Abstract

The combinational inhibitory effects of *Lactobacillus*, 1-monoglycerides, and grapefruit seed extract against *Gardnerella vaginalis* KCTC 5097

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The objective of this study was to observe the antibacterial effects of lactobacilli, 1-monoglycerides (MGs) and grapefruit seed extract (GSE) against the vaginosis-associated pathogen *Gardnerella vaginalis*. Five lactobacilli were selected from 26 lactobacilli by observing the effect of pH (pH=7) adjusted lactobacilli cell-free culture

supernatant (CFCS) on the viability of *G. vaginalis*. Among them, 3 strains of *Lactobacillus* showed relatively high inhibition on the growth of *G. vaginalis* KCTC 5097 during the co-culture of *Lactobacillus* and *G. vaginalis.* In addition, the effects of MGs and GSE on the bacterial growth curve were monitored at various levels. Glycerol monolaurate (GML) and GSE had MIC (minimum inhibitory concentration) values of 78.125 µg/mL and 20 ppm, respectively, against *G. vaginalis* at which the growth of the selected 3 *Lactobacillus* strains was inhibited to a much lesser degree. In the co-culture assay of *Lactobacillus* and *G. vaginalis* in the presence of GML and GSE, the growth of *G. vaginalis* was considerably suppressed whereas that of *Lactobacillus* was minimally affected. Taken together, the selected *Lactobacillus*, GML and GSE could be potential therapeutic candidates to suppress the colonization of *G. vaginalis* in the vaginal niche.

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G. vaginalis: *Gardnerella vaginalis* KCTC 5097

GML: Glycerol monolaurate

MGs: 1- monoglycerides

GSE: Grapefruit seed extract

CFCS: cell-free culture supernatant

MIC: Minimum inhibitory concentration

BV: Bacterial vaginosis

BHIS: Brain heart infusion supplement

qPCR: Quantitative Real-time polymerase chain reaction

1. Introduction

The human vagina is colonized by various microbes. Under healthy conditions, the vaginal flora is dominated by *Lactobacillus,* and the dominance of *Lactobacillus* in the vagina has been associated with a reduced risk of bacterial vaginosis (1, 2, 3). Bacterial vaginosis (BV), formerly known as nonspecific vaginosis, is the most common vaginal infection in women. BV mainly affects the vagina, urethra bladder, and the skin in the genital area. BV is associated with pregnancy complications (4, 5, 6), increased risk of gynecologic and obstetrical complications, and other infections (7). During BV infection, the *Lactobacillus* population decreases and bacterial groups such as the anaerobic bacterium *Gardnerella vaginalis* increase (8). Additional bacterial groups associated with BV include *Bacteroides* spp*., Peptostreptococcus* spp*., Mycoplasma hominis,* and *Prevotella* spp. (9, 10).

Antibacterial medications are the standard treatment for BV. While effective, these broad-spectrum drugs do not completely inhibit BV-associated pathogens. Subsequently, there is a high BV recurrence rate of ~20% (11, 12). The emergence of antibiotic resistance amongst pathogenic bacteria and the high recurrence of BV have led to a major research effort to find alternative antibacterial therapeutics to which bacteria do not easily develop resistance. Accordingly, antimicrobial

combinations, known as multiple antimicrobial hurdles, would be a desirable option in the treatment of BV, which rely on the use of multiple stress factors that simultaneously deplete various resources of a target cell, making microbial adaptation processes more challenging (11,13). In addition, there is a rapidly growing demand for environmentally friendly, safe natural antimicrobials over pharmaceutical antibiotics. (14). The antimicrobial properties of food-grade materials such as grapefruit seed extract (GSE), 1-monoglycerides (MGs), and lactic acid bacteria have been investigated by a number of studies worldwide (15-17). In the present study, these substances were assessed on their activities to inhibit the survival of *G. vaginalis* because of their food-grade status and differential mode of antimicrobial action.

Lactobacilli administered orally or intravaginally have been tested for their effectiveness in colonizing the vagina and curing women with BV, or preventing its recurrence (16-18). The present study evaluated the ability of 26 *Lactobacillus* strains to inhibit *G. vaginalis*. One of the main goals was to select strong *G. vaginalis*-inhibiting strains with the putative ability to protect the vagina.

MGs were shown to inhibit food-borne pathogens or spoilage gram-positive and gram-negative bacteria (*Bacillus cereus, B. subtilis, Enterococcus faecalis, Micrococcus luteus, Staphylococcus aureus, Citrobacter freundii, Escherichia coli, Proteus mirabilis, Salmonella enterica* ser. *enteritidis* and *Pseudomonas*

aeruginosa) (16, 19, 20). MGs may be therefore alternatives or adjuncts to antibiotics against *G. vaginalis.*

The antimicrobial activity GSE was reported to be due to flavonoids, such as naringin, limonoid, quercetin, kaempferol, citric acid, and other compounds (21，22). GSE has been found to be an effective broad-spectrum antimicrobial with activity against *Salmonella*, *E. coli*, *Candida,* herpes, influenza, and fungi (23-25). On the basis of these previous reports on GSE as a food additive, our attention was drawn to the possibility that GSE could be used to confer antimicrobial activity against *G. vaginalis* and its possible application in the treatment of BV.

