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RESEARCH ARTICLE

Antiviral Activity of Yogurt against Enterovirus 71 in Vero Cells

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Abstract Five yogurts were fermented with each bacteria strain. The viability and pH of each yogurt during fermentation or storage were evaluated, and then the cytotoxicity and antiviral activity against enterovirus (EV) 71 of cell-free supernatants (CFS) of the metabolites of each yogurt were compared with those of de Man, Rogosa, and Sharpe (MRS) broth fermented with the same bacteria. As the results, the number of viable bacteria for each yogurt after fermentation or during storage always remained higher than 5 log CFU/mL and the pH of those ranged from 4 to 6. The CFS of all yogurts showed antiviral activity over 45% against EV71, while it didn't exhibit cytotoxicity in Vero cells. Specially, the CFS of yogurt fermented with *Lactobacillus plantarum* and *Bifidobacterium bifidum* exhibited high anti-EV71 activity of 92.74 and 90.44%, respectively. In contrast, the CFS of each MRS broth fermented with the same bacteria showed low antiviral activity of less than 30%.

Keywords: yogurt, cell-free supernatant, antiviral activity, enterovirus 71, cytotoxicity

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Introduction

Enterovirus 71 (EV71) is a positive-stranded RNA virus that belongs to the enterovirus genus of the Picornaviridae family (1). EV71-infected children can develop severe neurological complications, which can lead to rapid clinical deteriorations and even death (2-5). In 1998, an EV71 infection epidemic affected more than 100,000 young children in Taiwan, from which approximately 400 children were hospitalized for pulmonary edema and neurogenic shock, ultimately resulting in 78 deaths (6-8). At this time, neither vaccine nor therapeutic treatment was available.

Lactic acid bacteria, including lactobacilli and bifidobacteria, are the most common bacterial species considered as potential probiotics (9). To date, probiotics are mainly selected from the *Lactobacillus* and *Bifidobacterium* genera. Numerous health benefits have been attributed to selected probiotic strains, including well-documented lactose intolerance alleviation, prevention, and reduction of symptoms associated with rotavirus and antibiotic associated diarrhea, allergy prevention, hypocholesterolemic, anti-carcinogenic, antimutagenic effects, and immunomodulation (10). These health benefits have been attributed to shifts in the intestinal microbial balance (11). The viability and survival of probiotic bacteria during dairy processing and storage are important for providing desired health benefits (12, 13). A previous study also showed that supernatants of MRS broth fermented with probiotic bacteria produced metabolites with an antiviral potential against vesicular stomatitis virus (VSV) (14).

In this study, we investigated the cytotoxicity, antiviral activity, and effect on enterovirus (EV) 71-induced cytopathic effects (CPE) of the cell-free supernatants (CFS) of the metabolites of each yogurts by *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Streptococcus thermophilus,* and *Bifidobacterium bifidum* were compared with those of de Man, Rogosa, and Sharpe

(MRS) broth fermented with the same bacteria.

Materials and Methods

Virus, cell line, and reagents The EV71 was obtained from Chungcheongnam-do Health and Environment Research Institute in Korea, and was propagated in African green monkey kidney (Vero) cells at 37°C. Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic solution. Antibiotic-antimycotic solution, trypsin-ethylenediaminetetraacetic acid (EDTA), FBS, and MEM were supplied by Gibco BRL (Invitrogen Life Technologies, Karlsruhe, Germany). The tissue culture plates were purchased from Falcon (BD Biosciences, San Jose, CA, USA). Sulforhodamine B (SRB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade.

