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Analysis of Antibacterial, Antioxidant, and *In Vitro* Methane Mitigation Activities of Fermented *Scutellaria baicalensis* Georgi Extract*

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발효 황금 뿌리 추출물의 항균, 항산화 효과 및 메탄가스 저감 효과 *In Vitro*

Tabita Dameria Marbun · 송재용 · 이기환 · 김수연 · 강주희 · 이상무 · 최영민 · 조상범 · 배귀석 · 장문백 · 김은중

This study was conducted to investigate the antibacterial, antioxidant, and *in vitro* greenhouse gas mitigation activities of fermented *Scutellaria baicalensis* Georgi extract. Seven starter cultures were used, comprising four of lactic acid bacteria and three of *Saccharomyces cerevisiae*. Ten grams of *S. baicalensis* Georgi powder was diluted in 90 mL autoclaved MRS broth. Each seed culture was inoculated with 3-10% (v/v) *S. baicalensis* Georgi MRS broth and incubated at 30℃ for 48 h. Among the starter cultures used, only *Lactobacillus plantarum* EJ43 could withstand the fermentation conditions. This fermentation broth was dried and extracted with ethanol to assess its antibacterial, antioxidant, and *in vitro* methane mitigation activities. The extract of *S. baicalensis* Georgi fermented by *L.*

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plantarum EJ43 (SBLp) showed higher antibacterial activity (bigger clear zone) compared to the unfermented *S. baicalensis* Georgi extract (SB0). SBLp also presented 1.2 folds higher antioxidant activity than SB0. During *in vitro* rumen fermentation, SBLp showed reduction in methane production compared to SB0 or the control. In conclusion, fermentation by *L. plantarum* EJ43 may enhance antibacterial and antioxidant activities of *S. baicalensis Georgi* and decrease enteric methane production.

Key words : *antioxidant activity, fermentation, Lactobacillus plantarum, methane, Scutellaria baicalensis Georgi*

Ⅰ. Introduction

Antibiotics have been used in the livestock industry not only to treat various diseases but also to improve animal productivity (Landers et al., 2012). However, overuse of antibiotics has caused numerous problems, particularly for public health, such as microbial resistance in animal products and outbreak of multidrug resistant pathogenic microorganisms. Because of these problems, the use of antibiotics has been banned, except in the treatment of diseases (Mathew et al., 2007). Numerous studies have been conducted to develop natural products that may substitute the use of antibiotics (Cowan, 1999).

Plant extract has been considered as a potential natural antibiotic alternative (Cowan, 1999). *Scutellaria baicalensis* Georgi is one of the medicinal herbs used in ancient and modern China and other Asian countries (Gao et al., 1999). It has flavonoid compounds, including baicalein, baicalin, oroxylin A, wogonin, and norwogonin, which have broad biological activities. Several reports have described their strong antibacterial, antiviral, antifungal, anti-inflammatory, and notably antioxidant activities (Gao et al., 2001; Gao et al., 1999; Guo et al., 2011).

Fermentation is a biological process driven by microorganisms, and it may alter the biological, chemical, and physical characteristics of a plant. Various factors determine the effectiveness of the fermentation process, and starter culture strain is one of them. The effectiveness of the factor can vary depending on the purpose of fermentation. The present study analyzed the antibacterial and antioxidant activities of *S. baicalensis* Georgi and reduction in enteric methane production in the rumen.

Methane is produced during rumen fermentation, because it can consume the hydrogen produced during degradation of feed and prevent its accumulation, to maintain the pH of the rumen microorganism niche (Morgavi et al., 2010). However, methane production is also

regarded as energy loss in the context of whole body metabolic status (Johnson and Johnson, 1995). As rumen methane emission was identified as one of the factors contributing to global warming by enhancing the greenhouse effect, many studies have been conducted to identify, quantify, and inhibit methanogens and methanogenesis through methane mitigation schemes (Martin et al., 2010). The present study investigated the effect of different starter cultures on the effectiveness of *S. baicalensis* Georgi fermentation, to select the most appropriate starter culture, and evaluated the improvement in biological activities of *S. baicalensis* Georgi extract after fermentation.