The differential mechanisms of action of these antimicrobials make them strong candidates for synergistic activity against *G. vaginalis*. The objective of this study was to verify the antimicrobial activity of the three antimicrobials, which could be used as multiple hurdles to allow for the use of each compound in amounts lower than their individual effective concentrations against *G. vaginalis*. This is the first report investigating the synergy of three natural antimicrobials against *G. vaginalis.*

2. Materials & Methods

2.1. Microorganisms and culture conditions.

G. vaginalis KCTC 5097 was purchased from Korean Collection for Type Cultures. It was grown in BHI medium supplemented with yeast extract(1%), maltose(0.1%), glucose(0.1%) and 10% horse blood serum(BHIS medium) under anaerobic conditions at 37℃ for 24 h (26). The experimentqal 26 lactobacilli were obtained from the Food Microbiology Lab., Department of Food and Nutrition in Seoul National University (Seoul, Korea). These strains were grown in MRS medium(Difco, Detroit, MI, USA) under anaerobic conditions at 37℃ for 18 h.

2.2. Chemicals

GSE was purchased from Seoul Food R&D CO,.LTD (Korea, 40% of GSE and 60% of glycerin) and glycerol monolaurate, glycerol monocaprylate and glycerol monocaprate were purchased from Tokyo Chemical Industry CO,.LTD (Tokyo, >97.0%).

2.3. Methods

2.3.1.   Effect of lactobacilli CFCS on the viability of *G. vaginalis*

The effect of pH (pH=7) adjusted and not adjusted lactobacilli cell-free culture supernatants (CFCS) of the experimental 26 lactobacilli on the viability of *G. vaginalis* were assessed. *G. vaginalis* (1×10^7,700 µL) was cultured in the presence of the CFCSs (700 µL) for 24 h. The CFCSs were obtained by centrifuging at $10,000 \times g$, for 30 min at 4℃. After being centrifuged, CFCSs were passed through a sterile 0.2 µm Acrodisc Syringe Filter (USA). The pH of the CFCSs was adjusted to 7.0 by using 5 M NaOH solution in order to eliminate the effect of lactic acid on *G. vaginalis*. As a control, aliquots of MRS broth, treated as filtered supernatants, were used (27).

2.3.2   Inhibitory activity of selected lactobacilli on the growth of *G. vaginalis* in the co-culture assay

The ability of lactobacilli to inhibit the growth of *G. vaginalis* was observed by colony count assays. It was performed by incubating *G. vaginalis* with the lactobacilli culture at 37℃. The control consisted of non-cultured MRS. Initially and at predetermined intervals, aliquots were removed，serially diluted, and plated on BHIS agar plate and MRS agar plate.

2.3.3   Antibacterial activity of MGs and GSE

The antibacterial activity of various concentrations of MGs, GSE, and their combinations were assessed by monitoring the bacterial growth curve at various levels. A macro-broth-dilution technique was used to determine the susceptibility of the bacteria to MGs and GSE. Susceptibility was expressed as minimum inhibitory concentration (MIC). The inhibition was assessed by monitoring the bacterial growth curve at various levels on 96 well plates.

2.3.3.1.   Preparation of antimicrobial solutions

MGs solutions were prepared by dissolving 5 mg/mL of MGs followed by heating (80℃, 30 min). Then the samples were filter-sterilized (0.45 μm Acrodisc Syringe Filter) and then diluted in MRS to obtain the desired concentrations. The MGs solution were made freshly for each experiment.

Stock solution of GSE was prepared in sterilized water to obtain a 500 ppm working solution. Then it was diluted in sterilized water to obtain the desired concentrations.

2.3.3.2.　　Determination of MICs

The antimicrobial activities of MGs and GSE were analyzed in triplicate by using microtiter plate growth assays in 96-well plates. Each well contained 100 μL antimicrobial solution and was inoculated with 100 μL of 1: 100-diluted working culture to yield $10^5 \sim 10^6$ CFU/mL. The tested antimicrobial concentrations ranged from 2.5 mg/mL to 9.75 μg/mL for MGs, and from 20 ppm to 0.125 ppm for GSE. Inoculated plates were incubated at 37℃ for 24 h, and the optical density at 595 nm of each well was monitored every 3 h after an automatic 10-s shake.

