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Secretion dynamics of soyasaponins in soybean roots and effects to modify the bacterial composition

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Abstract

Soyasaponins are triterpenoid saponins widely found in legume plants. These compounds have drawn considerable attention because they have various activities beneficial for human health, and their biosynthesis has been actively studied. In our previous study, we found that legume plants including soybean secrete soyasaponins from the roots in hydroponic culture throughout the growth period, but the physiological roles of soyasaponins in the rhizosphere and their fate in soil after exudation have remained unknown. This study demonstrates that soyasaponins are secreted from the roots of field-grown soybean, and soyasaponin Bb is the major soyasaponin detected in the rhizosphere. In vitro analysis of the distribution coefficient suggested that soyasaponin Bb can diffuse over longer distances in the soil in comparison with daidzein, which is a typical isoflavone secreted from soybean roots. The degradation rate of soyasaponin Bb in soil was slightly faster than that of daidzein, whereas no soyasaponin Bb degradation was observed in autoclaved soil, suggesting that microbes utilize soyasaponins in the rhizosphere. Bacterial community composition was clearly influenced by soyasaponin Bb, and potential plant growth–promoting rhizobacteria such as *Novosphingobium* were significantly enriched in both soyasaponin Bb–treated soil and the soybean rhizosphere. These results strongly suggest that soyasaponin Bb plays an important role in the enrichment of certain microbes in the soybean rhizosphere.

KEYWORDS

bacterial communities, *Novosphingobium*, root exudates, soyasaponin, soybean rhizosphere

1 | INTRODUCTION

The term "rhizosphere" was coined by L. Hiltner in 1904 and refers to "the zone of soil surrounding the root which is affected by it" (Hartmann, Rothballer, & Schmid, 2008, Hiltner, 1904). Plant roots function as an anchor that supports the plant body and absorb nutrients and water; they also secrete a variety of plant-derived metabolites into the rhizosphere, which include low-molecular weight compounds, such as amino acids, sugars, phenolics, terpenoids, and lipids, and high-molecular weight compounds, such as proteins, polysaccharides, and nucleic acids, depending on the growth stage and environmental conditions (Massalha, Korenblum, Tholl, & Aharoni, 2017). The amount of these root exudates is large (up to 40% of all carbon fixed by photosynthesis can be released from plant roots

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into the rhizosphere) and depends on the plant species, developmental stage, and environmental conditions (Grayston, Vaughan, & Jones, 1997; Lynch & Whipps, 1990). Root exudates are involved in many biological processes such as nutrient acquisition (Canarini, Kaiser, Merchant, Richter, & Wanek, 2019) and interactions with other organisms, including microbes in the rhizosphere (Huang et al., 2014). For example, organic acids such as malate and citrate mobilize inorganic phosphorus from soils to plant roots (Carvalhais et al., 2011; Giles et al., 2017; Krishnapriya & Pandey, 2016; Lyu, Tang, Li, Zhang, & Rengel, 2016) by solubilizing P bound to iron or aluminum oxides (Otani, Ae, & Tanaka, 1996). Flavonoids secreted by legume plants act as signaling compounds to initiate symbiosis with rhizobia (Abdel-Lateif, Bogusz, & Hocher, 2012). Some flavonoids are also involved in the interaction of plant roots with plant growth–promoting rhizobacteria (PGPR), mycorrhizal fungi, pathogens, nematodes, and other plants (Hassan & Mathesius, 2012; Sugiyama & Yazaki, 2014). A group of plant hormones, strigolactones, secreted by various plant species, stimulate mycorrhizal hyphal branching (Akiyama, Matsuzaki, & Hayashi, 2005).

Soybean (*Glycine max*), one of the major crops worldwide, secretes primary metabolites such as sugars and amino acids (Tawaraya et al., 2014; Timotiwu & Sakurai, 2002) and also specialized metabolites, for instance isoflavones and soyasaponins, into the rhizosphere (Sugiyama, 2019). Isoflavones are crucial compounds by which soybean plants establish symbiosis with rhizobia via the induction of nodulation genes (Kosslak, Bookland, Barkei, Paarent, & Appelbaum, 1987; Smit, Puvanesarajah, Carlson, Barbour, & Stacey, 1992). Isoflavone secretion from soybean roots is developmentally regulated to maintain the rhizosphere environment (Sugiyama, Yamazaki, Hamamoto, Takase, & Yazaki, 2017; Sugiyama et al., 2016). We previously found that soybean roots selectively release particular saponin molecules into root exudates, depending on their growth stages (Tsuno, Fujimatsu, Endo, Sugiyama, & Yazaki, 2018). Although hydroponically grown soybean secretes equivalent amounts of soyasaponins and isoflavones into the hydroponic medium, the secretion of soyasaponins in field-grown soybean and their function in the soybean rhizosphere remain to be elucidated.

Rhizosphere microbial communities have remarkable effects on plant growth, health, and production by affecting nutrient uptake and resistance to stresses and by suppressing diseases (Berendsen, Pieterse, & Bakker, 2012; Finkel, Castrillo, Paredes, González, & Dangl, 2017; Hacquard et al., 2015). The environment, including climate and soil type, as well as plant species, genotype, and growth stage influences the composition of the rhizosphere microbiome. Recent advancement in sequencing technologies has accelerated studies on the relationships between root exudates and soil microbiota, and links between root-secreted metabolites and microbial communities have been reported (Herz et al., 2018; Hu et al., 2018; Yuan et al., 2018; Zhalnina et al., 2018). Disruption of genes responsible for synthesis of specialized metabolites such as triterpenes, sesterterpenes, and coumarins in *Arabidopsis* revealed the functions of these metabolites in modulating microbial communities (Chen et al., 2019; Huang et al., 2019; Stringlis et al., 2018; Voges, Bai, Schulze-Lefert,

& Sattely, 2019). In soybean, changes in rhizosphere microbial communities during development and continuous cropping have been analyzed (Hara, Matsuda, & Minamisawa, 2019; Liu, Hewezi et al., 2019; Liu, Pan et al., 2019; Sugiyama, Ueda, Zushi, Takase, & Yazaki, 2014). We recently reported that daidzein, the major isoflavone secreted from soybean roots, diffuses within a few millimeters from the root surface and shapes the soybean rhizosphere bacterial communities (Okutani et al., 2020). To expand our understanding of the function of specialized metabolites in the soybean rhizosphere, here we analyzed the exudation and fate of soyasaponins in the rhizosphere and characterized their effects on microbial communities. By integrating the field and in vitro studies, we decipher the role of soyasaponins in the enrichment of particular bacterial families, including Sphingomonadaceae and Caulobacteraceae, in the soybean rhizosphere.

