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Antibacterial Effect of Eucalyptus Oil, Tea Tree Oil, Grapefruit Seed Extract, Potassium Sorbate, and Lactic Acid for the development of Feminine Cleansers

Young Sam Yuk*

Lecturer, Department of Clinical Medical Science, Graduate School of Health and Welfare, Dankook University, Cheonan 31116, Republic of Korea
E-mail: y60320@gmail.com

Abstract

Purpose*: It has been reported that the diversity and abundance of microbes in the vagina decrease due to the use of antimicrobial agents, and the high recurrence rate of female vaginitis due to this suggests that a new treatment is needed.*

Methods*: In the experiment, we detected that 10% potassium sorbate solution, 1% eucalyptus oil solution, 1% tea tree oil solution, 400 μL/10 mL grapefruit seed extract solution, 100% lactic acid, 10% acetic acid solution, and 10% lactic acid solution were prepared and used.*

After adjusting the pH to 4, 5, and 6 with lactic acid and acetic acid in the mixed culture medium, each bacterium was inoculated into the medium and incubated for 72 h at 35°C. Incubate and 0 h each. 24 h. 48 h. The number of bacteria was measured after 72 h.

Results*: In the mixed culture test between lactic acid bacteria and pathogenic microorganisms, lactic acid bacteria showed good results at pH 5–5.5. Potassium sorbate, which has varying antibacterial activity based on the pH, killed pathogenic bacteria and allowed lactic acid bacteria to survive at pH 5.5.*

Conclusion*: The formulation ratio obtained through this study could be used for the development of a feminine cleanser that can be used as a substitute for antibacterial agents. Further, the findings of this study may be able to solve the problem of antimicrobial resistance in the future.*

Keywords: *Essential oil, Microbiome, Antibacterial, Lactic acid bacteria, Phytochemical*

1. Introduction

Essential oils are effective in improving insomnia, depression, eczema, acne, headache, menstrual pain, antibacterial activity, and drowsiness [1-8]. Essential oils are also known to be effective against vaginitis [9]. There are many types of lactic acid bacteria in the healthy vagina, and these bacteria play an important role in maintaining a healthy vaginal environment [10, 11]. The microflora in a healthy vagina is mainly composed of gram-positive bacillus of the genus *Lactobacillus* [12–14]. It has been reported that the diversity and

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Corresponding Author: y60320@gmail.com
Tel: +82-41-550-1451, Fax: +82-41-559-7934
Lecturer, Department of Clinical Medical Science, Graduate School of Health and Welfare, Dankook University, Cheonan 31116, Republic of Korea

abundance of microbes in the vagina decrease due to the use of antibacterial agents, and the high recurrence rate of vaginitis because of this suggests that a new treatment is needed [15]. A significant amount of research has been conducted in this regard.

In this study, among the essential oils that have antibacterial effects against pathogenic bacteria, *Eucalyptus globulus* oil (eucalyptus oil) and *Melaleuca alternifolia* oil (tea tree oil), which are effective against *Candida albicans*, the causative agent of vulvar candidiasis and have been studied previously, grapefruit seed extract, which has antibacterial effects on bacteria and viruses, and potassium sorbate, which is mixed with *Allium hirtifolium* Boiss, which has an inhibitory effect on potent fungi, were used as raw materials[16-18]. These essential oils can be used as a substitute for antibacterial agents once the optimal blending ratio that allows beneficial lactic acid bacteria to survive and inhibits pathogenic bacteria in the vagina is determined, and this is what this study aimed to do. Ultimately, we intend to develop a broad-spectrum antibacterial phytochemical-based vaginal cleanser.

2. Experiments

2.1 Materials

2.1.1 Test Strains. The antibacterial activities of eucalyptus oil, tea tree oil, grapefruit seed extract, and potassium sorbate were tested against *Candida albicans*, the causative agent of vulvar candidiasis, and lactic acid bacteria, which maintain vaginal health (Table 1).