Ⅱ. Materials and Methods

1. Microorganisms, fermentation, and extraction conditions

A total of seven starter strains (four lactic acid bacteria and three *Saccharomyces cerevisiae*), namely *Lactobacillus brevis* SB35, *L. brevis* SB36, *Lactobacillus plantarum* EJ40, and *L. plantarum* EJ43 and *Saccharomyces cerevisiae* EJ44, EJ47, and EJ50, were used for the fermentation of *S. baicalensis* root material. These microorganisms were chosen because they are frequently in demand in the animal industry for various purposes, such as silage preparation and/or in feed additives as probiotics. All strains were kindly donated by the MK-Bioscience Co. Ltd (Suwon, South Korea). Prior to the main experiments, all microorganisms were maintained in MRS broth (Difco, Maryland, USA) and incubated at 30℃ with agitation (150 rpm) for 24 h.

S. baicalensis was purchased from a medicinal plant market (Kyungdong market) in Seoul, South Korea and was ground to 2 mm size by a cutter miller (HR2860, Phillips, South Korea). Ten grams of ground *S. baicalensis* was added to 90 mL MRS broth in a 250 mL Erlenmeyer flask and autoclaved at 121℃ for 15 min. After cooling to room temperature, the mixture was inoculated with 3% (v/v) seed cultures and incubated at 30℃ for 48 h. Cell growth was measured by viable cell count at the end of incubation. Culture broth was dried at 60℃ for about 24 h. The dried culture powder was ground using a mortar and pestle, and 20 mL of 99.9% ethanol (Samchun Chemical, Pyeongtaek, South Korea) was added to 2 g of the powder. Extraction was performed at 30℃ and 150 rpm agitation for 24 h. The extract was filtered through a filter paper (Whatman No. 1, Whatman International Ltd., Maidstone, UK), and the solvent in the filtrate was evaporated using a rotary evaporator (N-110, EYELA, Japan). The concentrated extract was redissolved in 2 mL ethanol and stored at -20℃.

2. Viable cell count

To evaluate the suitability of the starter culture strains for the fermentation of *S. baicalensis*, the cell growth in starter cultures was estimated by viable cell count analysis. After 48 h incubation, the culture broth was serially diluted with 0.85% (w/v) NaCl solution (Daejung Chemical & Metal Co. Ltd., Siheung, South Korea), and the diluted broths were spread on MRS plate medium. The number of colonies that appeared on the plate after 24 h of incubation at 30℃ was counted. The cell growth was represented as log10 colony forming units (CFU)/mL, by logarithmic transformation.

3. Antibacterial and antioxidant activity tests

Antibacterial activity of the extract was determined using the agar well diffusion method. For pathogenic bacteria, *Staphylococcus aureus* (wild type), *Listeria monocytogenes* KACC0550, *Salmonella gallinarum* ATCC9184, and *Mannheimia haemolytica* (wild type) were used, and they were grown in Luria-Bertani (LB) broth (Difco, Maryland, USA) at 37℃ in a shaking incubator for 24 h. A culture broth aliquot (0.1 mL) was added to sterilized 0.8% agar (Difco, Maryland, USA) solution, which was maintained at 50℃ to prevent solidification, and the suspension was immediately poured onto LB plate and cooled. An extract aliquot (30 μL) was dropped onto a sterilized paper disk (8 mm diameter, Advanctec, Japan), which was placed on the LB plate after ethanol was allowed to evaporate from it for 30 min. The assay plate was incubated at 37℃, and the diameter of the clear zone around the disk was measured.

Antioxidant activity was examined using DPPH (2,2-di (4-tert-octylphenyl)-1-picrylhydrazyl, Sigma, USA) to measure the free radical scavenging activity. Diluted extract (0.5 mL) was mixed with 2.0 mL of DPPH solution (0.2 M in ethyl alcohol) and incubated at room temperature for 15 min. Following this, 2.5 mL distilled water was added to the mixture, and its optical density was measured at 525 nm using a spectrophotometer (Optizen, South Korea). Antioxidant activity was expressed as EC50, the concentration of extract showing 50% free radical scavenging activity, where lower EC50 values refer to better antioxidant activity.

4. Thin layer chromatography (TLC)-DPPH screening assay

A compound in the extract, relevant to antioxidant activity, was further monitored using TLC, based on the method by Masoko and Eloff (2007). The TLC plate used was aluminum-back

TLC silica gel F254 plate (Fisher Scientific, South Korea). It was developed under three elution systems: ethyl acetate/methanol/water (EMW, 40:5:4.5, for polar/neutral), chloroform/ethyl acetate/formic acid (CEF, 5:4:1, for intermediate polarity/acidic), and benzene/ethanol/ammonium hydroxide (BEA, 90:10:1, for non-polar/basic). After development, the plate was dried under a fume hood, and the chromatogram was sprayed with 0.2% DPPH solution (w/v, in methanol).