2 | MATERIALS AND METHODS

2.1 | Study site, soybean cultivation, and sampling

Field experiments were conducted at the Kao Corporation, Ichikai-Machi, Tochigi, Japan (36°31′ 22″ N, 140°03′41′ E). A new field was prepared, and soybean was grown there for the first time in 2017. Soybean seeds (*Glycine max* cv. Enrei) were purchased from Tsurushin Shubyo (Matsumoto, Japan) and sown on July 5, 2018. Plants were irrigated as needed, and emerging weeds were removed by hand monthly. Bulk soil samples were collected before sowing the seeds. Bulk and rhizosphere soils for root exudate extraction and analysis of rhizobacteria were collected on July 23 (stage V5), August 6 (V11), August 20 (R3), and September 19 (R6; stages are indicated according to Fehr & Caviness, 1977). Bulk soil (soil not adherent to roots) was obtained at least 20 cm from the plants as described previously (Sugiyama et al., 2014). Bulk soils from four different spots were combined into one sample. One composite rhizosphere soil sample (soil adherent to the roots after gentle shaking) was obtained from the roots of 5–9 plants with the use of sterile brushes. Three composite samples each for bulk and rhizosphere soil were obtained per sampling. All samples were transferred to the laboratory in a cool (0–10°C) container within 10 min. The samples were homogenized, passed through a 1.5-mm sieve, and small root fragments were removed from rhizosphere soil samples. Each sample was then divided into two equal portions; one was lyophilized (FDU-2110, Eyela, Tokyo, Japan) and stored at −20°C until root exudate extraction, and the other one was stored at −20°C until analysis of the microbiome using 16S amplicon sequencing. Chemical properties of the bulk soil collected before sowing were analyzed using standard methods in the Agricultural Product Chemical Research Laboratory of the Federation of Tokachi Agricultural Cooperative Association (Obihiro, Japan). The bulk soil had a phosphate absorption coefficient of 1964 mg/100 g, cation exchange capacity of 45.6 mg/100 g, lime saturation degree of 55.7%, base saturation degree of 76.4%, and pH of 5.9; the

soil contained 0.53% total nitrogen, 12.8 mg/100 g hot water-extractable nitrogen, 1.06 mg/100 g NH_4-N, 18.32 mg/100 g NO_3-N, 162.9 mg/100 g K_2O, 711.7 mg/100 g CaO, 120.9 mg/100 g MgO, 0.70 ppm B, 0.32 ppm Cu, 20.35 ppm Zn, 48.2 mg/100 g P, 66.85 ppm Mn, and 12.1% soil corrosion. No fertilizers or pesticides were applied during the growth season.

2.2 | Chemicals

Daidzein was purchased from Tokyo Chemical Industry. Formic acid of LC-MS grade was purchased from Sigma-Aldrich (St. Louis). Soyasaponins Ba and Bb and soyasapogenols A and B were purchased from Tokiwa Phytochemical. Soyasaponins Bc, Be, A1, and A2 were purchased from AnalytiCon Discovery, and soyasaponins Aa and Ab from Indofine Chemical Company. Acetonitrile and methanol (both of HPLC grade) were purchased from Kanto Chemical. All water used in these experiments was Milli-Q water (Merck Millipore).

2.3 | Purification of soyasaponin Bb

Soyasaponin Bb was purified by column chromatography as follows. Soybean extract B-50 containing 31% (w/w) of soyasaponin Bb was purchased from J-Oil Mills, Inc. B-50 (500 mg) was dissolved in 20 ml of 20% (v/v) acetonitrile and filtered through a 0.45-μm polyethersulfone membrane in a 25-mm syringe filter (Agilent Captiva Econofilter, Agilent Technologies). The filtrate was fractionated by octadecyl-silica (ODS, C_{18}) chromatography on an YFLC AI-580 flash chromatography system (Yamazen) equipped with an injection column (26 × 150 mm, 55 g gel) and a Universal column ODS premium 3L (50 × 190 mm, 140 g gel, 30 μm, 120 Å). After loading the sample, the column system was first eluted with 20% (v/v) acetonitrile in water containing 0.01% TFA for 5 min, then with a linear gradient of acetonitrile from 20% to 40% (v/v) in 20 min, and finally with 40% acetonitrile for 30 min, with a flow rate of 60 ml min^{-1}. The eluate was collected in 80 ml fractions. Soyasaponin Bb was eluted at about 35 min. Each fraction collected around the retention time was analyzed using the HPLC system described below, and fraction No. 28 was found to be the most pure in comparison with the soyasaponin Bb standard. Fraction No. 28 was concentrated under reduced pressure, and lyophilized to give 49.2 mg (9.8% yield) of white powder (pure soyasaponin Bb).

Each fraction after separation of B-50 was analyzed on an Agilent 1260 Infinity HPLC system (Agilent Technologies), which consisted of a binary pump (1260 Bin pump G1312B), a degasser (1260 μ-degasser G1379B), a column thermostat (1260 TCC G1316A), an autosampler (1260 ALS G1329B), and a diode array detector (1260 DAD VL G1315D). Chromatography was performed by injecting a 5-μl sample onto an L-column2 ODS (50 mm × 3 mm, 2 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan) at 40 °C. Elution was monitored at 210 nm. The mobile phase consisted of (A) water containing 0.01% (v/v) TFA, and (B) acetonitrile. The program was as follows: isocratic elution at 40% B, 0–5 min; 100% B, 5.01–7 min; and 40% B, 7.01–10 min. The flow rate was 0.5 ml/min.

2.4 | Extraction of root exudates from bulk and rhizosphere soils

Soyasaponins and soyasapogenols were extracted from 100 mg samples of lyophilized soil with 500 μl of 50% (v/v) acetonitrile for 10 min at 50°C. This extraction was repeated twice. All the supernatants were combined and filtered through a DISMIC-13HP 0.45-μm syringe filter (Advantec, Tokyo, Japan). Each sample was diluted to 5 ml in a measuring flask with 50% (v/v) acetonitrile and additionally diluted if necessary before LC-MS/MS analysis, which was carried out as described previously (Tsuno et al., 2018). In brief, each sample was analyzed using a Shimadzu Nexera UHPLC system (Shimadzu, Kyoto, Japan), coupled to an AB SCIEX TripleQuad^{TM} 4500 triple quadruple tandem mass spectrometer (AB Sciex). Samples were separated on a Capcell Core C18 reversed-phase column (50 mm × 2.1 mm, 2.7 μm; Shiseido) and a Capcell Core C18 guard column (5 mm × 2.1 mm, 2.7 μm; Shiseido) at 40°C. The LC mobile phase consisted of (A) water containing 0.1% (v/v) formic acid and (B) acetonitrile. The gradient program was isocratic at 10% B, 0–1 min; linear 10–47.5% B, 1–7 min; linear 47.5–85% B, 7–9 min; isocratic at 100% B, 9–10 min; and isocratic at 10% B, 10–11 min. The injection volume of each sample was 5 μl, and the flow rate was 0.5 ml min^{-1}. Soyasaponins and sapogenins were detected based on multiple reaction monitoring (MRM) method and quantified using standard solutions.