2.3.3.3.　　Determination of synergy between GML and GSE

The interaction between GSE and the GML was tested via a "checkerboard" assay that allowed for testing of two antimicrobials at various concentrations at the same time. The checkerboard assays were performed by the method of Badaoui Najjar *et al*. (28) with the following modifications. In the experiment, a sterile 96-well microplate was prepared so that GSE (vertical columns) would be combined with the chosen GML (horizontal rows). Using a stock solution of a 10-fold-higher concentration than its respective MIC, each compound was aliquoted into the appropriate row or column. Each plate was designed to test concentrations directly above, equal to, and, below the individual MIC of each

antimicrobial. *G. vaginalis* cells were grown for 24 h and prepared as previously described; 100 μL of this preparation was added to each well. The first row and column of the microplate served as controls (no antimicrobials), as did a row of MRS alone, a row of water alone and growth medium alone. Each plate was run using the same equipment and under the same conditions as described in the previous section. Each assay was performed in triplicate. Results are recorded as 'No Inhibition' (NI), 'Delayed Growth' (DG), 'Partial Inhibition' (PI) and finally 'Complete Inhibition' (CI).

In order to assess possible synergistic activity, the Fractional Inhibitory Concentration (FIC) index was calculated according to the formula:

$$FICindex = FIC_{GML} + FIC_{GSE} = [GML]/MIC_{GML} + [GSE]/MIC_{GSE}$$

Respectively, [GML] and [GSE] indicate the concentrations of GML and GSE used for partial inhibition; MIC_{GML} and MIC_{GSE} indicate the minimum inhibitory concentrations of GML and GSE. The resulting FIC index of <0.5 indicates synergy, at 0.5-0.75 partial synergy, 0.75-1.0 additive effects, above 1.0 indifference and above 4.0 antagonism (29).

2.3.4. Co-culture assay of *Lactobacillus* and *G. vaginalis* in the presence of GML and GSE

The combined effects of the selected 4 lactobacilli, GML, and GSE on *G. vaginalis* were determined using Quantitative Real-time polymerase chain reaction (qPCR) assays

2.3.4.1. Sample preparation

G. vaginalis was incubated with the three *Lactobacillus* culture at 37℃ in the presence of 39 μg/mL GML and 10 ppm GSE in the BHIS medium. The organisms without antimicrobials served as a control. All of the experiments were performed in triplicate and repeated three times to confirm the results. The samples were divided into aliquots and stored at -20℃ until genomic DNA was extracted for PCR assays.

2.3.4.2. qPCR assay

Total genomic DNA of each harvested well was extracted from aliquots by using a Doctor Protein Cell DNA Purification mini Kit (MG med, Korea). Extraction was performed according to the manufacturers' instructions and the total bacterial DNA

was eluted with 100 µl of sterile water. DNA extracts were aliquoted and stored at -20℃.

The oligonucleotide primers for *G. vaginalis* were used as published. (F-GV 5`-TTACTGGTGTATCACTGTAAGG-3`; R-GV 5`-CCGTCACAGGCTGAACAGT-3`) The *Lactobacillus* primers (F-LBF 5`-ATGGAAGAACACCAGTGGCG-3`; R-LBR 5`-CAGCACTGAGAGGCGGAAAC-3`) were used to detect all *Lactobacillus* strains in this study (30). No cross-reactivity was detected with DNA from any of the tested species.

To construct standard curves for the real time PCR's, *L. bulgaricus* KCTC 3188, *L. rhamnosus* GG and *L. rhamnosus* BH09 were cultured on MRS broth at 37℃ for 18 h and *G. vaginalis* was cultured on BHIS broth at 37℃ for 24 h. DNA was extracted from 1 mL of bacterial suspension. The standard curve was prepared by serial tenfold dilution of this DNA extract. The DNA concentration was determined by using the Qubit 2.0 Fluorometer (Life Technologies, USA). The number of cells in each dilution was calculated taking into account the genome size of the bacterial species.

For real-time PCR, SYBR green qPCR mix (2×)(MG med, Korea) was used and the extracted DNA samples were amplified by ABI Stepone System(USA) with a thermocycle profile as follows: stage 1, 95℃ (5 min); stage 2 consisting of 50

cycles of 95℃ (30 s), 55℃ (30 s)and 72℃ (30 s) for *G. vaginalis*; and stage 1, 95℃ (10 min); stage 2 consisting of 45 cycles of 95℃ (5 s), 60℃ (30 s) for lactobacilli.

The final results were expressed as copies of microorganism DNA per mL of bacterial suspension. The quantitative result obtained with the qPCR was expressed in number of copies/mL and was back calculated taking into account the volume extracted, the DNA extract volume obtained, and volume of DNA amplified.

3. Data analysis.

The kinetic growth curve data from all assays were analyzed using Microsoft Excel. Results are expressed as the mean standard error of the mean (SD). Student's *t*-test was used for statistical comparisons ($P<0.05$).