5. *In vitro* fermentation characteristics

In vitro rumen fermentation was conducted according to the method by Johnson (1966), with three experimental treatments: 1) control (CON) with no extract added, 2) 10 μL supplementation with the extract of unfermented *S. baicalensis* root (SB0), and 3) 10 μL supplementation with the extract of *S. baicalensis* root fermented with *L. plantarum* EJ43 (SBLp). Ground orchard grass hay (0.5 g) was placed in a culture bottle and 50 mL of rumen inoculum, mixture of McDougal's buffer (1948) and filtered rumen fluid (1:9), was added to it. The rumen contents were obtained from a local slaughterhouse, immediately transferred to our laboratory in a thermal flask, and filtered through eight layers of muslin in a CO_2 enriched environment. After 10 μL *S. baicalensis* Georgi extract was added to the mixture, the bottle was capped and incubated at 39℃, in triplicates. Gas production was measured at 3, 6, 9, 12, and 24 h using a glass syringe (Sercrim Labtech Co. Ltd., South Korea). The produced gas was collected in an aluminum bag to determine the concentration of methane and carbon dioxide.

At the end of the incubation, all bottles were opened and stored in an ice-filled container to stop microbial fermentation. Supernatant was collected, by transferring the bottle contents to a nylon bag (R510, Ankom Technology, USA), for pH measurement and then centrifuged (5 min at 10,000 rpm, 1730MR, Labogene, South Korea) and stored at -20℃ for further analysis. Dry matter digestibility (DMD) was measured at 0 and 24 h, using a nylon bag to transfer any residues from the incubation bottle, which was washed with tap water until the water ran clear. Volatile fatty acid (VFA) concentration was determined according to the method by Erwin et al. (1961). A 1 mL aliquot of sample pre-treated with 0.2 mL of 25% metaphosphoric acid (Wako, Japan) was injected into the gas chromatograph (450-GC, Bruker Inc., Germany). VFA standard solution (Sigma Aldrich, USA) and BR-Wax fame (BR87503, Germany) column were used. Injector and detector (flame ionized detector) were aligned to 250℃, whereas oven temperature was 100℃. Ammonia-N was analyzed according to the method by Chaney and Marbach (1962).

6. Statistical analysis

The results of *in vitro* fermentation study were analyzed using the general linear model of SPSS 19 software (IBM Corporation), with the effect of supplementation as the main effect in the model. Further differences between means were subjected to Duncan's multiple range test, and significant effect of treatment were declared at $P < 0.05$, with tendencies at $0.05 < P < 0.10$.

Ⅲ. Results and Discussion

1. Cell viability

The bacterial growth in the MRS medium containing *S. baicalensis* Georgi (10%, w/v) was screened. Viability was only observed in *S. baicalensis* fermented with *L. plantarum* EJ43, with cell counts being approximately 107 CFU/mL. Therefore, in this experiment, it was concluded that *L. plantarum* EJ43 could ferment *S. baicalensis* Georgi root, and further extraction was performed only with this culture.

2. Antibacterial activity

The result of antibacterial assay is presented in Table 1. The extract of *S. baicalensis* fermented with *L. plantarum* EJ43 was examined, because only this treatment allowed for cell viability. This extract showed antibacterial activity against S. aureus, L. monocytogenes, and M. haemolytica, but not against *S. gallinarum*. Mukherjee and Ramesh (2015) reported that *L. plantarum* strongly inhibits *S. aureus* through inhibitory substances, such as bacteriocins. *S. baicalensis* was also reported to possess antibacterial activity against *S. aureus*. In this study, it is uncertain whether the antibacterial activity of *S. baicalensis* was improved by fermentation with *L. plantarum* EJ43, because the activities of control and treatments were not different.

The highest antibacterial activity was observed against *L. monocytogenes*, a pathogenic bacterium related to food contamination. Grazing animals are usually infected by ingesting this microorganism from contaminated vegetation and soil. *Listeria* contamination in silage resulted in latent infection, reported in some herds or flocks, and only a few animals showed clinical manifestation (Nightingale et al., 2004). In ruminants, listeriosis causes septicemia, abortion, and latent infection. *Listeria* may be shed for more than 1 month, via the vagina and milk. Recently,

Table 1. Antibacterial activity of the extracts of *Scutellaria baicalensis* root, with or without fermentation with *Lactobacillus plantarum* EJ43

	Treatment	
Pathogenic bacteria	SB0	SBLp
	Clear zone (mm)	
Staphylococcus aureus	8.5	8.5
Listeria monocytogenes	9.0	10.0
Mannheimia haemolytica	9.5	9.0
Salmonella gallinarum	ND	ND