2.5 | Treatment of bulk soil with soyasaponin Bb

The experiment was conducted at two concentrations of soyasaponin Bb using bulk soil collected before sowing.

1. Low concentration: We placed 1.30 g of fresh soil (23% moisture, i.e., 1.00 g of dry weight) in a 5 ml tube, and added 20 μg of purified soyasaponin Bb in 40 μl of water, followed by 200 μl of water to adjust soil moisture to 35%, and vortexed the mixture thoroughly. The tubes were capped and kept in the dark at 23°C. After 3 days from the first treatment, 12.5 μg of soyasaponin Bb in 25 μl of water was added to each tube, and the mixture was vortexed thoroughly and then kept in the dark without a lid until moisture in the soil returned to 35%. After 6, 9, and 12 days, the same treatment as on third day was performed. After 15 days, the tubes were frozen in liquid nitrogen and stored at − 20°C until DNA extraction. For control treatment, same treatment without soyasaponin Bb was performed using pure water.

2. High concentration: Treatment was performed as in (1), with the following modifications: 40 μg of soyasaponin Bb in 80 μl of water was initially added, 160 μl of water was added to adjust soil

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moisture to 35%, and 35 µg of soyasaponin Bb in 70 µl of water was added after 3, 6, 9, and 12 days, instead of 25 µl for low concentration samples. For control treatment, same treatment without soyasaponin Bb was performed, using pure water.

2.6 | Analysis of soyasaponin Bb and daidzein degradation in bulk soil

We placed 130 mg of fresh soil collected before sowing in a 2-ml tube, added 1 µg of soyasaponin Bb or daidzein in 10 µl of water, and vortexed the mixture thoroughly. The tubes were kept in the dark at 23°C. After 0, 2, 4, and 7 days, the tubes were frozen in liquid nitrogen and lyophilized (FDU-2110, Eyela). Dry soil samples were used for extraction described above and the extracted compounds were quantified by LC-MS/MS (Tsuno et al., 2018). For degradation analysis of soyasaponin Bb in autoclaved soil, a portion was taken from the same batch of soil described above and autoclaved at 120°C for 15 min. We performed the treatment as described above, except using autoclaved soil.

2.7 | Analysis of soyasaponin Bb and daidzein adsorption in bulk soil

We placed 1.30 g of fresh soil (1 g dry weight) in a 20-ml glass tube, added 9.7 ml of water and 10, 50, or 100 nmol of soyasaponin Bb or daidzein in 10 µl of DMSO, and stirred the mixture with a magnetic stirrer (RCX-1000D, Eyela) at 250 rpm for 15 min. The content of each tube was filtered through a 0.45-µm polytetrafluoroethylene membrane filter in a 13-mm syringe filter (DISMIC-13HP) and diluted with 50% acetonitrile for further quantitative analysis by LC-MS/MS (Tsuno et al., 2018). The adsorption of soyasaponin Bb and daidzein on bulk soil was calculated by subtracting the amount of soyasaponin Bb or daidzein in the aqueous phase from the added amount in the initial solution (Liang et al., 2011). Based on the obtained adsorption isotherm, the distribution coefficients (K_d, $m^3 \, kg^{-1}$ soil dry weight) of soyasaponins Bb and daidzein were calculated as the ratio of the measured soyasaponin Bb or daidzein concentration in the filtrate to its content in the soil, respectively.

2.8 | DNA Extraction, PCR, and sequencing

Each soil sample was lyophilized in a freeze dryer (VD-250R, Taitec, Saitama) and ground in a homogenizer (Shake Master Neo; BMS). DNA extraction and amplicon sequencing of the 16S rRNA gene were performed by Bioengineering Lab. Co. Briefly, DNA was extracted using an MPure bacterial DNA extraction kit (MP Bio Japan) and quantified using Synergy H1 (Bio Tek) and QuantiFluor dsDNA System (Promega). A DNA library was prepared using a two-step PCR method (Sinclair, Osman, Bertilsson, & Eiler, 2015).

In the first PCR, the V4 region was amplified in a 10-µl reaction mixture containing 1 ng template DNA, 1 × Ex buffer (TaKaRa, Shiga, Japan), 500 nM forward and reverse primers, 0.2 mM dNTPs, and 0.5 U of ExTaq HS (TaKaRa). Non-barcoded V4 forward primer mix (515f: 5′-ACACTCTTTCCCTACACGACGCTCTTC CGATCT-NNNNN-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer mix (806r: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTC CGATCT-NNNNN-GGACTCHVGGGTWTCTAAT-3′) were used, where NNNNN represents a random sequence of five5 nucleotides for quality improvement. The conditions of the first PCR were as follows: denaturation at 94°C for 2 min; 25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. PCR products were purified using AMPure XP reagent (Beckman Coulter).

In the second PCR, products from the first PCR were amplified as above except that the reaction mixture contained 10 ng of the template (first-PCR product) and the barcoded forward primer (5′-AATGATACGGCGACCACCGAGATCTACAC-[Index 2]-ACACTCTTTCCCTACACCGACGC-3′) and reverse primer (5′-CAAGCAGAAGACGGCATACGAGAT-[Index 1]-CTGACTGGAGTTCAGACGTGTG-3′). Index pair sequences are listed in Table S1. The conditions of the second PCR were as follows: denaturation at 94°C for 2 min; 10 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. PCR products were purified using AMPure XP reagent, and their quality and quantity were checked on a Fragment Analyzer using a dsDNA 915 Reagent Kit (Advanced Analytical Technologies).

DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to the manufacturer's instructions (Illumina). Reads with the wrong sequence of the start region were filtered with the FASTX Toolkit (fastq_barcode_splitter, http://hannonlab. cshl.edu/fastx_toolkit/). Among the selected reads, chimeras were identified by the QIIME2 pipeline (Bolyen et al., 2019) and omitted (Table S2). Sequences were clustered into operational taxonomic units (OTUs) according to a 97% identity cut-off using the QIIME2 workflow script with default parameters and EzBioCloud 16S reference database (https://www.ezbiocloud.net/), which has a higher accuracy of taxonomic identification than other existing databases (Park & Won, 2018). The filtered datasets were then normalized by transforming the number of OTU counts to relative abundance values. The raw read sequences were deposited into the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) database (accession: DRA009509). Abundance of *Novosphingobium* and *Phenylobacterium* in the soybean rhizosphere and daidzein-treated soils was analyzed using sequences previously deposited in DRA (DRA008649; Okutani et al., 2020).