Table 1. Strains and media used in this study

Strain	Medium
Lactobacillus plantarum (KCTC 3104)	MRS agar
Streptococcus thermophilus (KCTC 37119)	MRS agar
Lactobacillus rhamnosus (KCTC 3237)	MRS agar
Lactobacillus acidophilus (KCTC 3140)	MRS agar
Candida albicans (KCTC 7752)	PDA
Escherichia coli 0157:H7 (ATCC 43895)	TSA

2.1.2 Preparation of the Test Solution. In this experiment, 10% potassium sorbate (Sigma-Aldrich St. Louis, MO, USA) solution, 1% eucalyptus oil (Biochemica) solution, 1% tea tree oil (AEOC) solution, 400 μL/10 mL grapefruit seed extract (Chemeibuero) solution, 100% lactic acid, 10% acetic acid (Sigma-Aldrich, St. Louis, MO, USA) solution, and 10% lactic acid (Sigma-Aldrich, St. Louis, MO, USA) solution were prepared and used.

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2.2 Methods

2.2.1 Mixed Culture of Lactic Acid Bacteria and Pathogenic Microorganisms. The mixed culture of lactic acid bacteria and pathogenic microorganisms was prepared as per the method described by Denkova et al. [19] and Cunha et al. [20]. To culture the pathogenic microorganisms with lactic acid bacteria, a mixed culture of *E. coli* and lactic acid bacteria was set up using MRS broth (Difco, MI, USA), which is a medium for lactic acid bacteria, and tryptic soy broth (Difco, MI, USA), which is a culture medium for *E. coli*. After the same amount of mixing, 10 μL of lactic acid bacteria was inoculated with 10^7 CFU/mL 10 μL each, and 10 μL of 10^7 CFU/mL in *E. coli*, and the mixed culture of *C. albicans* and lactic acid bacteria was MRS broth (Difco, MI, USA) and *C. albicans* medium potato dextrose broth (Difco, MI, USA) were inoculated with 10 μL of 10^7 CFU/mL and 10 μL of 10^7CFU/mL for *C. albicans*. After adding the prepared essential oil, microaerobic (10% CO_2) culture was performed at 35°C for 72 h (*C. albicans* was cultured aerobically), and *E. coli* was cultured in tryptic soy agar (Difco, MI, USA). *C. albicans* growth was measured in potato dextrose agar (Difco, MI, USA), and lactic acid bacteria growth was measured on MRS agar (Difco, MI, USA).

2.2.2 Comparison of Bacterial Growth in Mixed Culture at Appropriate pH with in the Presence of Lactic Acid and Acetic Acid.

The pH was adjusted to 4, 5, and 6 with lactic acid and acetic acid in the mixed culture medium. Each bacteria was inoculated into a medium and cultured at 35°C, and the number of bacteria was measured at 0, 24, 48, and 72 hours.

2.2.3 Comparison of Growth of *L. plantarum*, *C. albicans*, and *E. coli* Cultured in the Presence of Potassium Sorbate.

We added 2 ml/200 ml and 1 ml/200 ml of potassium sorbate 10% solution to the mixed culture medium and adjusted the pH with lactic acid. Then, we inoculated 10^6 CFU/mL of each bacteria into a medium and cultured at 35°C, and the number of bacteria was measured at 0, 24, 48, and 72 hours.

2.2.4 Comparison of the Growth of *L. plantarum*, *C. albicans*, and *E. coli* in a Mixed Solution of Grapefruit Seed Extract, Eucalyptus Oil, and Tea Tree Oil. Grapefruit seed extract, eucalyptus oil, and tea tree oil were added to the mixed culture medium at a certain ratio (Table 3), and 10^6 CFU/mL of each bacteria was inoculated into a medium and cultured at 35°C. The number of bacteria was measured at 0, 24, 48, and 72 hours.

Table 3. The blending ratio of grapefruit seed extract, eucalyptus oil, and tea tree oil that satisfies the conditions for inhibiting pathogenic microorganisms and promoting the growth of lactic acid bacteria, which is a required for vaginal cleansers (pH 5.5)

Grapefruit seed extract (400 μL/10 mL)	Eucalyptus oil	Tea tree oil
20 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
30 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
40 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
	25 μL	25 μL
50 μL/200 mL	15 μL	15 μL

	12.5 μL	12.5 μL
	25 μL	25 μL
60 μL/200 mL	25 μL	25 μL
	15 μL	15 μL
	12.5 μL	12.5 μL
	10 μL	10 μL

2.2.5 Comparison of the Growth of *L. plantarum*, *C. albicans*, and *E. coli* in a Mixed Solution of Potassium Sorbate, Grapefruit Seed Extract, Eucalyptus Oil, and Tea Tree Oil. Potassium sorbate, grapefruit seed extract, eucalyptus oil, and tea tree oil were added to the mixed culture medium in a certain ratio (Table 4), and 10^6 CFU/mL of each bacteria was inoculated into a medium and cultured at 35°C. The number of bacteria was measured at 0, 24, 48, and 72 hours.

