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Uncovering potential 'herbal probiotics' in Juzen-taiho-to through the study of associated bacterial populations

Diego Montenegro [a,†], Kriti Kalpana [a,†], Christine Chrissian [a], Ashutosh Sharma [a], Anna Takaoka [a], Maria Iacovidou [a], Clifford E. Soll [a], Olga Aminova [b], Adriana Heguy [b], Lisa Cohen [b], Steven Shen [b,c], Akira Kawamura [a,*]

[a] Department of Chemistry, Hunter College of CUNY, 695 Park Avenue, New York, NY 10065, USA
[b] Genome Technology Center, NYU Langone Medical Center, 550 First Avenue, New York, NY 10016, USA
[c] Department of Biochemistry and Molecular Pharmacology, Center for Health Informatics and Bioinformatics, NYU School of Medicine, 227 East 30th Street, New York, NY 10016, USA

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ABSTRACT

Juzen-taiho-to (JTT) is an immune-boosting formulation of ten medicinal herbs. It is used clinically in East Asia to boost the human immune functions. The active factors in JTT have not been clarified. But, existing evidence suggests that lipopolysaccharide (LPS)-like factors contribute to the activity. To examine this possibility, JTT was subjected to a series of analyses, including high resolution mass spectrometry, which suggested the presence of structural variants of LPS. This finding opened a possibility that JTT contains immune-boosting bacteria. As the first step to characterize the bacteria in JTT, 16S ribosomal RNA sequencing was carried out for *Angelica sinensis* (dried root), one of the most potent immunostimulatory herbs in JTT. The sequencing revealed a total of 519 bacteria genera in *A. sinensis*. The most abundant genus was *Rahnella*, which is widely distributed in water and plants. The abundance of *Rahnella* appeared to correlate with the immunostimulatory activity of *A. sinensis*. In conclusion, the current study provided new pieces of evidence supporting the emerging theory of bacterial contribution in immune-boosting herbs.

Bacteria are everywhere. From scorching desert to deep ocean trench, they have adapted to every niche of the terrestrial biosphere.[1,2] Some gained the ability to live in other organisms for mutual benefits (mutualism), harmless cohabitation (commensalism) or destructive invasion (parasitism). Among such bacteria are those that colonize plants. Their presence has been known. Yet they were difficult to characterize until recently when powerful sequencing tools began to unravel unique plant-microbe interactions.[3–9] Sequencing studies confirmed what has long been suspected: Plants are loaded with diverse communities of bacteria.

The finding of diverse microbial communities in plants has important implications not only in plant biology but also in herbal medicine. It means that humans have been consuming plant-colonizing bacteria together with medicinal herbs. Those bacteria are probably harmless to humans; plants with toxic bacteria would not have been selected for herbal medicine. But, are they important for the therapeutic effects of medicinal herbs? Do they produce molecules that are beneficial to humans? Clues to address such questions came from recent studies on *Echinacea*, an immune-boosting herb. Pasco and co-workers observed that bacterial loads in *Echinacea* correlated with immunostimulatory activities.[10] In addition, the activity of *Echinacea* was depleted by a polymyxin-B affinity-resin, which removes lipopolysaccharides (LPSs) of gram-negative bacteria.[11,12] These data argued for the bacterial contribution in some immune-boosting herbs. However, the theory was disputed in other studies. Studies on Yamoa™ (the ground bark of *Funtumia elastica*)[13] and the fruit of Acai (*Euterpe oleracea*)[14] argued against the bacterial contribution, because these herbs showed immunological activities different from those of typical LPSs.

Our group has been studying Juzen-taiho-to (JTT), which is an immune-boosting herbal formulation with an ideal balance of safety and efficacy.[15] JTT is used clinically in East Asia to strengthen the immune functions of patients with various diseases, including cancer,[16,17] hepatitis C,[18] and otitis.[19] The formulation is a mixture of ten different herbs (see Table S1 in Supplementary data). The active factors in JTT have not yet been clarified due to its chemical complexity.

We suspected that bacteria might contribute to the activity of JTT for several reasons. First, JTT has been known for 'LPS-like'

* Corresponding author.
† These authors contributed equally to this manuscript.