Quantification of bacterial number was performed using Quantitative PCR Kit (TaKaRa) according to the manufacturer's instructions (Keymer & Kent, 2014). Real-time quantitative PCR was performed in a 96-well plate using an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific) with the following

conditions: an initial denaturation at 95°C for 30 s, 35 cycles of 95°C for 5 s, 60°C for 30 s.

2.9 | Statistical analysis

To evaluate species richness and diversity (α-diversity), we calculated the number of OTUs, CHAO1, and ACE using the "estimateR" function, and Shannon index (H) and Simpson's reciprocal index (invsimpson, 1/D) using the "diversity" function in the 'vegan' package (Oksanen et al., 2013) in R (R Core Team, 2016).

To compare community-level differences (β-diversity) between taxa identified in each soil, non-metric multidimensional scaling (NMDS) ordinations using Bray–Curtis distance were constructed with the "metaMDS" function in the "vegan" package (Oksanen et al., 2013); default settings were used except trymax = 20. Before the analysis, the raw OTU counts were rarefied to the lowest number of reads in a sample (n = 24,678) using the "rarefy" function in the "vegan" package. To simplify comparisons, two-dimensional ordinations were selected. Families, genera, and OTUs with differential abundance between bulk and rhizosphere soils at each growth stage or in soyasaponin Bb-treated and non-treated soils were identified with a negative binomial generalized linear model in the "edgeR" package with an FDR-corrected p-value below 0.05 (Robinson, McCarthy, & Smyth, 2009). Before the analysis, taxa were filtered based on their read counts, that is, only the taxa detected by at least one read count in at least 50% of the samples were retained. Venn diagrams were generated using the Venn diagram package (Chen & Boutros, 2011) in R.

2.10 | Accession numbers

Sequence data from this article can be found in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA) database under accession number DRA009509.

3 | RESULTS

3.1 | Soyasaponin secretion into rhizosphere soils during soybean growth

Soyasaponins, commonly found in legume plants, are composed of soyasapogenol (aglycone) and oligosaccharide moieties. They are classified into four subgroups depending on their aglycone structures, with soyasaponin groups A, B, and E being the glycosides of soyasapogenols A, B and E, respectively, and group DDMP soyasaponins being the glycosides of soyasapogenol B, consisting of C-22 chains bound to DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one) residues (Shiraiwa & Kurosawa, 2001). Since it has been demonstrated that the secretion of soyasaponins from soybean roots altered throughout the growth periods in hydroponic cultures
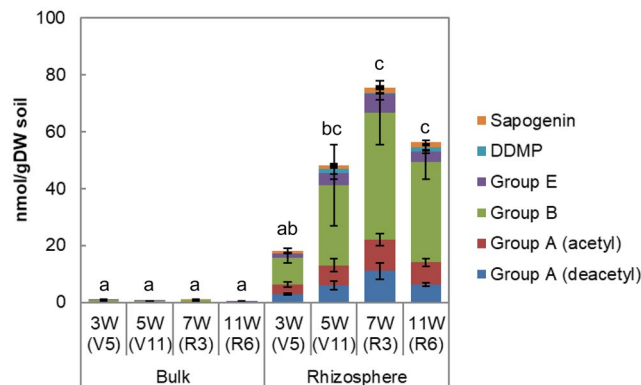

FIGURE 1 Contents of soyasaponins and sapogenins in bulk and rhizosphere soils throughout soybean growth stages in the field. The contents of group A, B, and E soyasaponins are sum of several soyasaponins data shown in Figure S2, and those of DDMP soyasaponin and sapogenin are soyasaponin βg and soyasapogenol B data, respectively. Data are means ± SD (n = 3). Significant differences (P < 0.05; Tukey–Kramer test) are indicated with different letters. V5, V11, R3, and R6, soybean growth stages; W, weeks after sowing

with higher secretion during vegetative (V) stages than reproductive (R) stages (Tsuno et al., 2018), we analyzed soyasaponins in both bulk and rhizosphere soils at two stages of vegetative growth (V5 and V11) and two stages of reproductive growth (R3 and R6). Remarkable amounts of soyasaponins were detected in the soybean rhizosphere soil but not in the bulk soil during all growth stages (Figure 1), suggesting that soybean secreted soyasaponins throughout the growth period under field conditions, as observed in hydroponic cultures (Tsuno et al., 2018). Sapogenins were also detected, but the amount was less than 4.0 % of soyasaponins throughout the growth stages (Figure 1). The amount of soyasaponins in rhizosphere soil was highest at the R3 stage and lowest at the V5 stage. The composition of soyasaponins was similar at all growth stages tested, with group B soyasaponins representing ca. 60% of the total soyasaponins, followed by group A, E, and DDMP soyasaponins. Soyasaponin Bb represented 65%–70% of group B soyasaponins, whereas soyasaponins Ab and deacetyl Af represented 24%–27% and 26%–31% of group A soyasaponins, respectively (Figure 1; Figures S1 and S2). For sapogenins, only soyasapogenol B was detected (Figure S2). Because soyasaponin Bb was the main soyasaponin in the soybean rhizosphere (Figure S2), we used soyasaponin Bb for further studies.

3.2 | Degradation of soyasaponin Bb in bulk soil

We analyzed the degradation rate and the distribution coefficient of soyasaponin Bb in field soils. Soyasaponin Bb was gradually degraded in bulk soil, whereas the degradation was not observed in autoclaved bulk soil even after 7 days (Figure 2), suggesting that soyasaponin Bb was degraded by soil microbes. After 7 days, 13.2% soyasaponin Bb remained, and the half-life was 2.4 days
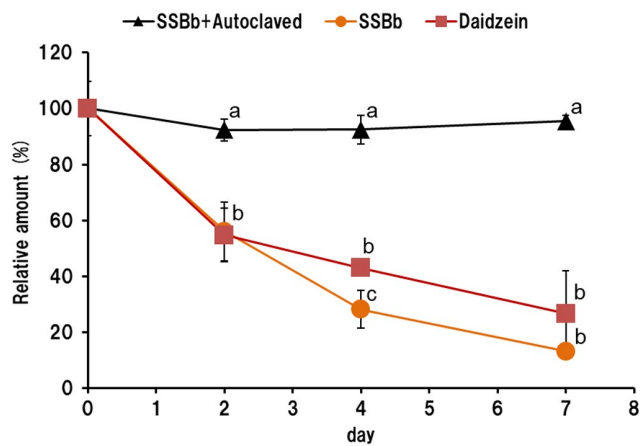
FIGURE 2 Degradation of soyasaponin Bb (SSBb) and daidzein in field soil. Bulk soil collected at sowing was used to analyze degradation of each compound within 7 days, in addition to the autoclaved bulk soil with SSBb. Values are means ± SD of three (SSBb and daidzein) or five (SSBb + Autoclaved) replicates. Significant differences ($P < 0.05$; Tukey–Kramer test) on the same day (except day 0) are indicated with different letters.