Table 4. The blending ratio of potassium sorbate, grapefruit seed extract, eucalyptus oil, and tea tree oil that inhibits pathogenic microorganisms and allows lactic acid bacteria to grow (pH 5.5)

Potassium sorbate (10%)	Grapefruit seed extract (400 μL/10 mL)	Eucalyptus oil	Tea tree oil
0.2 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL
0.25 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL
0.4 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL
1 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL

2.2.6 Comparison of *Str. thermophilus, L. plantarum, C. albicans, E. coli* Growth with Potassium Sorbate, Grapefruit Seed Extract, Eucalyptus Oil, and Tea Tree Oil Mixture at pH 5.0, 5.5, and 6.0.

In the mixed culture medium, 0.2 ml/200 ml of 10% solution of potassium sorbate, 20 μl of grapefruit seed extract (400 μl/10 ml), 10 μl of eucalyptus oil, and 10 μl of tea tree oil were added, and the pH was adjusted with lactic acid. After inoculation of about 10^6 of each bacteria, the samples were cultured at 35°C, and the number of bacteria were measured on day 0, 1, 3, and 6.

3 Results

3.1 Comparison of Bacterial Growth in Mixed Culture with Appropriate pH in the Presence of Lactic Acid and Acetic Acid

The effects of lactic acid and acetic acid on bacterial growth are shown in Figs 1 and 2. In Fig. 1, it can be seen that *Str. thermophilus* was killed, and there was little change in the number of other lactic acid bacteria. However, at pH 5 and 6, the growth was not affected by acetic acid, and the growth of lactic acid bacteria at pH 4 was not greater than that at pH 5 and pH 6. In Fig. 2, it can be seen that acetic acid had a greater inhibitory effect on *C. albicans* than lactic acid at pH 4, and that *C. albicans* died in the culture medium mixed with *L. plantarum* at pH 5. No inhibitory effect was observed at pH 6 when mixed with *Str. thermophilus*. Lactic acid was found to have no significant effect on *C. albicans* growth at pH 4, 5, and 6.