Production of yogurt Cows' milk was provided by Seoul milk (Korea) and MRS agar was supplied by Gibco BRL. *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, and *Bifidobacterium bifidum* were provided by Bision Corporation (Seongnam, Korea). Each organism was stored at –70°C prior to use. Sterile 10 mL portions of MRS broth were inoculated with each organism and incubated for 1 day at 40°C. Activated organisms after 3 successive transfers were used for the production of yogurt. Yogurts were prepared in 5 sterilized glass containers, where 100 mL of commercial cows' milk was inoculated with 3×10^5 CFU/mL of *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *S. thermophilus,* and *B. bifidum.* All experiments were conducted under aseptic conditions in a laminar flow. Inoculated cows' milk batches were incubated at 40°C for 1 day. The products were removed from the incubator and stored at 4°C for 12 days.

Bacterial growth and viability during fermentation and cold storage The colony counts of yogurt were determined just after inoculation, during fermentation and monitored thereafter at 2 hr intervals. Counts on MRS agar were determined at 1, 3, 6, 8, and 12 days of cold storage. Viability of bacteria was expressed as log colony forming units (CFU)/mL.

Measurement of pH The pH of samples was determined using a pH meter (Model 420; Thermo Orion, Minneapolis, MN, USA). The pH of all yogurt batches was recorded at the end of fermentation and subsequently at 1, 3, 6, 8, and 12 days of cold storage.

Preparation of the metabolites for measurement antiviral activity and cytotoxicity Cows' milk and MRS broth were inoculated with each bacteria, flasks were cooled after 1 day and stored at 4°C. Cell-free supernatants (CFS) of the metabolites were obtained when 1 mL of each samples was diluted with 1 mL MEM to adjust to pH 7.0 value and filtered using 0.22-μm syringe filter (Millipore Corp., Bedford, MA, USA). The CFS of yogurt and MRS broth were kept frozen (–20°C).

Cytotoxicity assay Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. Next day, medium was removed and then washed with 1× phosphate buffered saline (PBS). The 96-well plates were exposed to 5 CFS (3 wells/CFS) in maintenance medium for 2 days at 37°C, in parallel with the virus-infected cell cultures. For each CFS, 3 wells were used as controls (non-CFS-treated cells). After 2 days of incubation, cytotoxicity was evaluated by SRB assay as previously described (15). Cytotoxicity was presented as % of control.

Assay of antiviral activity The antiviral activities of the CFS against EV71 were determined by a CPE reduction method. One day before infection, Vero cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. Next day, medium was removed and then washed with 1×PBS. Subsequently, 0.09 mL of the diluted virus suspension containing 50% cell culture infective dose ($CCID_{50}$) of the virus stock was added to produce appropriate CPE within 2 days after infection, followed by the addition of 0.01 mL of CFS. The wells were used as the virus controls (virus-infected non-CFS-treated cells) and the cell controls (non-infected non-CFS-treated cells). The culture plates were incubated at 37°C in 5% CO_2 for 2 days until appropriate CPE was achieved. Subsequently, the 96-well plates were washed once with 1×PBS, and 100 μL of cold (–20°C) 70% acetone was added on each well and left standing for 30 min at –20°C. After the removal of 70% acetone, the plates were dried in a dry oven for 30 min, followed by the addition of 100 μL of 0.4%(w/v) SRB in 1% acetic acid solution to each well, and left standing at room temperature for 30 min. SRB was then removed, and the plates were washed 5 times with 1% acetic acid before oven-drying. The plates were dried in a dry oven. After drying for 1 day, the morphology of the cells to observation the effect of CFS on EV71-induced CPE was observed under microscope at 32×40 magnification (Axiovert 10; Zeiss, Wetzlar, Germany), and images were recorded. Bound SRB was then solubilized with 100 μL of 10 mM unbuffered Tris-base solution, and the plates were left on a table for 30 min. The absorbance was then read at 540 nm by using a VERSAmax microplate reader (Molecular

Devices, Palo Alto, CA, USA) with a reference absorbance at 620 nm. The results were then transformed into percentage of the controls, and the percent protection achieved by the test CFS in the EV71-infected cells was calculated using the following formula:

$$\frac{(OD_t)_{EV71}-(OD_c)_{EV71}}{(OD_c)_{mock}-(OD_c)_{EV71}} \times 100 \text{ (expressed in \%)}$$

where $(OD_t)_{EV71}$ is the optical density measured with a given CFS in EV71-infected cells; $(OD_c)_{EV71}$ is the optical density measured for the control untreated EV71-infected cells; and $(OD_c)_{mock}$ is the optical density measured for control untreated mock-infected cells. Antiviral activity was presented as % of control.