4. Results

4.1. Effect of lactobacilli CFCS on the viability of *G. vaginalis*

In this study, the inhibitory activity of 26 experimental *Lactobacillus* strains, previously isolated from human intestine and various foods, were assessed. *In vitro* tests with non-adjusted pH CFCSs from the experimental *Lactobacillus* cultures exhibited strong antimicrobial activity by killing *G. vaginalis* after 2 h of incubation (data not shown). However, among the 26 strains exerting an inhibitory activity from the pH adjusted (pH=7) CFCSs, different levels of efficacy were observed. Only 5 out of the 26 examined strains showed a strong activity against *G. vaginalis* after 24 h of incubation and thus, were further tested.（Table 1）

Table 1. Test microorganisms, culture conditions and inhibitory activity of the pH adjusted lactobacilli CFCSs against *G. vaginalis*

Test microorganisms	Culture conditions	Inhibitory activity	Test microorganisms	Culture conditions	Inhibitory activity
Lactobacillus plantarum KFRI 708	MRS 37℃	/	*Lactobacillus delbrueckii* KCTC 1047	MRS 37℃	/
Lactobacillus casei 704	MRS 37℃	+++	*Lactobacillus helveticus* LHB-02	MRS 37℃	+
Lactobacillus rhamnosus GG	MRS 37℃	+++	*Lactobacillus brevis* R01	MRS 37℃	+
Lactobacillus acidophilus KCTC 3154	MRS 37℃	/	*Lactobacillus brevis* IFO 12005	MRS 37℃	++
Lactobacillus reuteri KCTC 53608	MRS 37℃	/	*Lactobacillus brevis* GABA 100	MRS 37℃	+++
Lactobacillus bulgaricus KCTC 3188	MRS 37℃	+++	*Lactobacillus brevis* 221	MRS 37℃	++
Lactobacillus cremoris	MRS 37℃	+	*Lactobacillus brevis* 239	MRS 37℃	++
Lactobacillus delbueckii subsp. Delbrueckii KCCM 354886	MRS 37℃	++	*Lactobacillus brevis* 651	MRS 37℃	/

Table 1. Test microorganisms, culture conditions and inhibitory activity of the pH adjusted lactobacilli CFCSs against *G. vaginalis*（continue）

Lactobacilllus cremoris ATCC 19257	MRS 37℃	+	*Lactobacillus brevis* 805	MRS 37℃	++
Lactobacillus casei KFRI 196	MRS 37℃	++	*Lactobacillus brevis* 806	MRS 37℃	/
Lactobacillus casei KCTC 911	MRS 37℃	/	LR 822	MRS 37℃	/
Lactobacillus casei KCTC 3110	MRS 37℃	/	*Lactobacillus casei* IBS 041	MRS 37℃	/
Lactobacillus rhamnosus BH09	MRS 37℃	+++	*Lactobacillus fermentum BH03*	MRS 37℃	++

＊Collections: KTCC-Korean Collection for Type Cultures, Korea; ATCC-American Collection for Type Cultures, USA; KCCM-Korean Culture Center of Microorganisms, Korea; KFRI-Korean Food Research Institute, Korea ＊Inhibitory activity : +++ strong inhibitory, ++ medium inhibitory, / no inhibitory .

4.2. Inhibitory activity of selected lactobacilli on the growth of *G. vaginalis* in the co-culture assay

The 5 selected *Lactobacillus* strains were examined for their inhibitory activity against *G. vaginalis* in co-culture condition. The inhibitory activity was measured after 24 h of co-culture as previously described. The results reported in Table 2 show that 3 strains, *L. bulgaricus* KCTC 3188, *L. rhamnosus* GG and *L. rhamnosus* BH09, strongly reduced the viability of *G. vaginalis,* and thus, chosen for the combinatory study with the other antimicrobials.

Table 2. Inhibitory effect of selected lactobacilli on the growth of *G. vaginalis* in co-culture condition

Test strains	Viable cell numbers of *G. vaginalis* (Log₁₀CFU/mL)
Control	9.40 ± 0.11
Lactobacillus bulgaricus KCTC 3188	$7.57 \pm 0.15^{*}$
Lactobacillus rhamnosus BH09	$7.43 \pm 0.20^{*}$
Lactobacillus rhamnosus GG	$7.40 \pm 0.22^{*}$
Lactobacillus casei 704	$8.69 \pm 0.14^{*}$
Lactobacillus brevis GABA 100	9.00 ± 0.65

Each value shown is the mean ± SD from three experiments. Values that are significantly different from the control value are indicated by an asterisk ($P < 0.05$).

4.3. Determination of MICs

The MICs of GML, glycerol monocaprate and glycerol monocaprylate against *G. vaginalis* were determined with the broth microdilution method in BHIS broth. As seen in Table 3, all of the tested MGs were able to effectively inhibit the growth of *G. vaginalis*. GML proved to be the most effective MG with a MIC of 78.125 μg/mL (Figure1), while glycerol monocaprate and glycerol monocaprylate had MICs of 156.25 μg/mL and 312.5 μg/mL, respectively. The MIC values of GML against *L. bulgaricus* KCTC 3188, *L. rhamnosus* GG and *L. rhamnosus* BH09, were 5 mg/mL, 2.5 mg/mL, and 2.5 mg/mL, respectively (Table 4).

