Bactericidal Activity of Soymilk Fermentation Broth by *In Vitro* and Animal Models

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ABSTRACT Soybean fermentation broth (SFB) exhibits potent antibacterial activity against different species of bacteria in *in vitro* assays and animal models. Four isoflavone compounds—daidzin, genistin, genistein, and daidzein—of SFB were analyzed and quantified by high-performance liquid chromatography. In the *in vitro* test, daidzin and daidzein had more potent antibacterial activity than genistin. The minimum inhibition concentration values for these bacteria of SFB ranged from 1.25% to 5%, and the minimum bactericidal concentration values of strains ranged from 2.5% to 10%, depending on the species or strain. Vancomycin-resistant *Enterococcus faecalis* (VRE) strains were also tested for susceptibility to SFB in two species of animal model: the Sprague–Dawley rat and the BALB/c mouse. SFB-fed Sprague–Dawley rats showed excellent elimination efficiency against VRE, close to 99% compared with the phosphate-buffered saline–fed control group. In the BALB/c mouse model, SFB antibacterial activity was 65–80% against VRE compared with the control. In conclusion, SFB contains natural antibacterial substances such as daidzin, genistin, and daidzein that inhibit bacterial growth.

KEY WORDS: • *animal models* • *antibacterial activity* • *isoflavones* • *soybean fermentation broth* • *vancomycin-resistant Enterococcus faecalis*

INTRODUCTION

THE HISTORY OF HUMANKIND can be regarded from a medical point of view as a struggle against infectious diseases. At the beginning of the 21st century, the occurrence of drug-resistant bacterial infections has become common, and research into the development of new antimicrobial drugs appears to be spreading into new clinical niches. Contamination with microorganisms resistant to multiple antibiotics represents a threat to clinics.¹ However, natural products discovery programs serve an important role in current development of new drugs, including antimicrobial drugs.² One category of natural phytochemicals, isoflavones, is plentiful in soybeans, and their various chemical structures give rise to variable bioactivities in biological systems.^{3–5} Isoflavones inhibit tyrosine kinases, have antioxidant activity, bind to and activate peroxisome proliferator regulators α and γ , inhibit enzymes in steroid biosynthesis, strongly

influence natural killer cell function and the activation of specific T-cell subsets, and inhibit metastasis.³ Isoflavones and their metabolites are well absorbed and undergo an enterohepatic circulation.

The lactic acid bacteria have cancer chemopreventive properties and act through diverse mechanisms, including alteration of the intestinal microflora, enhancement of the host's immune response, and antioxidative and anti-proliferative activities.⁶ Some reports also claim that soymilk fermented with probiotic bacteria has some advantages: a reduced content of oligosaccharides, enhanced antioxidant activities, and improved flavor and sensory characteristics.^{7,8} There is evidence suggesting that combining several probiotic bacteria will achieve stronger effects than single-strain probiotics.⁹

Soybean fermentation broth (SFB) was fermented by a coculture system of *Lactobacillus* and yeast, which consists of a mixture of soybean extracts and the secondary metabolites of these microorganisms. Moreover, SFB had been used as a traditional remedy in clinical trials to prevent cancer and cardiovascular disease progression, as an adjuvant drug for the reduction of chemotherapy side effects, for the prevention of nosocomial infection, etc. After taking SFB, most cancer patients experience positive

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responses, including immune function enhancement, appetite improvement, and fatigue reduction during chemotherapy or radiotherapy.¹⁰ One report also claimed that SFB has potent antioxidant activity.¹¹

In this study, the antibacterial effect of SFB was evaluated using an *in vitro* assay and two different types of animal models: one for short-term observation during acute peritonitis in the Sprague–Dawley rat model and the other to prove the cumulative effect of the effective compound(s) in serum of a BALB/c mouse model.