Results and Discussion

Cell growth during fermentation *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *S. thermophilus,* and *B. bifidum* grew well during fermentation. After cows' milk was inoculated with each bacteria, their numbers steadily increased during the 1 day fermentation period. Growth of *L. acidophilus* in cows' milk was the slowest of all bacterial strains. However, their growth patterns followed different trajectories. Growth of *B. bifidum* was faster than the other 4 bacteria, whereas little growth of the other 4 bacteria was shown in the first 8 hr (Fig. 1). After 1 day of fermentation, the number of *L. rhamnosus* was highest, while *L. rhamnosus* outnumbered *L. acidophilus* by about 3 fold. The final number of *L. plantarum*, *S. thermophilus* and *B. bifidum* were similar at approximately 8 log CFU/mL.

pH change during fermentation The initial pH of cows' milk was 7.1. The pH of each yogurt after 1 day of fermentation was between 4.0 and 6.0. Changes in pH of each yogurt before and after fermentation are presented in Fig. 2. The changes in pH during fermentation were found to vary with each bacterial strain. Yogurt with *L. rhamnosus* exhibited the highest pH decline during fermentation. After 1 day, the yogurt containing *B. bifidum* was the only sample to maintain pH 6.0.

Viability of bacteria and pH changes during cold storage Overall, the viability of all bacteria in each yogurt decreased during cold storage (Fig. 3). After 12 days of cold storage, yogurt fermented with *L. rhamnosus* exhibited counts over 8 log CFU/mL, while yogurt fermented with *B. bifidum* exhibited counts over 7 log CFU/mL. Other yogurt samples exhibited counts over 5 log CFU/mL. After 12 days of cold storage, the pH of each yogurt ranged from 4 to 6, without greatly changing from the 1st stage of cold (Table 1).

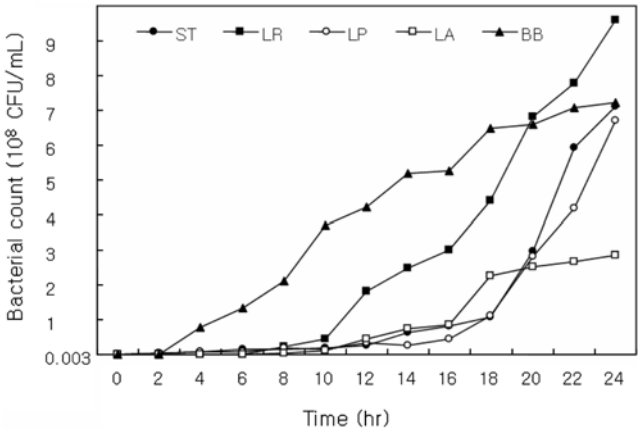

Fig. 1. Growth of bacteria during fermentation at 40°C. ST, *Streptococcus thermophilus*; LR, *Lactobacillus rhamnosus*; LP, *Lactobacillus plantarum*; LA, *Lactobacillus acidophilus*; BB, *Bifidobacterium bifidum*