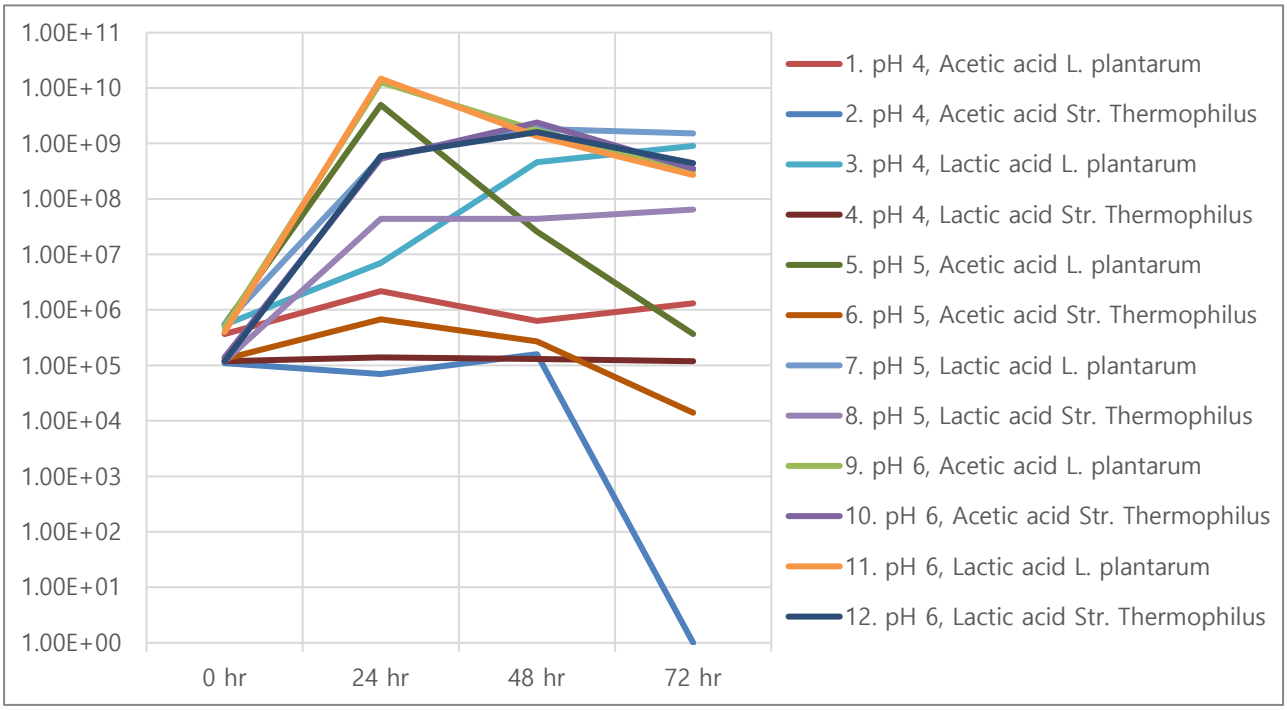

Figure 1. Growth of lactic acid bacteria in mixed culture of *C. albicans* and lactic acid bacteria at the appropriate pH and in the presence of lactic acid and acetic acid

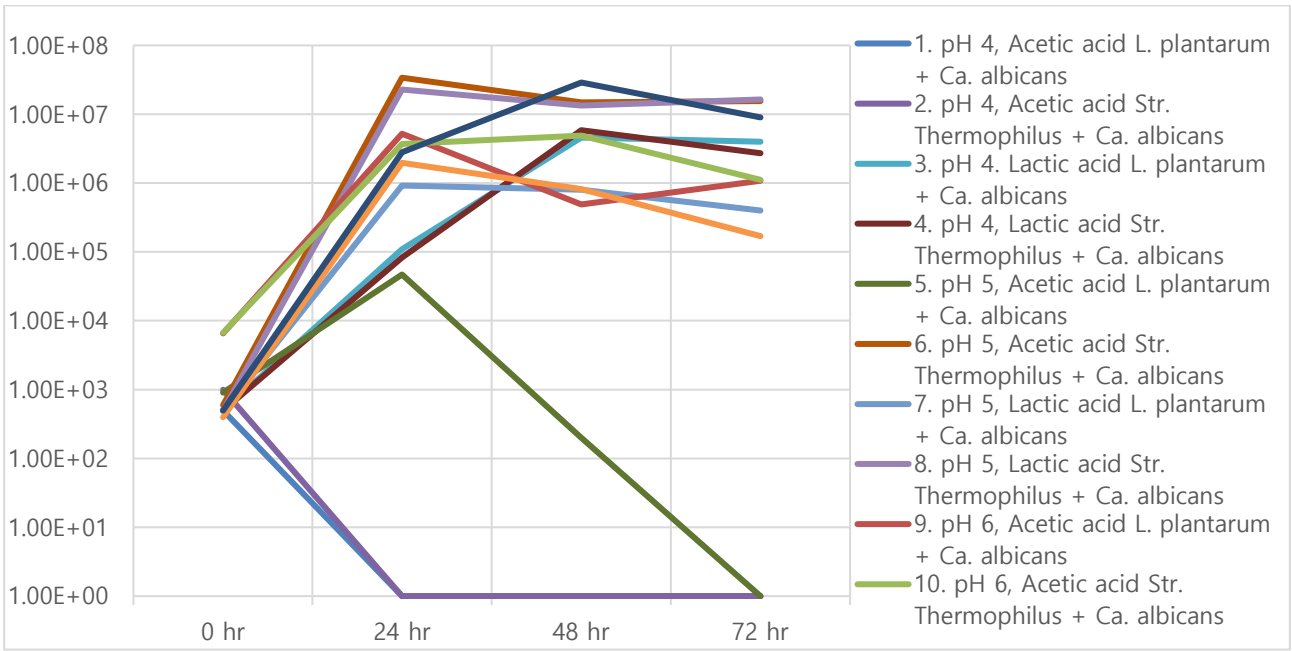

Figure 2. Growth of *Candida albicans* in mixed culture of *C. albicans* and lactic acid bacteria at the appropriate pH in the presence of lactic acid and acetic acid

3.2 Comparison of the Growth of *L. plantarum*, *C. albicans*, and *E. coli* in the Presence of Potassium Sorbate

Table 2 shows the effect of potassium sorbate on the growth of *L. plantarum*, *C. albicans*, and *E. coli*. At pH 6.7, potassium sorbate had no inhibitory effect on *C. albicans*, *E. coli*, or *L. plantarum*. At pH 5, the growth of *C. albicans*, *E. coli*, and *L. plantarum* was inhibited, and at pH 5.5, *C. albicans* and *E. coli* were inhibited but *L. plantarum* was not.