ND, Not detected; SB0, supplemented with the extract of *Scutellaria baicalensis* root; SBLp, supplemented with the extract of *S. baicalensis* root fermented by *Lactobacillus plantarum* EJ43.

lactic acid producing bacteria have been used as potential biological agents against *L. monocytogenes* in contaminated food. This anti-*Listeria* action of the bacteria has been ascribed to lactic acid and bacteriocin production (Wilson et al., 2005). Bacteriocins are bacterial proteinaceous toxins that display bactericidal activity against similar or closely related bacteria. The anti-*Listeria* bacteriocin works by dissipating both proton motive force and membrane potential (Bruno and Montville, 1993).

The antioxidant activity is shown in Fig. 1. The extract of *S. baicalensis* fermented by *L. plantarum* EJ43 (SBLp) showed 1.2 folds lower EC50 than the control (SB0).

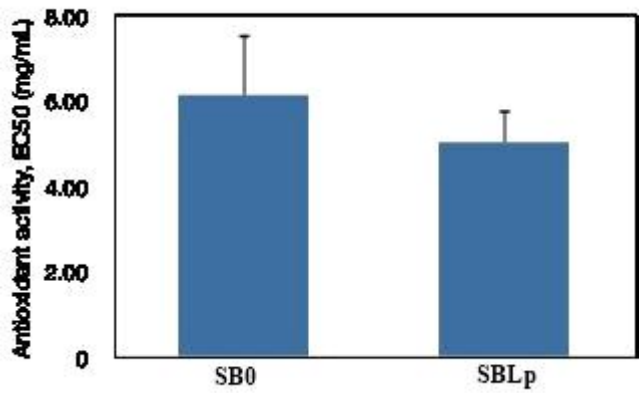
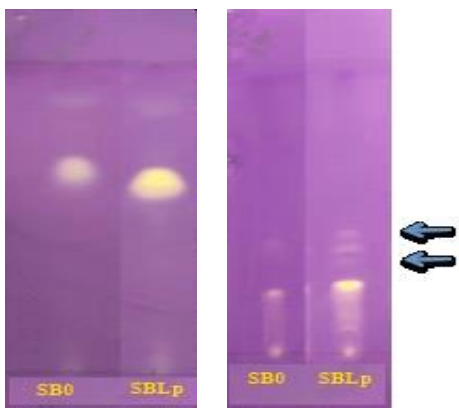


Fig. 1. Antioxidant activity of ethanol extracts of *Scutellaria baicalensis* (SB0) and *S. baicalensis* fermented by *Lactobacillus plantarum* EJ43 (SBLp).

3. TLC-DPPH assay

In this study, three different elution systems were applied to separate a compound in the extract relevant to antioxidant activity. To identify the constituent related to the antioxidant activity, TLC plate was stained with DPPH solution. No new spots were detected in EMW system (data not shown), delayed migration of spot was observed in CEF elution system (Fig. 2A), whereas distinct new spots were detected in BEA elution system (Fig. 2B). As a result, it was found that TLC-DPPH with CEF and BEA could be used for the detection of newly synthesized and/or newly altered compounds, and fermentation with *L. plantarum* EJ43 could change unknown compounds in *S. baicalensis* that may have potential to antioxidant activity.



(A) (B)

Fig. 2. Antioxidant component developed on a thin layer chromatography plate using elution solvent composed of chloroform/ethyl acetate/formic acid (5:4:1) [A] and benzene/ethanol/ammonium hydroxide (90:10:1) [B]. In [A] and [B], the developed chromatogram was treated with 0.2% 2,2-di (4-tert-octylphenyl)-1-picrylhydrazyl solution, and bright spots represent the components that have antioxidant activity. SB0 indicates *Scutellaria baicalensis* extract, whereas SBLp indicates the extract of *S. baicalensis* fermented by *Lactobacillus plantarum* EJ43.

4. *In vitro* fermentation parameters

The effects of *S. baicalensis* extract on pH, ammonia-N concentration, and DMD *in vitro* are presented in Table 2. The ruminal pH was close to neutral at the end of 24 h incubation; hence, no detrimental effect on rumen microorganisms is expected *in vitro*. The tests for ammonia-N

concentration showed somewhat different results. *S. baicalensis* fermented by *L. plantarum* EJ43 extract showed the highest production of ammonia-N among all the treatments, with a value of 26.23 mg/100 mL ($P < 0.05$). DMD showed significant difference among treatments ($P < 0.05$).