In order to investigate the compounds effective for antibacterial activity in SFB, three isoflavones—daidzin, genistin, and daidzein—of SFB were detected and quantified by high-performance liquid chromatography (HPLC). Elements of SFB were also analyzed by inductively coupled plasma (ICP) mass spectrometry (MS), including trace elements, heavy metals, and other chemicals. The bacterial strains tested *in vitro* included two Gram-positive and four Gram-negative bacteria from the ATCC Cell Bank and 10 clinical isolates of bacteria, including eight strains of *Helicobacter pylori* and two drug-resistant strains: vancomycin-resistant *Enterococcus faecalis* (VRE) and oxacillin-resistant *Staphylococcus aureus* (ORSA).

MATERIALS AND METHODS

Tested species of bacteria

Salmonella typhimurium (ATCC14028), *Bacillus subtilis* (CCRC10447), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Proteus vulgaris* (ATCC13315), and *S. aureus* (ATCC25923) were purchased from the ATCC Cell Bank. Two of the clinical bacterial species (VRE and ORSA) were isolated from clinical patients in the Hospital of Taipei Medical University. The minimum inhibition concentration (MIC) of vancomycin for this VRE isolate is between 62.5 µg/mL and 125 µg/mL, and that of oxacillin for the ORSA strain is between 200 µg/mL and 400 µg/mL. The other clinical bacterial strain, *H. pylori*, was isolated from clinical patients with gastrointestinal disease in the Hospital of Taipei Medical University and designated C-98, C-99, D-3, D-4, D-16, E-46, E-85, and G-22.

Animals

For the peritonitis model, male Sprague–Dawley rats weighing 200–250 g were divided into three groups (two experimental groups and one control group of eight rats each) and housed four per cage. All animals had access to chow and water *ad libitum* throughout the experiment. Each group was fed with 4 mL per rat of 1% or 10% SFB solution or phosphate-buffered saline (PBS) buffer, respectively, once per day for 4 days. The VRE inoculums in capsules were implanted into the peritoneal layer of rats on the third day.

In the mouse model, 30 BALB/c mice (weighing about 25 g, 6 weeks old) were randomly divided into three groups (10 mice each), including one control (PBS buffer) and two experimental groups fed with 0.4 mL per rat of 1% or 5%

SFB solution once per day, continuously for 2 weeks. The sera of mice were taken from the eye vein on Days 7 and 14, respectively.

SFB preparation

SFB, a concentrated solution of fermented soybean extract, was provided by Microbio Co., Ltd. The microorganisms used in the fermenting process included *Lactobacillus paracasei*, *Lactobacillus burglarius*, and *Saccharomyces cerevisiae*, which are often found in the human intestinal tract or in some traditional fermented products. The final products with a weight of 1.136 g/mL were subjected to a sterilization process to ensure that they did not carry any food-borne pathogens. The moisture, crude protein, crude fat, crude fiber, carbohydrate, and ash contents in the SFB were 71.49%, 5.45%, 0.16%, 0.15%, 17.6%, and 5.15%, respectively.¹¹

Elemental components of SFB measured by ICP-MS

Some submajor elements (except C, H, O, N and P), important trace elements, and heavy metal ions in SFB were analyzed using ICP-MS (Perkin Elmer) (simultaneous ICP-AES Allied Analytical System Jarrel-Ash and ICP-mass spectrometer, model ICAP 9000, model OPT-DV 3000, and ICP-MS model Elam 5000).

The isoflavones of SFB quantified by HPLC

The concentrations of isoflavones in SFB were quantified by HPLC using the method of Hvattum and Ekeberg,¹² with an Agilent XDB-C18 column (150 mm × 24.6 mm) and Agilent 1100 series liquid chromatography pump (Agilent Technologies, Santa Clara, CA, USA). The solvent system was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). For quantification, a quadrupole mass spectrometer (model API3200, AB Sciex, Foster City, CA, USA) equipped with a turbo ion spray ionization source was used. Analyst version 1.5.1 software (Applied Biosystems) was used for instrument control, data acquisition, and data analysis. Four standard compounds were purchased: genistein and daidzein from Sigma Chemical Co. (St. Louis, MO, USA) and daidzin and genistin from Fluka (Buchs, Switzerland).