Table 2. Growth of *Candida albicans*, *Escherichia coli*, and *Lactobacillus plantarum* according to changes in pH and potassium sorbate concentration

Strain	pH	Potassium sorbate 10%	24 h	48 h	72 h
C. albicans	6.7	1 mL/200 mL	+	+	+
	5.5	1 mL/200 mL	-	-	-
	5.0	1 mL/200 mL	-	-	-
E. coli	6.7	1 mL/200 mL	+	+	+
	5.5	1 mL/200 mL	-	-	-
	5.0	1 mL/200 mL	-	-	-
L. plantarum	6.7	1 mL/200 mL	+	+	+
	5.5	1 mL/200 mL	-	+	+
	5.0	1 mL/200 mL	-	-	-

3.3 Comparison of the Growth of *L. plantarum*, *C. albicans*, and *E. coli* in a Mixed Solution of Grapefruit Seed Extract, Eucalyptus Oil, and Tea Tree Oil

Table 3 shows the mixing ratio of grape fruit seed extract, eucalyptus oil, and tea tree oil that inhibits pathogenic microorganisms but allows the survival of lactic acid bacteria. Of the 14 formulations, two solutions (grapefruit seed extract (20 μL), eucalyptus oil (12.5 μL), and tea tree oil (12.5 μL) and grapefruit seed extract (60 μL), eucalyptus oil (10 μL), and tea tree oil (10 μL)) turned out to be the most suitable.

Table 3. The blending ratio of grapefruit seed extract, eucalyptus oil, and tea tree oil that satisfies the conditions for inhibiting pathogenic microorganisms and promoting the growth of lactic acid bacteria, which is a required for vaginal cleansers (pH 5.5)

Grapefruit seed extract (400 μL/10 mL)	Eucalyptus oil	Tea tree oil
20 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
30 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
40 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
	25 μL	25 μL

50 μL/200 mL	15 μL	15 μL
	12.5 μL	12.5 μL
	25 μL	25 μL
60 μL/200 mL	25 μL	25 μL
	15 μL	15 μL
	12.5 μL	12.5 μL
	10 μL	10 μL

3.4 Comparison of the Growth of *L. plantarum*, *C. albicans*, and *E. coli* in a Mixed Solution of Potassium Sorbate, Grapefruit Seed Extract, Eucalyptus Oil, and Tea Tree Oil

Table 4 shows the mixing ratio of potassium sorbate, grapefruit seed extract, eucalyptus oil, and tea tree oil that inhibits pathogenic microorganisms and does not inhibit lactic acid bacteria growth. Among the eight blending ratios, a mixture of 0.2 mL of potassium sorbate, 20 μL of grapefruit seed extract, 10 μL eucalyptus oil, and 10 μL tea tree oil and lactic acid at a pH of 5.5 was found to be the most suitable as a vaginal cleanser.

Table 4. The blending ratio of potassium sorbate, grapefruit seed extract, eucalyptus oil, and tea tree oil that inhibits pathogenic microorganisms and allows lactic acid bacteria to grow (pH 5.5)

Potassium sorbate (10%)	Grapefruit seed extract (400 μL/10 mL)	Eucalyptus oil	Tea tree oil
0.2 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL
0.25 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL
0.4 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL
1 mL/200 mL	20 μL	10 μL	10 μL
		12.5 μL	12.5 μL

3.5 Comparison of *Str. thermophilus*, *L. plantarum*, *C. albicans*, and *E. coli* Growth with a Potassium Sorbate, Grapefruit Seed Extract, Eucalyptus Oil, and Tea Tree Oil Mixture at pH 5.0, 5.5, and 6.0

The results of the growth of *Str. thermophilus*, *L. plantarum*, *C. albicans*, and *E. coli* in a mixture of potassium sorbate (0.2 ml), grapefruit seed extract (20 μl), eucalyptus oil (10 μl), and tea tree oil (10 μl) are shown in Fig. 3. At pH 5, *C. albicans* and *E. coli* died; the abundance of *L. plantarum* increased on the 3rd day and decreased on the 6th day; and the abundance of *Str. thermophilus* decreased by 10^2 on the 3rd day and by 10^2 on the 6th day. *C. albicans* and *E. coli* died even at pH 5.5, while *L. plantarum* did not decrease in abundance compared to its abundance at pH 5. However, *Str. thermophilus* showed a similar pattern to that seen for this bacterium at pH 5, while at pH 6, it showed a similar pattern to that seen at pH 5.