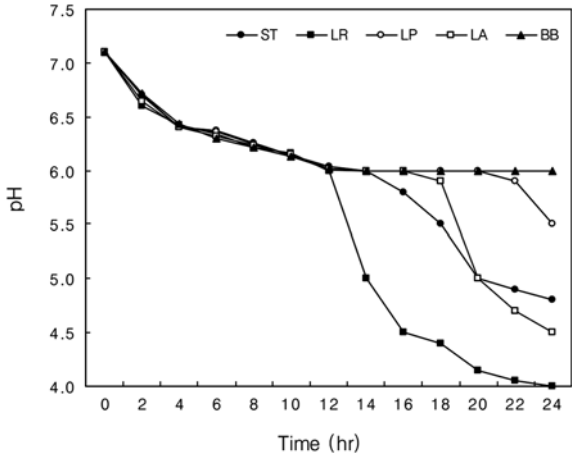

Fig. 2. pH changes during fermentation at 40°C. ST, *Streptococcus thermophilus*; LR, *Lactobacillus rhamnosus*; LP, *Lactobacillus plantarum*; LA, *Lactobacillus acidophilus*; BB, *Bifidobacterium bifidum*

Antiviral activity and cytotoxicity of cell-free supernatants of the metabolites against enterovirus 71 The CFS of each yogurt and MRS broth were tested for antiviral activity against EV71. The CFS of the 5 yogurts showed antiviral activity over 45% against EV71 without exhibiting cytotoxicity in Vero cells (Table 2). In particular, the CFS of yogurt fermented with *L. plantarum* and *B. bifidum* exhibited high anti-EV71 activity of 92.74 and 90.44%, respectively. In contrast, the CFS of each MRS broth showed low antiviral activity of less than 30%.

Effect of CFS on EV71-induced CPE Each CFS of yogurt and MRS broth displayed typical spread-out shapes and normal morphology in Vero cells (Fig. 4C, 4E, 4G, 4I, 4K, 5C, 5E, 5G, 5I, and 5K). Infection with EV71, in the

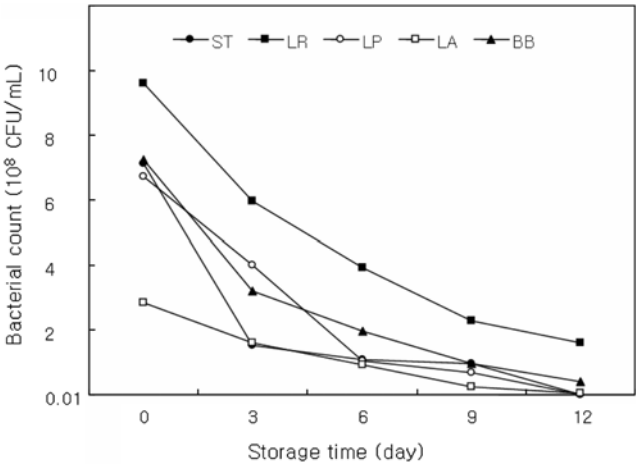

Fig. 3. Viability of bacteria during cold storage. ST, *Streptococcus thermophilus*; LR, *Lactobacillus rhamnosus*; LP, *Lactobacillus plantarum*; LA, *Lactobacillus acidophilus*; BB, *Bifidobacterium bifidum*

absence of the CFS, resulted in severe CPE (Fig. 4B and 5B). Addition of the CFS of yogurt on EV71-infected Vero cells inhibited the formation of visible CPE (Fig. 4D, 4F, 4H, 4J, and 4L). The morphology of EV71-infected Vero cells in the presence of CFS of yogurt fermented with *L. plantarum* or *B. bifidum* powerfully prevented CPE (Fig. 4D and 4H), but the increasing CPE of Vero cells after infection with EV71 wasn't greatly decreased from that of EV71 by addition of CFS of MRS broth (Fig. 5D, 5F, 5H, 5J, and 5L).