Total VFA production numerically decreased when the extract of *S. baicalensis* was supplemented, although there was no statistical difference among the treatments (Table 2). This result did not match with that of DMD, which was highest for SB0. The reason for such differences remains unclear.

The total gas production by SB0 and SBLp was lower than that by CON (Table 3). However, SBLp also lowered methane production, indicating that the extract of this particular plant after fermentation may be used for methane control in the diet of ruminants, although further studies are warranted.

Table 2. Effect of fermented *Scutellaria baicalensis* root extract on rumen fermentation characteristics *in vitro*

Characteristic	Treatment			SEM	P-value
	CON	SB0	SBLp		
pH	6.77	6.75	6.78	0.01	0.554
Ammonia-N (mg/100 mL)	22.48[b]	12.67[a]	26.23[c]	2.04	0.001
Dry matter digestibility (%)	60.75[a]	65.12[b]	60.68[a]	0.78	0.001
Volatile fatty acids (VFA) (mM)					
Acetate	48.25	46.52	45.97	0.79	0.535
Propionate	14.17	13.85	13.36	0.29	0.583
Isobutyrate	1.00	0.86	0.84	0.03	0.105
Butyrate	12.54[b]	10.39[a]	10.61[a]	0.38	0.051
Isovalerate	2.17	1.92	1.94	0.07	0.291
Valerate	1.33	1.21	1.20	0.04	0.351
Total VFA	79.46	74.75	73.91	1.59	0.353
A:P ratio	3.41	3.36	3.44	0.02	0.470

CON, treatment with no supplement; SB0, supplemented with the extract of *S. baicalensis* root; SBLp, supplemented with the extract of *S. baicalensis* root fermented by *Lactobacillus plantarum* EJ43

A:P ratio, acetate:propionate ratio

SEM = standard error of the mean

[a, b, c] Different superscripted letters in the same row indicate significant difference ($P < 0.05$).

Methanogenesis in the rumen utilizes hydrogen and carbon dioxide produced during carbohydrate fermentation, which leads to the formation of VFAs. Formate formed during acetate production may be involved in methanogenesis, even though it is usually directly degraded into hydrogen and carbon dioxide (Hungate et al., 1970). VFAs, the end product of rumen fermentation, show a positive correlation with bacterial growth (Hungate et al., 1971). In terms of hydrogen transaction, acetate and butyrate production are considered hydrogen production reactions, whereas propionate production is considered a hydrogen consuming reaction (Martin et al., 2010; Moss et al., 2000). It is difficult to estimate such a relationship in our study, as the results of VFA and gas production did not reflect each other. Nevertheless, the extract of fermented *S. baicalensis* root showed antibacterial and antioxidant effects and reduced methane production *in vitro*. Therefore, it may be suggested that this extract can be potentially developed as an alternative feed additive, to replace antibiotics in animal industry. Further research is necessary to identify the compound observed during the TLC analysis, and *in vivo* experiments are required to examine the efficacy of the extract.

Table 3. Effect of fermented *Scutellaria baicalensis* root extract on gas production and methane mitigation *in vitro*

Component	Treatment			SEM	P-value
	CON	SB0	SBLp		
Total gas production (mL)	110.67^{b}	100.00^{a}	96.67^{a}	2.29	0.003
CH_4 (mL)	8.52^{ab}	10.11^{b}	7.63^{a}	0.46	0.056
H_2 (mL)	0.20	0.24	0.23	0.01	0.499

CON, treatment with no supplement; SB0, supplemented with the extract of *S. baicalensis* root; SBLp, supplemented with the extract of *S. baicalensis* root fermented by *Lactobacillus plantarum* EJ43; SEM=standard error of the mean

$^{a, b, c}$ Different superscripted letters in the same row indicate significant difference ($P < 0.05$).

Ⅳ. Conclusions

In this study, only *L. plantarum* EJ43 showed viable cell activity during fermentation of *S. baicalensis* Georgi root. Extract from this fermentation showed potential antibacterial activity against pathogenic bacteria. It also showed higher antioxidant activity compared to the extract of unfermented *S. baicalensis* Georgi. The reason may be changes in the antioxidant compounds, as

deduced from TLC assay. During *in vitro* rumen fermentation, addition of fermented *S. baicalensis* Georgi significantly reduced methane production, compared to that on addition of unfermented *S. baicalensis* Georgi and control. Further studies are warranted to identify unknown antioxidant compounds in the extract of fermented *S. baicalensis*, and *in vivo* evaluation is necessary to confirm the effects of *S. baicalensis* extract.

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