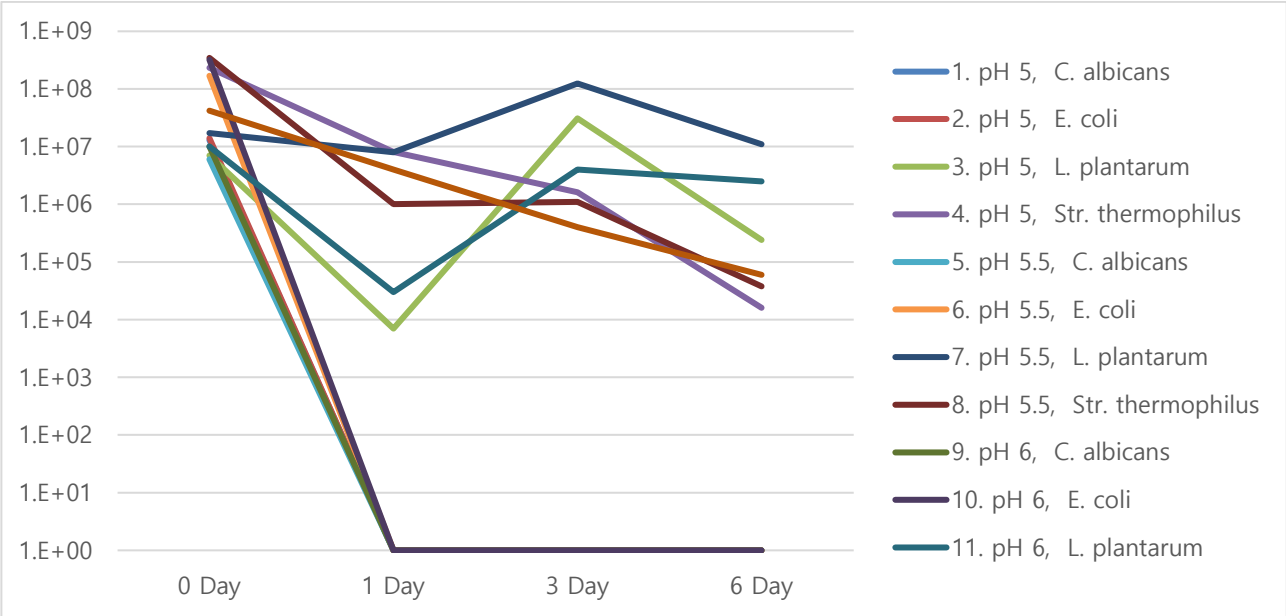

Figure 3. Comparison of microbial growth in the presence of a potassium sorbate (10%; 0.2 mL/200 mL), grapefruit seed extract (400 µL/10 mL; 20 µL), eucalyptus oil (10 µL), and tea tree oil (10 µL) mixture at different pH values

4. Discussion

Lactic acid bacteria, which have a therapeutic effect on bacterial vaginosis [21] and vulvar candidiasis, play an important role in maintaining vaginal hygiene. These bacteria can be used as a substitute alternative for fluconazole and azol families in vaginal washes [22].

In our study, lactic acid bacteria present in the vagina can grow and suppress the bacteria that cause vaginitis. We experimented with an essential oil of grapefruit seed extract, which is a safe and environmentally friendly substance, and potassium sorbate, which is used as an antibacterial agent in foods and pharmaceuticals. It was judged that lactic acid, which has almost no effect on the growth of *C. albicans*, is more suitable than acetic acid, which kills *C. albicans* and *Str. thermophilus*, as the raw material for pH titration during product production. It was shown that the number of bacteria at pH 4 was lower than that at pH 5 and 6, and the results showed that the growth of *C. albicans*, *Str. thermophilus*, and *L. plantarum* was also affected by acetic acid, but the effect was weak. It seems that there was some influence from pH.