Determination of MIC of SFB for various bacteria

The SFB solution was twofold serially diluted in a U-shape 96-well plate, and then an equal volume of tested bacteria suspension containing 3×10^5 colony-forming units (CFU)/mL was added to each well. After incubating at 37°C for 15 h the pattern of sediment on the bottom of the well was observed. For the *H. pylori* strains, the bacterial suspension was also adjusted to 3×10^5 CFU/mL. Then 0.1 mL of bacterial suspension was taken and plated out on chocolate agar plates containing various concentrations of SFB solution (twofold series dilution from 10% to 0.32%) or solution without SFB. The plates were incubated at 37°C for

4 days in a microaerophilic atmosphere with 10% CO₂ and 99% humidity. The MIC was determined.

Determination of minimum bactericidal concentration of SFB

Bacterial suspension (10⁶ CFU/mL) was cultured in Mueller–Hinton broth with various concentrations of SFB solution (twofold series dilution from 10% to 0.32%) and then incubated at 37°C for 24 h. Bacterial counts were obtained by counting CFU on a brain–heart infusion agar plate.

Preparation and implantation of inocula for the Sprague–Dawley rat model of peritonitis

The Sprague–Dawley rat peritonitis study was performed according to the methods of Dupont *et al.*¹³ and Montravers *et al.*¹⁴ In brief, VRE bacteria were grown in brain–heart infusion broth. Semisolid agar was prepared by adding 2% (wt/vol) agar to the diluted culture broth (10⁸ CFU/mL) associated with barium sulfate (10% [wt/vol]), and aliquots (0.5 mL) of the final product were packaged into a double gelatin capsule for peritoneal implantation.

On the third day of SFB feeding, the rats were anesthetized with an intramuscular injection of ketamine (30 mg/kg of body weight), and the gelatin capsule was implanted into the pelvic peritoneal cavity through a midline abdominal incision. The wound was closed with a musculoperitoneal layer and a skin layer by using interrupted nylon sutures. After implantation of inocula, the animals were returned to separate cages; they were observed and weighed daily until sacrifice.

Twenty-four hours after inoculation, the animals were sacrificed. Twenty milliliters of sterilized PBS buffer was injected into the intraperitoneum of each rat. During midline laparotomy, the peritoneal cavity was washed with PBS buffer. The VRE number in the peritoneal washing was determined by colony count on brain–heart infusion agar containing 20 µg/mL vancomycin.

Determination of the antibacterial activity of sera from the BALB/c mouse model

This animal model was designed to elucidate the antibacterial effect of SFB during ingestion time in sera of tested animals. The sera of mice were taken from the eye vein twice, on Days 7 and 14 after ingestion, respectively. Each serum was separated into two aliquots, which were preincubated at 37°C for 1 h in the presence of 20 µg/mL vancomycin to eliminate vancomycin-sensitive bacteria. One of these two serum aliquots was preheated at 56°C for 30 min to eliminate the antibacterial effect of the complement in sera. Each serum sample was mixed with VRE (1.5 × 10⁵ cells/mL). Then the bacterial number was incubated at 37°C for 8 h and determined by colony count on a trypticase soy agar plate containing 20 µg/mL vancomycin. The result was expressed as a percentage as the control group value.

Statistical methods

Student's *t* test and one-way analysis of variance test were used for statistical analysis in this study. Values of *P* < .05 and *P* < .01 by Student's *t* test were considered as statistically significant and very significant differences, respectively. Significant differences by one-way analysis of variance test were indicated by letters.

RESULTS

The elements of SFB by ICP-MS

The concentrations of essential trace elements and three heavy metals in SFB are listed in Table 1. One of the important trace elements, zinc, was present in high concentrations (19.9 ppm), whereas heavy metals such as Hg, Pb, and Cd appeared in low concentrations (<0.02 ppm). The recommended values for these heavy metal concentrations by the Food and Agriculture Organization and the World Health Organization in foods are about 0.2–0.3 ppm.¹⁵

Concentrations of four kinds of isoflavone compounds in SFB and their MIC values

Four isoflavones in SFB were quantified by HPLC and MS: daidzin, genistin, genistein, and daidzein. From the results in Table 2, daidzin was most abundant at 6.13 µg/mL among the four compounds, with genistin and daidzein at 0.95 and 2.85 µg/mL, respectively; genistein was undetectable. Daidzin and genistin had similar antibacterial activities (Table 3).