When the three *Lactobacillus* cultures were grown for 24 h in the presence of 78.125 μg /mL GML, no inhibition was noticed (data not shown).

The MIC values of GSE against *G. vaginalis*, as determined by the broth microdilution assay, are presented in Table 5. The MIC value of GSE against *G. vaginalis* was 20 ppm (Figure 2), whereas it was 62.4 ppm against the latic acid bacteria, *L. bulgaricus* KCTC 3188, *L. rhamnosus* GG and *L. rhamnosus* BH09. When the three *Lactobacillus* cultures were grown for 24 h in the presence of 20 ppm GSE, no inhibition was noticed (data not shown).

Table 3. MICs of MGs against *G. vaginalis*

Antimicrobial compound	MIC (μg/mL) for *G. vaginalis*
Glycerol monolaurate(GML)	78.125
Glycerol monocaprate	156.25
Glycerol monocaprylate	312.5

Each MIC assay tested a wide range of concentrations for each compound and was conducted in triplicate

Table 4. MICs of GML against *G. vaginalis* and lactic acid bacteria

Test strains	MIC for *G. vaginalis*
G. vaginalis KCTC 5097	78.125 µg/mL
L. bulgaricus KCTC 3188	5 mg/mL
L. rhamnosus GG	2.5mg/mL
L. rhamnosus BH09	2.5mg/mL

Each MIC assay tested a wide range of concentrations for each compound and was conducted in triplicate

4.4 Combinational effect of GML and GSE on the growth of *G. vaginalis*

The MICs of GML and GSE for *G. vaginalis* were determined to be 78.125 µg/mL and 20 ppm, respectively. When tested against *G. vaginalis*, specially selected partially inhibitory concentrations of GML (39.0 µg/mL) and GSE (10 ppm) caused little decrease in microbial growth (Fig 1 and 2). When combined, these concentrations of 39.0 µg/mL of GML and 10 ppm of GSE nearly completely inhibited *G. vaginalis* over a 24-h period with an FIC index of 1.0 indicating an additive effect according to the FIC calculations (Figure 3).

Table 5. MICs of GSE against *G. vaginalis* and lactic acid bacteria

Test strains	MIC (ppm) for *G. vaginalis*
G. vaginalis KCTC 5097	20.0
L. bulgaricus KCTC 3188	62.4
L. rhamnosus GG	62.4
L. rhamnosus BH09	62.4

Each MIC assay tested a wide range of concentrations for each compound and was conducted in triplicate
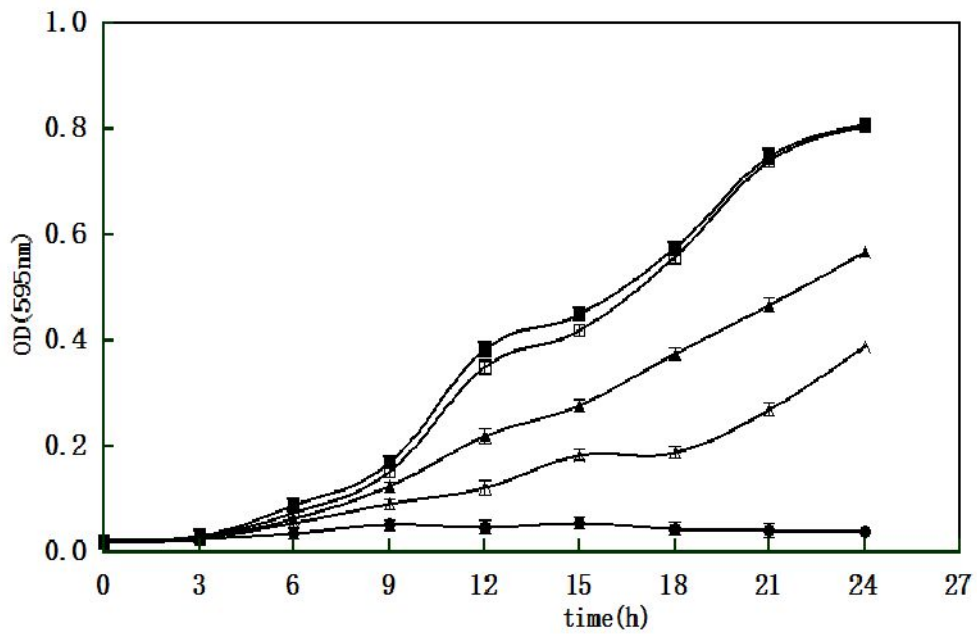
Figure 1. Growth kinetics of *G. vaginalis* in the presence of GML