Of the *C. albicans* and *L. plantarum* mixed culture and *C. albicans* and *Str. thermophilus* mixed culture, the culture containing *L. plantarum* had fewer *C. albicans* bacteria than the culture containing *Str. thermophilus*. This result was the same as that reported by Kim et al. (23) and Yuk et al. (24,25) (Figs 1, 2). *L. plantarum* is the third most used bacteria in the food and pharmaceutical industry and has a large antibacterial effect that depends on pH. In the case of alkali solutions, the antibacterial effect is reduced, and there are side effects when used at high concentration (26). Potassium sorbate solution has a high pH. The most appropriate conditions are acidic, where *C. albicans* and *E. coli* are suppressed at low concentrations, but *L. plantarum* growth is not affected at pH 5.5 and concentration 1 ml/200 ml (Table 2). A mixed solution of eucalyptus oil, tea tree oil, and grapefruit seed extract is environmentally friendly and has antibacterial effects. The mixing ratio of the seed extract, eucalyptus oil, and tea tree oil mixed solutions at pH 5.5 is grapefruit seed extract (20 µl), eucalyptus oil (12.5 µl), and tea tree oil (12.5 µl), and grapefruit seed extract (60 µl), eucalyptus oil (10

µl), and tea tree oil (10 µl) (Table 3). As a result of analyzing the results of the mixed solution of potassium sorbate, grapefruit seed extract, eucalyptus oil, and tea tree oil, we found that a mixed solution of potassium sorbate (0.2 ml), grapefruit seed extract (20 µl), eucalyptus oil (10 µl), and tea tree oil (10 µl) was acceptable. Therefore, it is considered that the ratio of lactic acid adjusted to pH 5.5 is the most suitable as a vaginal cleaner (Table 4). When potassium sorbate (0.2 mL) and grapefruit seed extract (20 µL) at pH 5, 5.5, and 6 were present in a solution of 10 µL eucalyptus oil and 10 µL tea tree oil, the abundance of *C. albicans*, *E. coli*, and *L. plantarum* were estimated. At pH 5.5, *C. albicans* and *E. coli* were killed, and *Str. thermophilus* was found to be more abundant than it was at pH 5, so the mixture at pH 5.5 of 0.2 mL potassium sorbate, 20 µL grapefruit seed extract, 10 µL eucalyptus oil, and 10 µL tea tree oil was found to be the most suitable for use in a vaginal cleanser. This reconfirmed our earlier results.

The mixed solution of two kinds of essential oils, grapefruit seed extract, and potassium sorbate (with adjusted pH with lactic acid) prepared at the specified mixing ratio allowed the growth of lactic acid bacteria. This mixture showed a very high inhibitory effect against pathogenic microorganisms. In order to develop an effective vaginal detergent, in vitro studies, such as additional safety tests and expansion of target pathogenic microorganisms, are necessary. In addition, in order to confirm whether the aforementioned solution can be used as a cleansing agent for human skin (face and body) in addition to its application as a vaginal cleanser, the inhibitory effect and stability tests on the skin flora should also be studied. For this, various antimicrobial tests of essential oils and recommendations for the use of an appropriate essential oil-containing vaginal cleansers are must be conducted. Such research will greatly enhance the development of essential oil-containing products in the future.

5. Conclusion

In the mixed culture test between lactic acid bacteria and pathogenic microorganisms, lactic acid showed good results at pH 5–5.5. Potassium sorbate, which showed a difference in antibacterial activity according to pH, kills pathogenic bacteria but not lactic acid bacteria at pH 5.5. The mixing ratio of the mixed solution of grapefruit seed extract, eucalyptus oil, and tea tree oil was determined at pH 5.5 as follows: potassium sorbate (0.2 ml), grapefruit seed extract (20 µl), eucalyptus oil (10 µl), and tea tree oil (10 µl). In the extract, eucalyptus oil, and tea tree oil mixed solution test and the test for measuring the number of viable cells, the optimum mixing ratio for the survival of lactic acid bacteria while killing pathogenic bacteria such as *C. albicans* was at pH 5.5 with potassium sorbate (0.2 ml), grapefruit seed extract (20 µl), eucalyptus oil (10 µl), and tea tree oil (10 µl). As a result of this, the formulation obtained in this experiment is considered to be high effective for the development of a vaginal cleaner that can be used as a substitute for antibacterial agents, and it is thought that it will be able to solve the problem of antimicrobial resistance in the future.

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Conflict of interest: The authors declare that there are no conflicts of interest

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