TABLE 1. CONCENTRATIONS OF ESSENTIAL AND POSSIBLE ESSENTIAL TRACE ELEMENTS AND THREE HEAVY METALS IN SOYMILK FERMENTATION BROTH

Chemical element	Concentration (µg/mL)
B	31.20
Si	22.21
Zn	19.90
Fe	7.89
Mn	4.72
Mo	4.15
Ni	1.40
Cr	0.39
Se	0.09
V	0.02
Co	0.05
Cu	0.02
As ^a	0.01
Br ^a	0.03
Sn ^a	0.02
Cd ^b	0.02
Hg ^b	0.01
Pb ^b	0.02

Compounds were quantified by inductively coupled plasma mass spectrometry.

^aPossible essential trace element.

^bHeavy metal.

TABLE 2. CONCENTRATIONS OF FOUR ISOFLAVONE COMPOUNDS IN SOYMILK FERMENTATION BROTH ANALYZED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS

Compound	Concentration ($\mu\text{g/mL}$, ppm)
Daidzin	6.13
Genistein	ND
Genistin	0.95
Daidzein	2.85

ND, not detectable (below the limit of 50 ng/g [ppb]).

TABLE 3. MINIMUM INHIBITION CONCENTRATION VALUES OF THREE ISOFLAVONE COMPOUNDS FOR VARIOUS BACTERIAL STRAINS

Microorganism	MIC value ($\mu\text{g/mL}$)		
	Daidzin	Genistin	Daidzein
<i>S. typhimurium</i>	64	64	64
<i>B. subtilis</i>	64	64	64
<i>E. coli</i>	64	64	64
<i>P. aeruginosa</i>	64	64	64
<i>P. vulgaris</i>	64	64	64
<i>S. aureus</i>	128	128	64
Vancomycin-resistant <i>E. faecalis</i>	32	32	16
Oxacillin-resistant <i>S. aureus</i>	128	128	128

MIC, minimum inhibition concentration.

MIC and MBC values of SFB

The MIC and MBC values of SFB against various bacterial species are shown in Tables 4 and 5, respectively. SFB exhibited potent antibacterial activity against 16 strains of bacteria. The MIC values of SFB ranged from 1.25% to 5% (Table 4), and the MBC values for eight tested bacterial strains ranged from 2.5% to 10% (Table 5), depending on the species of bacteria.

Antibacterial effect of SFB in the Sprague–Dawley rat model

Table 6 shows that the eradication efficiencies of rats fed with 1% or 10% SFB were close to 99%, as compared with

TABLE 4. MINIMUM INHIBITION CONCENTRATION VALUES OF SOYMILK FERMENTATION BROTH FOR VARIOUS BACTERIAL STRAINS

Microorganism	MIC value (%)
<i>S. typhimurium</i>	≤ 2.5
<i>B. subtilis</i>	≤ 2.5
<i>E. coli</i>	≤ 5
<i>P. aeruginosa</i>	≤ 2.5
<i>P. vulgaris</i>	≤ 2.5
<i>S. aureus</i>	≤ 2.5
Vancomycin-resistant <i>E. faecalis</i>	≤ 1.25
Oxacillin-resistant <i>S. aureus</i>	≤ 1.56
<i>H. pylori</i> (D-3, D-4, D-16, E-85)	≤ 1.25
<i>H. pylori</i> (C-98, C-99)	≤ 2.5
<i>H. pylori</i> (E-46, G-22)	≤ 5