All of the concentrations were tested in triplicates; ■, control experiments without GML. Δ, 9.75 μg/mL GML. ♦, 19.5 μg/mL GML. □, 39 μg/mL GML. ●, 78.125 μg/mL GML.
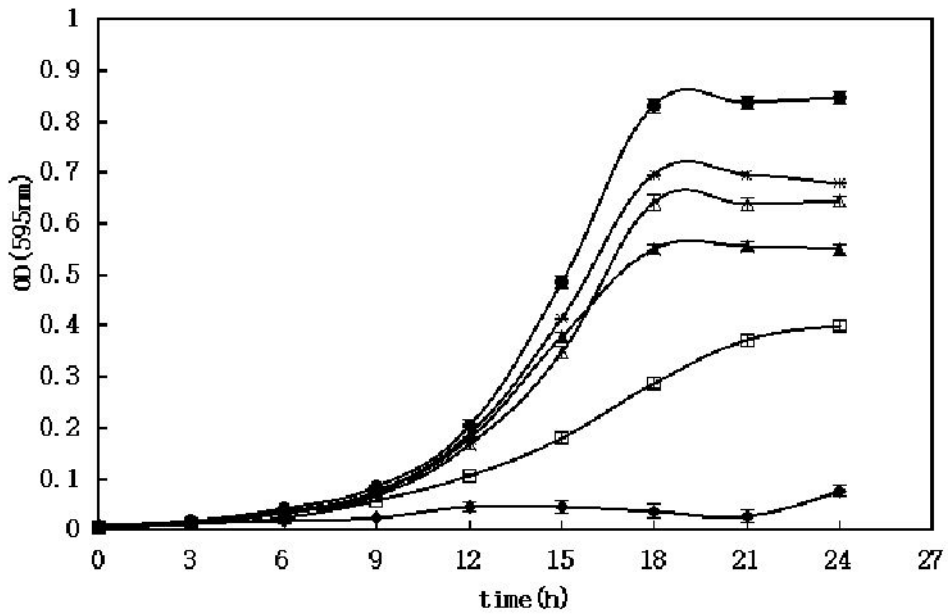
Figure 2. Growth kinetics of *G vaginalis* in the presence of GSE

All of the concentrations were tested in triplicates; ●, control experiments with 0 ppm GSE.

*, 1.25 ppm GSE. Δ, 2.5 ppm GSE. ▲, 5 ppm GSE. □, 10 ppm GSE. ♦, 20 ppm GSE.
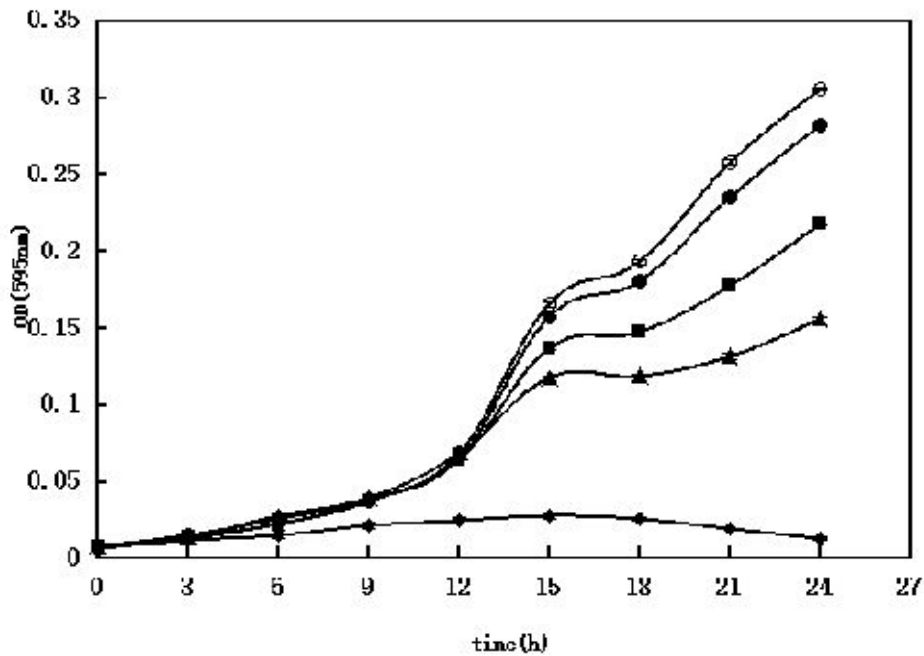
Figure 3. Effect of combination of 39 μg/mL GML with different concentrations of GSE on the growth of *G. vaginalis*.

All of the concentrations were tested in triplicates; ○, control experiments with 39 μg/mL GML and 0 ppm GSE. ●, 39 μg/mL GML and 1.25 ppm GSE. ■, 39 μg/mL GML and 2.5 ppm GSE. ▲, 39 μg/mL GML and 5 ppm GSE. ♦, 39 μg/mL GML and 10 ppm GSE.

4.5　The co-culture assay of *Lactobacillus* and *G. vaginalis* in the presence of GML and GSE

The number of *G. vaginalis* was measured by qPCR assays. When *G. vaginalis* was co-cultured with the three selected lactobacilli, a slight decrease in the level of viable *G. vaginalis* was observed (Table 6). The inhibitory activity was considerably increased when *G. vaginalis* was co-cultured with the three selected lactobacilli in the presence of 39 μg/mL of GML and 10 ppm of GSE. *L. rhamnosus* BH09 showed the highest inhibitory effect in the presence of GML and GSE.