FIGURE 3 Adsorption of soyasaponin Bb (SSBb) and daidzein in the field soil. The mixtures of bulk soil collected at sowing and water solution of each compound at different concentrations were analyzed to calculate distribution coefficient. Cs (mg/kg), the amount of each compound adsorbed in the soil phase; Cw (mg/L), the concentration of each compound in the aqueous phase; Kd (m^3 kg^{-1}), distribution coefficient. Values are means ± SD ($n = 3$)

assuming a first-order reaction. In the same bulk soil, daidzein was degraded slightly slower than soyasaponin Bb, with a half-life of 3.8 days (Figure 2).

In soyasaponin Bb, glucuronic acid and two sugars (galactose and rhamnose) are bound to soyasapogenol B (Figure S3). Therefore, the sugar moieties are likely to be eliminated by hydrolysis to deliver sapogenin as a degradation product of soyasaponin Bb. In the tested soils, soyasaponin Be (oxidized at position 22 of soyasaponin Bb), soyasaponin Bb' (with rhamnose cleaved off), and soyasapogenol B were found as metabolites of soyasaponin Bb (Figures S3 and S4). One day after the addition of soyasaponin Bb, the total number of moles of these three metabolites and remaining soyasaponin Bb was similar to that of soyasaponin Bb added. At 2 days after the addition, the total amount of saponins decreased significantly and was ca. 70 mol% of soyasaponin Bb added to soil. After 7 days, most soyasaponin Bb was metabolized to components other than these three metabolites (Figure S4). These results suggest that soyasaponin Bb was metabolized and utilized as a carbon source by soil microbes.

3.3 | Adsorption of soyasaponin Bb to soil

Distribution coefficient (Kd), defined as the ratio of concentrations in the adsorbed and solution phases, is a key parameter for assessing the transport of a compound in the soil (Okutani et al., 2020; Wang & Chen, 2020). The distribution coefficient of soyasaponin Bb was 0.0088 m^3kg^{-1}, and that of daidzein was 0.0474 m^3kg^{-1}, that is, 5.4 times that of soyasaponin Bb (Figure 3). These results indicate that soyasaponin Bb adsorbed to soil to a lesser extent than did daidzein. Because the degradation rates of soyasaponin Bb and daidzein
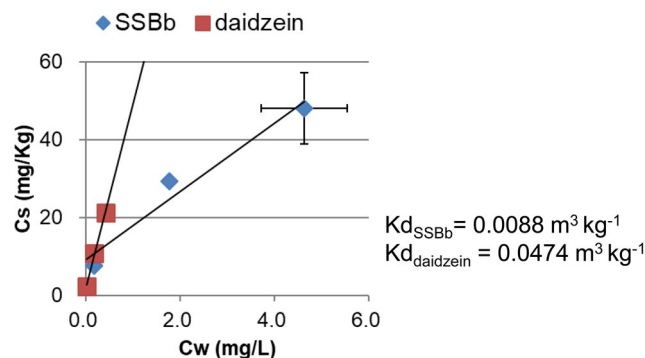
were similar, five-fold lower distribution coefficient of soyasaponin Bb suggests that soyasaponin Bb moves over longer distances in the soybean rhizosphere than does daidzein.

3.4 | Effect of soyasaponin Bb on bacterial community

Soyasaponin Bb was detected in rhizosphere soil throughout the growth period of the soybean (Figure 1, Figure S1) and was degraded by microbes (Figure 2). Soyasaponin Bb can diffuse over longer distances in the rhizosphere in comparison with daidzein (Figure 3). To evaluate the effect of soyasaponin Bb on microbial communities in the rhizosphere, we added it in vitro to the field soil every 3 days (five additions in total). The total amount of soyasaponin Bb added per soil dry weight was 70 µg g^{-1} (74.2 nmol g^{-1}) or 180 µg g^{-1} (190.0 nmol g^{-1}). The low and high concentrations were chosen on the basis of the calculated content of soyasaponin Bb in the rhizosphere during the growth (Figure S1).

Soyasaponin Bb treatment did not affect any of the α-diversity indices at either of the two concentrations (Figure S5), and slightly reduced the overall abundance of bacteria at low concentration and slightly increased that at high concentration (Figure S6). Evaluation of β-diversity using non-metric multidimensional scaling (NMDS) analysis showed that bacterial communities shifted from bulk to rhizosphere soil on the first NMDS axis (NMDS1; Figure 4). Among rhizosphere soil samples, the bacterial communities tended to change depending on the growth stage (second NMDS axis, NMDS2), but no such tendency was observed among bulk soil samples. Soil samples from in vitro studies were clustered near the bulk soil samples but slightly differed from them on NMDS2 (Figure 4). The bacterial communities of soyasaponin Bb-treated soils were not closer to those of the rhizosphere than bulk soil communities. Because different amount of water was added to Low-Cont and High-Cont samples,

the bacterial communities between these control samples were separated (Figure 4).
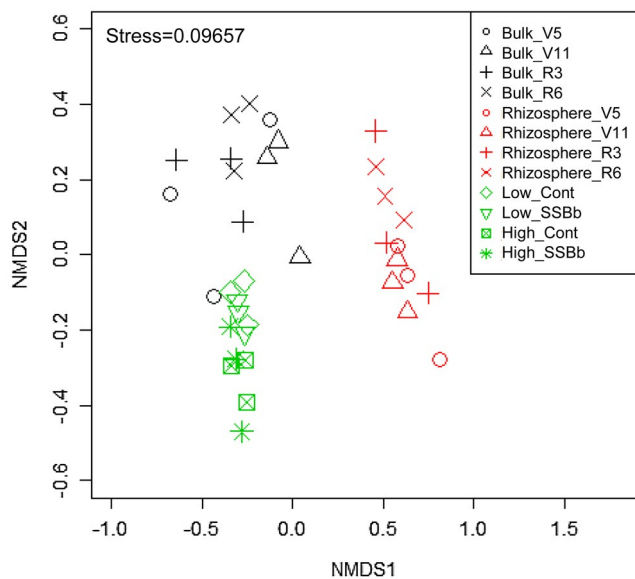