TABLE 5. MINIMUM BACTERICIDAL CONCENTRATION VALUES OF SOYMILK FERMENTATION BROTH FOR VARIOUS BACTERIAL STRAINS

Microorganism	MBC value (%)
<i>S. typhimurium</i>	≤ 10
<i>B. subtilis</i>	≤ 2.5
<i>E. coli</i>	≤ 5
<i>P. aeruginosa</i>	≤ 2.5
<i>P. vulgaris</i>	≤ 2.5
<i>S. aureus</i>	≤ 5
Vancomycin-resistant <i>E. faecalis</i>	≤ 5
Oxacillin-resistant <i>S. aureus</i>	≤ 2.5

MBC, minimum bactericidal concentration.

the control group (PBS-fed group), and was statistically significant. However, body weights were not different among the groups of Sprague–Dawley rats (data not shown).

Antibacterial effects in BALB/c mice

After the BALB/c mice were fed with either 1% or 5% SFB or PBS control for 2 weeks, the Day 14 sera of mice were collected individually and examined for incubation of antibacterial activity. Figures 1 and 2 show that the survival rates of VRE bacteria incubated with sera from mice fed with 1% or 5% SFB for 2 weeks were decreased to 58% and 35%, respectively, compared with the control group (Fig. 1). Furthermore, when one of the sera was heated at 56°C for 30 min to inactivate complement activity, survival rates of VRE bacteria were decreased to 23% and 20%, respectively, compared with the control group (Fig. 2). Based on these results, the antibacterial effect of the Day 7 sera were not detectable (data not shown). However, the body weights of BALB/c mice among the three groups were not different.

DISCUSSION

Research has demonstrated that nutrition plays a crucial role in preventing of chronic diseases, as most of them can be related to diet.^{16,17} Functional food enters the concept of considering food not only as a necessity for living but also as

TABLE 6. BACTERIAL TITERS OF VANCOMYCIN-RESISTANT *E. FAECALIS* IN THE PERITONEAL CAVITY IN SACRIFICED SPRAGUE–DAWLEY RATS FROM THREE EXPERIMENTAL GROUPS

Treatment	Titer (CFU/mL)
Control	$9.33 \pm 3.3 (\times 10^4)^a$
SFB solution	
1%	$7.31 \pm 3.1 (\times 10^2)^b$
10%	$1.16 \pm 0.22 (\times 10^2)^{bc}$

Data are mean $\pm$ SD values and were taken from at least five experiments. Differences were analyzed by the one-way analysis of variance test statistical method: ^{abc}significantly different.

CFU, colony-forming units; SFB, soybean fermentation broth.

FIG. 1. The survival rate of vancomycin-resistant *E. faecalis* (VRE) bacteria was determined according the number of bacteria growing in a trypticase soy agar plate. After SFB was fed for 8 weeks, VRE was incubated with plasma from different groups of BALB/c mice at 37°C for 8 h, and then cell growth was enumerated. The value for the control group was defined as 100% bacterial growth. Data are mean $\pm$ SD values. * $P < .05$ by Student's *t* test.

a source of mental and physical well-being, contributing to the prevention and reduction of risk factors for several diseases or enhancing certain physiological functions.^{16,18}

Micronutrients are very important to human health; micronutrient deficiencies and infectious diseases often coexist and exhibit complex interactions leading to a vicious cycle of malnutrition and infections.¹⁹ This study found that the elemental composition of SFB contains important trace elements (Table 1), including the immune system modulator zinc,²⁰ the anemia-related iron,²¹ the brain cell transporter and enzyme cofactor manganese,²² and the Wilson's disease-related molybdenum.²³

As in the study of Kataoka,²⁴ the soy sauce possesses antimicrobial activity against bacteria such as *S. aureus*,

FIG. 2. The survival rate of VRE was determined according the number of bacteria growing in a trypticase soy agar plate. After SFB was fed for 2 weeks, VRE was incubated in plasma from different groups of BALB/c mice at 37°C for 8 h, and then cell growth was enumerated. Before VRE was incubated, the plasma were heat-inactivated at 56°C for 30 min to inactivate the complement in this assay. The value for the control group was defined as 100% bacterial growth. Data are mean $\pm$ SD values. ** $P < .01$ by Student's *t* test.