We assessed cell growth and pH changes of cows' milk inoculated with lactic acid bacteria. The cytotoxicity, antiviral activity, and effect on virus-induced CPE of the CFS of each yogurt were compared with those of MRS broth. All cultured bacterial strains exhibited changes in cell growth and pH during fermentation and storage. However, there was notable difference between cell growth and pH changes. Regardless of their different growth trajectories, final bacterial count was similar in yogurt fermented with *L. plantarum*, *S. thermophilus,* and *B. bifidum* after 1 day. The pH of yogurt fermented with *L. plantarum* and *B. bifidum* were over 5.5 during fermentation and storage, whereas in *S. thermophilus* pH decreased to less than 5. Lactic acid bacteria were present in all yogurts at levels more than 8 log CFU/mL after fermentation and exhibited counts over 5 log CFU/mL during cold storage. Several studies have reported on the effects of factors such as acidity, pH, and concentrations of lactic and acetic acids during yogurt manufacture and storage (16-18). Some authors suggested the minimum level for probiotic microorganisms in fermented milks in order to produce therapeutic benefits was above 10^5 CFU/mL (19,20). Also, milk- and whey-derived products are known for their positive effects including modulation of the immune

Table 1. pH changes of yogurt during cold storage[1)]

Storage time (day)	ST	LR	LP	LA	BB
0	4.8	4	5.5	4.5	6
3	4.7	4	5.5	4.5	6
6	4.6	4	5.5	4.5	6
9	4.6	4	5.5	4.5	6
12	4.5	4.5	5.5	4	6

[1)]ST, yogurt fermented *Streptococcus thermophilus*; LR, yogurt fermented with *Lactobacillus rhamnosus*; LP, yogurt fermented with *Lactobacillus plantarum*; LA, yogurt fermented with *Lactobacillus acidophilus*; BB, yogurt fermented with *Bifidobacterium bifidum*

Table 2. Antiviral activity of lactic acid bacteria metabolic products against EV71 in Vero cells

Bacteria[1)]	Yogurt	MRS broth
ST	52.20±1.83[2)]	26.68±2.16
LR	47.78±4.73	26.64±0.17
LP	92.74±1.63	11.47±1.25
LA	57.09±3.96	30.91±0.06
BB	90.44±2.64	21.85±1.71

[1)]ST, metabolites of *Streptococcus thermophilus*; LR, metabolites of *Lactobacillus rhamnosus*; LP, metabolites of *Lactobacillus plantarum*; LA, metabolites of *Lactobacillus acidophilus*; BB, metabolites of *Bifidobacterium bifidum*
[2)]Values represent the means of 3 independent experiments. Antiviral activity was presented as % of control.

system (21). We examined the antiviral activity of CFS of the metabolites of each yogurt and MRS broth against EV71, while also examining the effect of these on EV71-induced CPE. SRB assays indicate that the tested CFS of yogurts is able to inhibit virus multiplication *in vitro*. Among the yogurts tested, CFS of yogurt fermented with *L. plantarum* or *B. bifidum* were the most potent inhibitors of EV71 infection. The CFS of yogurt fermented with *L. acidophilus, L. rhamnosus,* or *S. thermophilus* were also effective, showing antiviral activity between 45-60%. The increasing CPE of EV71-infected Vero cells was greatly decreased by addition of CFS of yogurt fermented with *L. plantarum* or *B. bifidum*, and addition of those in Vero cells did not exhibit cytotoxicity with normal morphology. But, the CFS of each MRS broth was showed low antiviral activity of less than 30%, and the increasing CPE of EV71-infected Vero cells wasn't greatly decreased from that of EV71 by addition of that. The CFS of each fermented yogurt, after storage for 12 days, exhibited similar antiviral activity to CFS at the 1st stage after fermentation against EV71 (data not shown).