FIGURE 4 β-Diversity of bacterial communities in bulk and rhizosphere soils throughout soybean growth stages (V5, V11, R3, and R6) and in soyasaponin Bb (SSBb)-treated bulk soil using non-metric multidimensional scaling (NMDS) ordination. NMDS plots of field soils and soils obtained from in vitro studies based on Bray–Curtis distance. Bulk soil collected at sowing was used for in vitro studies. Bulk or Rhizosphere_V5, V11, R3, and R6, bulk or rhizosphere soils collected at each growth stage; Low_Cont and High_Cont, the bulk soils treated with water obtained from in vitro studies; Low_SSBb and High_SSBb, the bulk soils treated with low and high amount of SSBb obtained from in vitro studies, respectively

To evaluate whether soyasaponin Bb treatment affected taxon composition of the bacterial communities, we compared taxa in all soil samples at the phylum level. Proteobacteria were predominant in all soils (bulk: 28.7%–32.8%; rhizosphere: 29.2%–40.8%; in vitro studies: 29.2%–36.8%), followed by Firmicutes (bulk: 10.4%–32.0%; rhizosphere: 18.6%–33.0%; in vitro studies: 13.3%–19.8%), and Actinobacteria (bulk: 11.6%–16.3%; rhizosphere: 14.4%–21.1%; in vitro studies: 16.9%–21.1%; Figure 5). Rhizosphere soils showed distinct changes during soybean growth, with Actinobacteria increasing from 15.9% at the V5 stage to 20.8% at the R6 stage; Acidobacteria increased from 3.9% to 5.2%, while Bacteroidetes decreased from 8.6% to 5.0% (Figure 5). It is to be noted that the relative abundance of Acidobacteria and Chloroflexi was lower in the rhizosphere soils than in bulk soils, while that of Actinobacteria and Bacteroidetes was higher. No statistical differences were observed between soyasaponin Bb-treated and untreated soils for abundant phyla (Figure 5), but Cyanobacteria was higher in low soyasaponin Bb-treated soil and AD3 and Verrucomicrobia were lower in high soyasaponin Bb-treated soil. These results suggest that soyasaponin Bb had little effect on the microbiome at the phylum level, but did not rule out the possibility that soyasaponin Bb influenced specific bacterial families or genera.

3.5 | Comparison of taxa affected by soyasaponin Bb common to soybean rhizosphere

To further analyze the effects of soyasaponin Bb on soil microbial composition, we analyzed enriched or depleted bacterial families in soyasaponin Bb treatment in bulk soils, and compared these families with those enriched or depleted in soybean rhizosphere. Significant
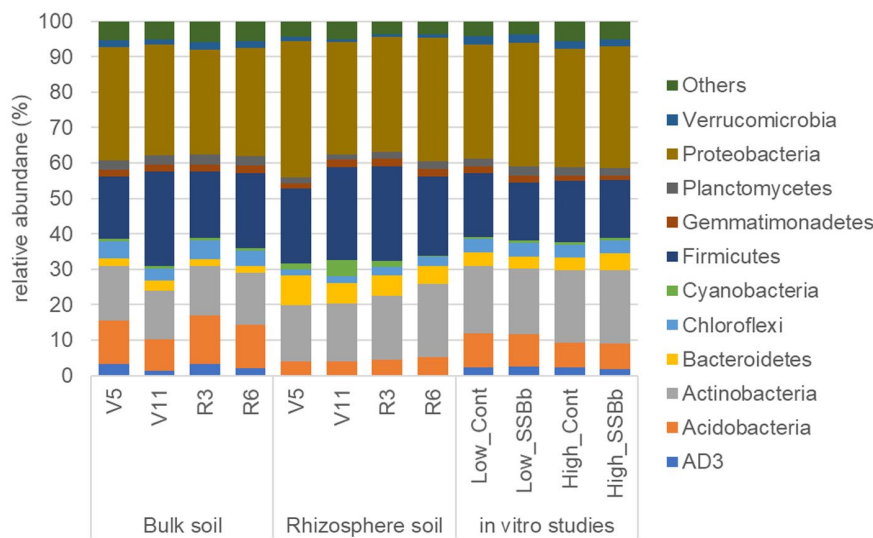