Shigella flexneri, *Vibrio cholerae*, *Salmonella enteritidis*, nonpathogenic *E. coli*, and pathogenic *E. coli* O157:H7. Soy isoflavones are present in soy foods as aglycones where genistein, daidzein, and glycitein make up 50%, 40%, and 10%, respectively, of the total soybean isoflavones.²⁵ Daidzein glucoside is known as puerarin and daidzin, and it is often the major isoflavone in dietary supplements. The ingredients of SFB include daidzin, daidzein, and genistin but not genistein (Table 2). According the *in vitro* data, three of the isoflavones exhibit antibacterial activity (Table 3).

However, there is substantial evidence that probiotics modulate *H. pylori* colonization on gastric mucosa. The important substances produced by probiotics modify the *H. pylori* eradication rate.²⁶ The result from this report indicates that SFB inhibits the growth of *H. pylori* and hence may also exert eradication effects. The pH of the original SFB was 3.9, and dilution up to 25% SFB did not alter the pH value of culture medium. The bacterial growth inhibition (MIC) and bactericidal effects (MBC) were not contributed by the acid pH of SFB, but from the ingredients that are active compounds with antibacterial effect in SFB. However, the antibacterial activity of SFB may be partially attributed to the three isoflavones (Tables 4–6).

It is an important concern that the resistance of pathogenic bacteria toward antibiotics is certainly increasing but that the rate of discovery and development of new and effective antibiotic compounds is declining.^{27,28} Among the clinically used antibiotics, over two-thirds have been discovered from natural sources or are the semisynthetic derivatives of natural antibiotics.²⁹ The search for new antibiotics is an important element in the fight against the threat posed by the increasing numbers of infections caused by antibiotic-resistant pathogens such as VRE or ORSA. The number of vancomycin-resistant isolates of *S. aureus* or of VRE causing disease is steadily increasing. However, treatment of VRE is more difficult as there are no effective alternative antibiotics available.³⁰

In the Sprague–Dawley rat animal model, the VRE bacteria implanted into the peritoneum directly represent a model of systemic sepsis induced in a very short period of time.³¹ The information provided by the peritoneal implantation of a septic capsule is interesting. In addition, this model also represents a compartmentalized sepsis with prolonged infectious and inflammatory responses that provides the means for studying the prolonged effect on the relationship between the host and the offending organisms. In the Sprague–Dawley rat model, in rats fed with 10% SFB and then challenged with VRE, about 99% of the VRE were eradicated (Table 6). It is interesting that SFB was endowed with potent antibacterial activity, although we have not yet determined the active compound(s) in SFB and its action mechanism and whether they exhibit antibacterial activity directly and/or modulate the immune system of Sprague–Dawley rats indirectly.

Based on the results from the short-term Sprague–Dawley rat study, we designed the mouse model for 2 weeks as a long-term model, trying to answer the question of “whether

the antibacterial effective compound(s) existing in SFB could accumulate in sera after ingestion by the animal.” The results shown in Figure 1 indicate that mice fed with SFB for 2 weeks accumulate enough active components in serum as demonstrated by the antibacterial assay. This phenomenon is especially obvious after elimination of the effective complement in sera by preheating at 56°C for 30 min (Fig. 2). However, a 1-week duration for SFB feeding is not enough to accumulate the effective components.

Similar studies have reported that the functional foods propolis and lemon juice are also proven to have antibacterial activity.^{32,33} The results in this study suggest that SFB may act as a functional food to maintain human health and prevent infections.

CONCLUSIONS

In summary, soymilk can be fermented by various strains of lactic acid bacteria and yeast. SFB exhibited strong antibacterial activity against various bacteria, including two antibiotic-resistant bacteria, VRE and ORSA, isolated from clinical patients. Except for four isoflavone compounds the other active compound or compounds with antibacterial activity in SFB have not yet been identified. However, from our results, it is interesting to demonstrate that the product SFB has potent antibacterial activity as demonstrated by *in vitro* assay and in two animal models.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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