Table 6. Inhibitory effect of three selected lactobacilli and antimicrobial combinations on the viability of *G. vaginalis* in co-culture conditions

Experimental *Lactobacillus* strains	Viable cell numbers of *G. vaginalis* ($\log_{10}$ copies/mL)			Viable cell numbers of *Lactobacillus* strains ($\log_{10}$ copies/mL)		
	Control	L+G[*]	L+G+C[*]	Control	L+G[*]	L+G+C[*]
L. bulgaricus KCTC 3188	8.26±0.01	7.92±0.01	7.49±0.01	9.52±0.01	9.24±0.06	9.27±0.01
L. rhamnosus GG	8.26±0.01	7.39±0.07	7.03±0.05	8.40±0.11	8.81±0.05	8.83±0.01
Lactobacillus rhamnosus BH09	8.26±0.01	7.60±0.03	6.93±0.11	9.70±0.08	9.52±0.03	9.56±0.02

Control: *G. vaginalis* cultured without antimicrobials in BHIS medium.

L+G: *G. vaginalis* co-cultured with *Lactobacillus* in BHIS medium.

L+G+C: *G. vaginalis* co-cultured with *Lactobacillus* in the presence of antimicrobials combination in BHIS medium.

Antimicrobials combination: 39 μg/mL GML and 10 ppm GSE

Each value shown is the mean±SD from three experiments. *Values are significantly different from the control value ($P < 0.05$).

5. Discussion

We performed extensive studies of the potential ability of *Lactobacillus* strains, GML and GSE to be used as topical bactericidal agents against *G. vaginalis*.

The antimicrobial activities of the *Lactobacillus* strains against *G. vaginalis* have been previously documented (18, 31, 32). Lactobacilli have been found to produce lactic acid, and some selected biotypes also secrete hydrogen peroxide and bacteriocins, molecules that are known for their ability to hinder the growth and persistence of specific target bacterial species (1,2) In the present study, we studied the bactericidal potency of the 26 food and intestinal lactobacilli isolates. The pH adjusted (pH=7) CFCSs of the five selected *Lactobacillus* strains showed differential degrees of inhibitory activity against *G. vaginalis*. The data reported here also show that, after co-culture, among the five lactobacilli examined, *L. bulgaricus KCTC* 3188*, L. rhamnosus GG* and *Lactobacillus rhamnosus BH09* showed relatively high inhibitory activity against *G. vaginalis*. Our findings are consistent with the above reports in that the killing activity displayed by the 3 lactobacilli are not only attributable to low pH, but also to the presence of other compound(s) located in their CFCSs.

As for the bactericidal effect of MGs against *G. vaginalis*, our present work showed that all of the three MGs showed inhibitory activity against *G. vaginalis*, but GML had the

highest antimicrobial activity with a MIC of 78.125 µg/mL. To our knowledge this is the first report for a comparison of inhibitory effect of 3 1-monoglycerides on *G. vaginalis*. However, the exact mechanism for the effect of 1-monoglycerides on bacteria which would explain this difference has not been fully elucidated. It has previously been reported that GML, generally recognized as safe for oral use by the FDA (33), has been used extensively in the food and cosmetic industries. Previous studies have shown that GML inhibits the cell growth and production of exotoxins of various gram-positive bacteria but does not inhibit the growth or metabolism of lactobacilli (34- 37). Long-term *in vivo* studies of monkeys show that 50 mg/mL of GML in intravaginal gels does not inhibit lactobacilli and is not proinflammatory (38). In our study reported here, we also found that GML (78.125 µg/mL) was fully inhibitory to *G. vaginalis*, and it did not exert adverse effects on the selected three lactobacilli.

Next, we showed the antimicrobial activity of grapefruit seed extract against *G. vaginallis* with a MIC of 20 ppm. GSE has been registered as a natural additive showing antimicrobial activity in the Korea Food Additive Standards Codex (39). To our knowledge, this is the first study to observe the antibacterial effect of GSE on *G. vaginalis*. Previously, the mechanism for the antibacterial activity of GSE was shown to disrupt the bacterial membrane and liberate the cytoplasmic contents within 15 min after contact (40).

While the ability to use considerably smaller amounts of each compound to inhibit *G vaginalis* growth was a promising result, our main interest lay in whether these interactions were the result of a synergy between GML and GSE. The interactions between GML and GSE were investigated with checkerboard 96-well plate assays. Some additive effect was noticed between the two antimicrobials. The mechanisms of GML and GSE were different as previously described; therefore, it is not surprising that the antimicrobial combination show additive effect against *G vaginalis*.