FIGURE 5 Bacterial community composition at phylum level in the bulk and rhizosphere soils during soybean growth, and in soyasaponin Bb (SSBb)-treated bulk soil. The composition at phylum level in each soil shown in Figure 4. Each bar represents the average relative abundance (*n* = 3). Others include archaea, unassigned reads, and bacteria with average relative abundance <1% in all samples. V5, V11, R3, and R6, growth stages; Low-Cont and HighCont, the bulk soils treated with water obtained from in vitro studies; Low-SSBb and High-SSBb, the bulk soils treated with low and high amount of SSBb obtained from in vitro studies, respectively
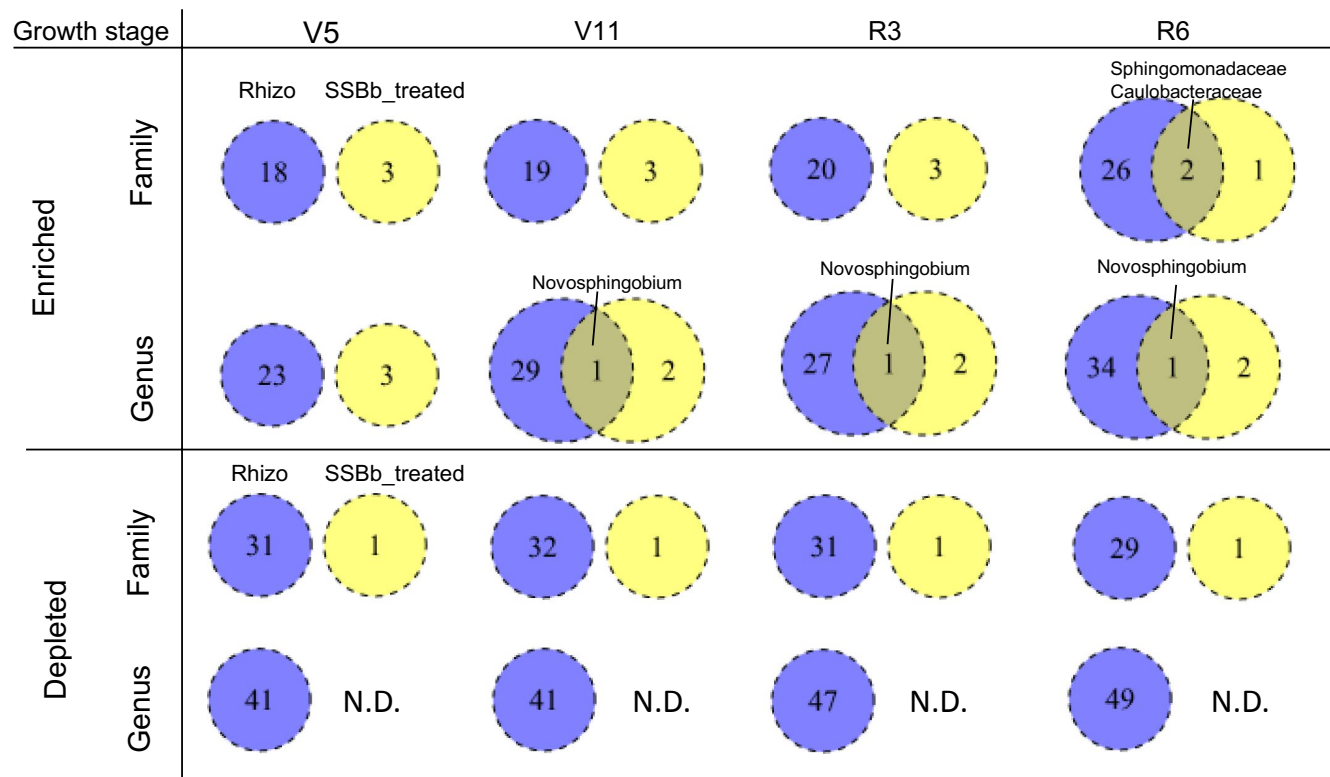
FIGURE 6 Comparison of bacterial communities between soyasaponin Bb (SSBb)-treated bulk soil and soybean rhizosphere soils at the family and genus levels. Venn diagrams show overlap of bacterial families and genera enriched or depleted in SSBb-treated bulk soil at the high concentration (yellow) and in the soybean rhizosphere (blue). Numbers in the circles are the numbers of bacterial families or genera. These families and genera were identified with a negative binomial generalized linear model (FDR-corrected $P < 0.05$). Rhizo, rhizosphere soil; SSBb-treated, SSBb-treated bulk soil; N.D., statistically significant families or genera were not detected
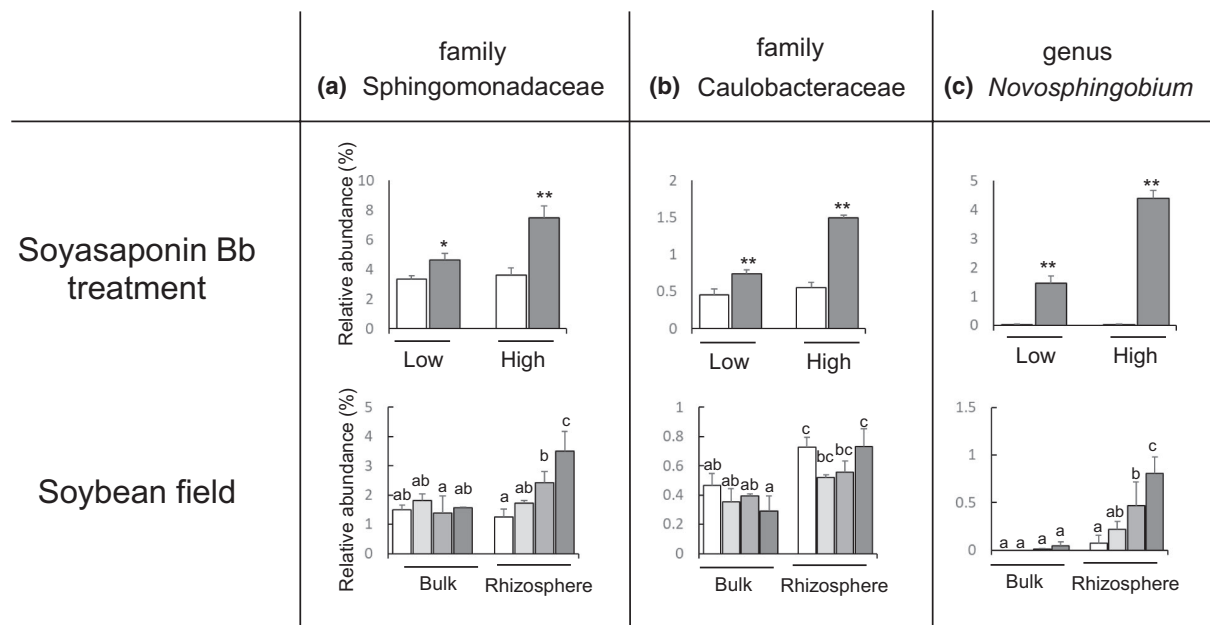
FIGURE 7 Relative abundance of (a) family Sphingomonadaceae, (b) family Caulobacteraceae and (c) genus *Novosphingobium* enriched in both soyasaponin Bb (SSBb)-treated bulk soil and soybean rhizosphere soils. Soyasaponin Bb treatment (upper graphs): these relative abundances in SSBb-treated bulk soil (■) at the low and high concentration versus control (□) are shown. Data are means ± SD ($n = 3$). Significant differences (*$P < 0.05$, **$P < 0.01$ vs. control; Student's t-test) are indicated. Soybean field (lower graphs): these relative abundances in bulk and rhizosphere soils at each growth stage (□ V5; ▨ V11; ▦ R3; ■ R6) are shown. Data are means ± SD ($n = 3$). Significant differences ($P < 0.05$; Tukey–Kramer test) are indicated with different letters

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changes were caused by soyasaponin Bb treatment at the high concentration. Three families were enriched in soyasaponin Bb-treated soil, and two of them, Sphingomonadaceae and Caulobacteraceae, were also enriched in soybean rhizosphere soil, although only at the R6 stage (Figure 6). In contrast, Enterobacteriaceae was depleted in soyasaponin Bb-treated soil but not in rhizosphere soil at all growth stages (Figure 6). Relative abundances of Sphingomonadaceae and Caulobacteraceae were increased not only by in vitro soyasaponin Bb treatment but also in rhizosphere soil at some growth stages (Figure 7a,b). These data suggest that microbes belonging to these families were affected by soyasaponin Bb in the soybean rhizosphere.

Three genera of microbes—unclassified genus belonging to Micrococcaceae, *Phenylobacterium*, and *Novosphingobium*—were enriched in soyasaponin Bb-treated soils, and *Novosphingobium* was also enriched in soybean rhizosphere soil at the R3 and R6 stages (Figure 6 and 7c). Similar results were obtained at the OTU level, that is, OTU_046 (annotated as a *Novosphingobium* species) was enriched in both soyasaponin Bb-treated and soybean rhizosphere soils except at the V5 stage (Figure S7). *Novosphingobium*, but not several other Sphingomonadaceae genera, was specifically enriched in both soyasaponin Bb-treated and rhizosphere soils (Figure S8). Among genera belonging to the other enriched family, the Caulobacteraceae, *Phenylobacterium* was enriched in both soyasaponin Bb-treated and soybean rhizosphere soils (Figure S9). These results suggest that soyasaponin Bb functions as an attractant for specific genera, such as *Novosphingobium* and *Phenylobacterium*, and not as a repellent in soybean rhizosphere.