Probiotic bacteria are known to aid lactose digestion and resistance to enteric pathogens, possess anti-colon cancer benefits, and help with allergy and immune system modulation (22). Specifically, whey proteins, principally immunoglobulin, exert their anti-human, and simian

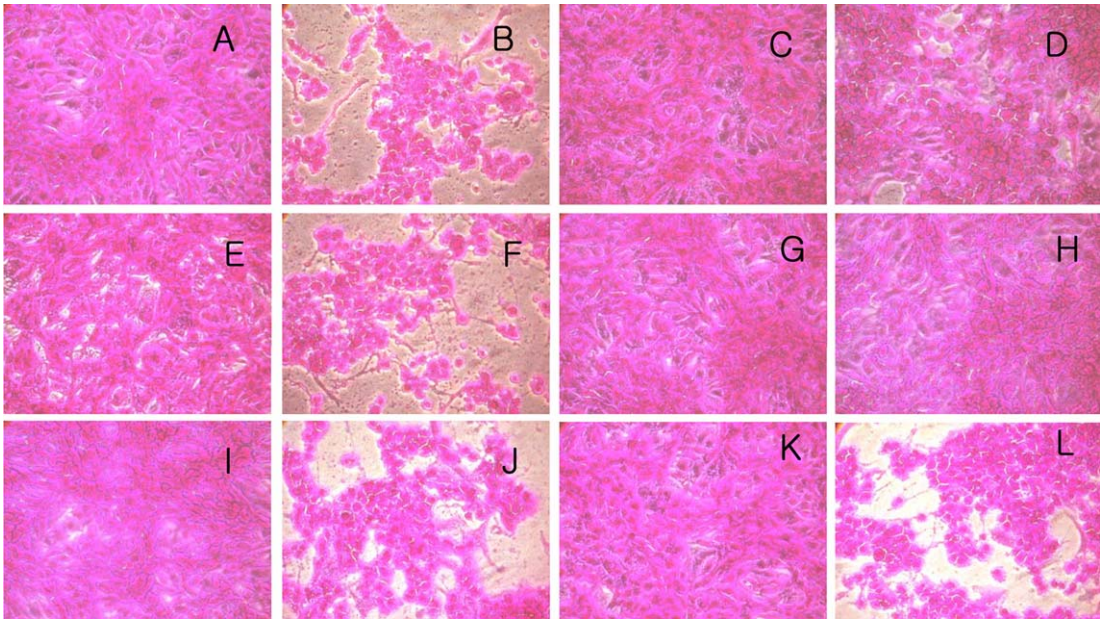

Fig. 4. Effect of cell free supernatants of the metabolites of yogurt on EV71-induced CPE. The 10 μL of CFS of yogurt were treated. After incubation at 37°C in 5% CO_2 for 2 days, inhibition of virus replication was evaluated by SRB assay, and images of cell morphology was taken a picture under microscope. (A) Noninfected cells, (B) EV71-infected cells, (C) Noninfected cells with CFS fermented with *B. bifidum*, (D) EV71-infected cells with CFS fermented with *B. bifidum*, (E) Noninfected cells with CFS fermented with *L. acidophilus*, (F) EV71-infected cells with CFS fermented with *L. acidophilus*, (G) Noninfected cells with CFS fermented with *L. plantarum*, (H) EV71-infected cells with CFS fermented with *L. plantarum*, (I) Noninfected cells with CFS fermented by *L. rhamnosus*, (J) EV71-infected cells with CFS fermented with *L. rhamnosus*, (K) Noninfected cells with CFS fermented with *S. thermophilus*, (L) EV71-infected cells with CFS fermented with *S. thermophilus*

