่มี Lactobacillus casei สายพันธุ์ Shirota มีประสิทธิผลในการป้องกันและรักษาแบคทีเรียดื้อยาที่อาศัยอยู่ในผู้ป่วยหรือก่อการติดเชื้อในผู้ป่วยที่อยู่ในโรงพยาบาลหรือไม่*