4 | DISCUSSION

Soyasaponins are commonly found in legumes and pulses and have biological activities beneficial for human health (Singh, Pal, Singh, & Kaur, 2017). Although the biosynthesis and distribution of soyasaponins in legume plants have been studied for decades (Kurosawa, Takahara, & Shiraiwa, 2002; Sayama et al., 2012; Shibuya, Nishimura, Yasuyama, & Ebizuka, 2010; Shimoyamada, Kudo, Okubo, Yamaguchi, & Harada, 1990; Shiraiwa, Harada, & Okubo, 1991; Yano et al., 2017), the effects of soyasaponins on soil microbes have not been reported so far. In our previous report, we discovered soyasaponins in root exudates of legume plants cultured hydroponically (Tsuno et al., 2018). The selective and developmentally controlled secretion of soyasaponins from legume roots suggests that soyasaponins affect their underground environment including soil microbes. To understand the rhizosphere, it is important to evaluate root exudates not only in hydroponic cultures but also in the field. Our purpose in this study was to evaluate root exudation of soyasaponins and their effects on rhizosphere microbes in the field.

Our analysis suggested that soyasaponins were secreted from the roots of field-grown soybean throughout the growth stages, but were undetectable in bulk soil. The group B saponins were predominant in the rhizosphere throughout the growth period, and the amount of soyasaponin Bb, a major saponin in the soybean rhizosphere, was similar to that of daidzein reported previously (Sugiyama et al., 2017). In contrast, Group A saponins were predominant during vegetative stages in hydroponic culture (Tsuno et al., 2018). This difference may be caused by differences in environmental conditions between the field and hydroponic culture. Future detailed analysis of group A saponins in soil may help us to understand this difference.

In the field soil, soyasaponin Bb was first degraded to soyasapogenol B with a loss of sugar moieties; soyasapogenol B was further slowly degraded, suggesting that it was used as a carbon source by soil microbes. The degradation rate and distribution coefficient suggested that its behavior in soil differs from that of daidzein, although the amounts of soyasaponin Bb and daidzein secreted at the R4 stage were similar (Okutani et al., 2020; Sugiyama et al., 2017; Sugiyama et al., 2016; Tsuno et al., 2018). Because it diffuses into a broader region of the rhizosphere than does daidzein, soyasaponin Bb may influence a larger area in the soil, where it may mediate the interactions with microbes to improve the rhizosphere environment for soybean roots.

In this study, the effect of soyasaponin Bb on soil microbes in the rhizosphere was characterized by sequencing of 16S rRNA genes. Soyasaponin Bb treatment did not make the microbial community of bulk soil closer to that in soybean rhizosphere soil, but specific microbes such as *Novosphingobium* and *Phenylobacterium* were enriched in both soyasaponin Bb-treated soil and soybean rhizosphere. In accordance with the changes of bacterial communities in the rhizosphere during the growth periods of soybean grown in the fields (Hara et al., 2019; Sugiyama et al., 2014), the relative abundances of Sphingomonadaceae and *Novosphingobium* gradually increased during the growth period, which resulted in the significant difference detected at R6 stage (Figure 7). These results indicate that soyasaponin Bb accumulated in the rhizosphere stimulates proliferation of a limited number of microbes to modulate the microbial community. *Novosphingobium* degrades polycyclic aromatic hydrocarbons (Segura, Hernández-Sánchez, Marqués, & Molina, 2017) and 1-methylphenanthrene (Sha, Zhong, Chen, Lin, & Luan, 2017), is resistant to the pesticide DDT (dichlorodiphenyltrichloroethane; Lin et al., 2014), and enriched by crude oil contamination (Yang, Wen, Zhao, Shi, & Jin, 2014). These findings suggest the involvement of *Novosphingobium* in the degradation of organic matter in soil, which results in the production of nutrients that can be absorbed by plant roots or in avoidance of toxicity of harmful compounds (McGuinness & Dowling, 2009). *Novosphingobium* also elicits induced systemic resistance in pepper (Hahm et al., 2012) and reduces salt stress in *Citrus* species (Vives-Peris, Gómez-Cadenas, & Pérez-Clemente, 2018), suggesting that it may be involved in stress control and functions as a potential PGPR.

Soyasaponin Bb is metabolized by human intestinal and fecal microorganisms (Chang, Han, Han, & Kim, 2009; Hu, Zheng, Hyde, Hendrich, & Murphy, 2004), but the microbes that metabolize

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soyasaponin Bb or soyasaponin Bb-metabolizing enzymes in soil are yet to be identified. Specific enrichment of microbes by soyasaponin Bb suggests that *Novosphingobium* possesses a gene set that allows the bacteria to use soyasaponin Bb as a carbon source. Because *Novosphingobium* was enriched in a growth stage–dependent manner, these bacteria may play important roles, especially at reproductive stages. It is noteworthy that the enrichment of *Novosphingobium* and *Phenylobacterium* in soybean rhizosphere does not depend on soil types or plant genotypes, because the enrichment of these genera was also observed in the rhizosphere of five soybean genotypes grown in agriculture soil and forest soil collected in Tennessee, USA (Liu, Hewezi et al., 2019), and also in the rhizosphere of soybean (cv. Shintambagro) grown in a farm in Kyoto, Japan (Okutani et al., 2020, Figure S10a). We previously showed daidzein enriched various bacteria in in vitro experiments, but daidzein did not enrich *Novosphingobium* and *Phenylobacterium* (Okutani et al., 2020; Figure S10b). These results suggest that, in addition to daidzein, soyasaponin Bb has roles in shaping microbial communities in the soybean rhizosphere.

In conclusion, this study revealed the exudation and functions of soyasaponins in a soybean field. In vitro analysis of degradation and adsorption suggests that soyasaponin Bb can diffuse over longer distances in the rhizosphere than daidzein. Moreover, soyasaponin Bb affected the bacterial communities by increasing the populations of potential PGPRs such as *Novosphingobium*, which is also enriched in the soybean rhizosphere. The secretion of sugar-containing compounds into soil appears to be a large carbon loss for plants, but this loss could be a trade-off to attract microbes with growth-promoting effects to the rhizosphere. Further studies on the physiological functions of these microbes in plant growth are needed to clarify the soyasaponin-mediated interactions between plants and rhizosphere microbes.

CONFLICT OF INTERESTS

No potential conflict of interest was reported by the authors.

AUTHOR CONTRIBUTIONS

T.F., K.E., K.Y., and A.S. designed the research; T.F. performed research; T.F. and A.S. analyzed data; K.E., K.Y., and A.S. supervised the research and provided technical assistance; T.F., K.E., K.Y., and A.S. wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.