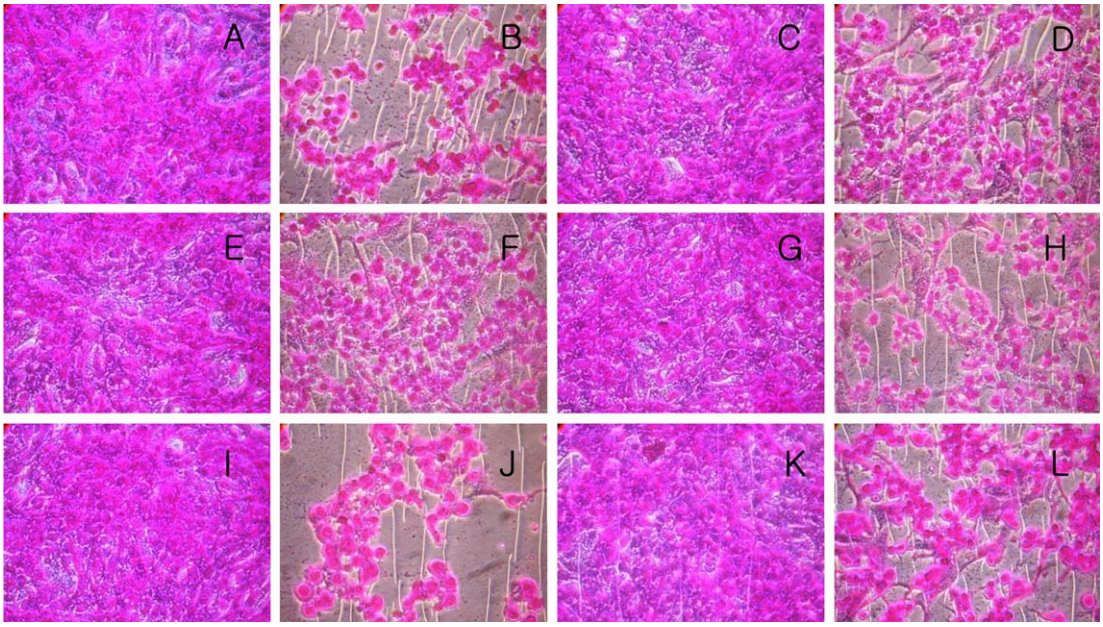

Fig. 5. Effect of cell free supernatants of the metabolites of MRS broth on EV71-induced CPE. The 10 μL of CFS of MRS broth were treated. After incubation at 37°C in 5% CO_2 for 2 days, inhibition of virus replication was evaluated by SRB assay, and images of cell morphology was taken a picture under microscope. (A) Noninfected cells, (B) EV71-infected cells, (C) Noninfected cells with CFS fermented with *B. bifidum*, (D) EV71-infected cells with CFS fermented with *B. bifidum*, (E) Noninfected cells with CFS fermented with *L. acidophilus*, (F) EV71-infected cells with CFS fermented with *L. acidophilus*, (G) Noninfected cells with CFS fermented with *L. plantarum*, (H) EV71-infected cells with CFS fermented with *L. plantarum*, (I) Noninfected cells with CFS fermented by *L. rhamnosus*, (J) EV71-infected cells with CFS fermented with *L. rhamnosus*, (K) Noninfected cells with CFS fermented with *S. thermophilus*, (L) EV71-infected cells with CFS fermented with *S. thermophilus*

rotavirus activity by neutralization of virus and toxins (23). Lactoferrin also possesses anti-HIV-1 activity by prevention of virus binding and inhibition of reverse transcriptase (24-26). These studies indicate that whey proteins exhibited also antiviral properties. Little, however, is known about the absorption of bioactive peptides with larger molecular weight in the intestine. It is well known that di- and tripeptides are easily absorbed in the intestine (27,28). Specifically, ACE-inhibitory tripeptide VPP has been reported to pass the membrane of Caco-2 cell monolayers in a significant amount, and paracellular diffusion has been proposed as the transport mechanism (29). According to present study, enzymes from bacteria starter cultures degrade proteins and peptides liberated by these enzymes also may differ from those liberated by digestive enzymes (30). To date, there is no approved antiviral agent for the treatment of enterovirus infections. The widespread use of lactic acid bacteria and bifidobacteria in fermented foods and dairy products has a long history of safety. In conclusion, yogurt fermented with these lactic acid bacteria may possess strong antiviral materials against EV71. We present a brief synopsis of important antiviral activities that are also attributed to many of these milk-derived materials. Further studies are necessary to isolate specific antiviral compounds and define their mechanism of action.

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