These optimal additive combinations were then tested against *G vaginalis* when co-cultured with the three selected *Lactobacillus* strains by qPCR assays. The results showe that the 3 selected lactobacilli with the antimicrobials combination of GML and GSE considerably inhibited the growth of *G vaginalis*, but not for lactobacilli. Although the exact mechanism of inhibitory action of lactobacilli, GML, and GSE on the growth of *G vaginalis* is not known, treatment with lactobacilli could lead to a decrease in the intracellular pH of *G vaginalis*, which can lead to the inactivation of intracellular enzymes (41), and the inhibition of the transport of the various nutrients (42). Therefore, the addition of lactobacilli into the combination of GML and GSE could have enhanced the anti-bactericidal properties of the antimicrobials agaisnt *G vaginalis*. Similarly, Wentao Xu et al have shown that lowering pH increased the inhibitory activity of GSE against a bacterial cocktail (43). In addition, Marshall reported that MIC values were lower when

GML was combined with selected organic acids, such as acetic acid, benzoic acid, or lactic acid, in dual combinations, and they also showed that the enhanced inhibitory effect of the combinations of GML with organic acids was influenced by both the pH and the type of organic acid used (44). The present studies showed that lactobacilli, GML, and GSE are strong multiple antimicrobial hurdles, which would be a desirable option in the treatment of BV.

In order to enumerate the number of bacterial cells, selective or differential medium can be used. However, no selective medium for counting *G. vaginalis* in the presence of a dominant number of *Lactobacillus* was available. Therefore, we adopted qPCR to quantify the number of *G. vaginalis* in the co-culture systems. Recently, PCR and real-time quantitative PCR have been used successfully to study the antimicrobial susceptibilities of other bacteria such as *Campylobacter jejuni*, (45) *St. aureus*, *St. epidermidis*, *Helicobacter pylori*, *Enterococcus faecalis,* and *Ent. faecium* (46). The flexibility of real-time PCR is further demonstrated by its ability to detect a specific target organisms in the presence of a variety of other organisms (47). These methods are simple, rapid, sensitive, reproducible, and specific and provide the possibility of automation.

There could be several reasons for false-negative PCR results, such as the presence of inhibitory substances in the enrichment broth contents (48). However, the corresponding results of the standard culture methods and real-time PCR method suggest that there were

no false-positives or false-negatives in the present study. The limitation of the qPCR assays is that damaged and dead cells can also be detected. Therefore, the data reported from the qPCR assays can be an overestimation. Taken together, the present results confirme that the combination of selective *Lacbacillus*, GML, and GSE is an efficient way to inhibit the growth of *G. vaginalis*.

6. Conclusion

Our results suggest that the three lactobacilli (*L. bulgaricus* KCTC 3188, *L. rhamnosus* GG and *Lactobacillus rhamnosus* BH09), GML, and GSE are effective natural antimicrobials, inhibiting the growth of *Gardnerella vaginalis*. In addition, the treatment at MICs against *G. vaginalis* of two antimicrobials have no adverse effect on the selected three lactobacilli. Moreover, in the co-culture assays of *G. vaginalis* and *Lactobacillus* in the presence of GML and GSE, the combination of three natural antimicrobials indeed provide the multiple hurdles required to effectively control the growth of *G. vaginalis*. In conclusion, we demonstrated that three lactobacilli (*L. bulgaricus* KCTC 3188, *L. rhamnosus* GG, and *Lactobacillus rhamnosus* BH09), GML and GSE could be potential candidates for the beneficial use in protecting against vaginal microbial infections.

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KOREAN ABSTRACT

본 연구는 세균성 질염의 주요 원인균으로 알려져 있는 *Gardnerella vaginalis* KCTC 5097 균주를 이용하여 유산균, 1−모노글리세리드와 자몽씨추출물(GSE)의 항균력을 관찰하였다. 먼저 26 가지 유산균의 cell free 배양액(pH=7) 의 항균성을 조사하였고 그들 중 5 종류를 선택하여 *G. vaginalis* 와 같이 배양하였다. 상대적으로 항균성이 강한 3 종류의 유산균은 *G. vaginalis* 의 성장을 저해하는 효과가 높았다. 또한 모노글리세리드및 자몽씨추출물 각각의 항균력을 평가하였으며, 모노글리세라이드 중 항균성이 제일 강한 Glycerol monolaurate (GML) 와 자몽씨추출물의 병용효과 및 시간대별 균 성장 곡선을 관찰하였다. 본 실험의 결과, *G. vaginalis* 에

대한 GML 와 GSE 의 MIC 는 각각 78.125 μg/mL 및 20 ppm 로 관찰되었다.

이들 결과들을 근거로 GML 와 GSE 첨가한 배지에서 G. vaginalis 와 선발

유산균들을 공동 배양 배양하였는데 *G. vaginalis* 의 성장은 효과적으로

억제되었고 유산균은 미미한 영향만 나타난 결과를 얻었다. 결론적으로, 선발

유산균, GML, GSE 의 조합물은 질염 완화에 효과적으로 사용될 가능성이

있을 것으로 사료된다.