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immunostimulatory activity;[20,21] both JTT and LPS induce similar gene expression profiles in monocytes.[22,23] Second, many of the component herbs of JTT are roots (Table S1), which directly interact with a wide variety of soil bacteria. Third, in our recent screening of JTT, we purified a glycolipid fraction with LPS-like activities, which include potent stimulation of primary macrophages and dendritic cells.[22] These activities were not due to contaminations of ubiquitous bacteria, such as *Escherichia coli*, because the purified fraction did not exhibit the endotoxicity of typical pathogenic/enteric bacteria.[22] One possible origin of the activity was plant-colonizing bacteria that produce structural variants of LPS.

To examine the possibility of bacterial contribution in JTT, we conducted a series of chemical and biochemical studies. The presence of LPS was examined not only by indirect methods, such as the polymyxin-B treatment, but also by direct measurements of their molecular formulas by mass spectrometry. In addition, Illumina sequencing was conducted to obtain the snapshots of bacterial communities in *Angelica sinensis*, one of the most potent component herbs in JTT. Collectively, these results support the notion that plant bacteria contribute to the activity of JTT.

Polymyxin-B affinity resin depleted the immunostimulatory activity of JTT: As the first step to examine the possible bacterial contribution in JTT, we treated JTT with an affinity-resin of polymyxin-B.[24] We reasoned that, if the activity comes from LPSs, it would be diminished by the polymyxin-B treatment. The immunostimulatory activity was analyzed by the qRT-PCR of intercellular adhesion molecule 1 (ICAM-1) in THP-1 monocytes; ICAM-1 is a biomarker of monocyte/macrophage stimulation.[25,26]

Mock-treated JTT (JTT treated with a streptavidin affinity resin: JTT Mock) and untreated JTT caused nearly 30-fold induction of ICAM-1 compared to the DMSO control (Fig. 1). On the other hand, the polymyxin-B treated JTT (JTT PmxB) induced a significantly lower level (12-fold) of ICAM-1 (Fig. 1). The residual activity of JTT PmxB could still be due to some LPS variants, because polymyxin-B binds only some, but not all, LPSs.[27] Alternatively, it is possible that the residual activity comes from other classes of molecules, such as saponins.[28,29] In any event, the polymyxin-B study was consistent with the notion that bacteria contribute to the immunostimulatory activity of JTT.

Lipid A (LA)-like factors are purified from JTT: Next, we subjected JTT to a purification protocol outlined in Scheme 1. This protocol was based on an established scheme to purify lipid A (LA) from bacterial cultures;[30] LA is the part of LPS responsible for the immunostimulatory activity (see Fig. S1 in Supplementary data). In this scheme, hydrophobic lipids are first removed by organic extraction. LPSs are extracted to the aqueous phase because of the large polysaccharide moiety. The subsequent acid hydrolysis releases

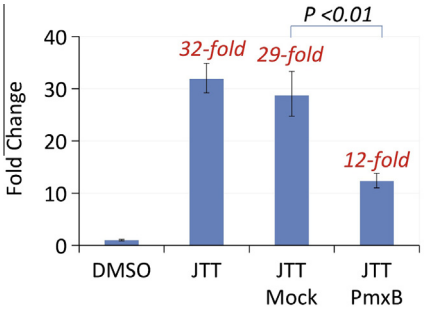
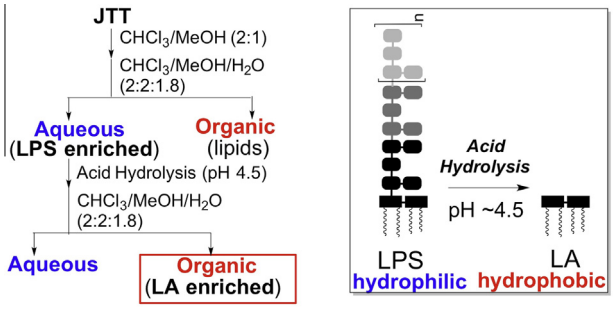


Figure 1. The activity of JTT is diminished by polymyxin-B treatment. ICAM-1 qRT-PCR of JTT treated with polymyxin-B (JTT PmxB) and streptavidin (JTT Mock), which examined non-specific binding. The polymyxin-B treatment significantly reduced the activity of JTT compared to the mock treatment. The mock treatment did not significantly alter the activity compared to the JTT sample with no treatment. THP-1 cells were treated with samples (100 μg/mL) for 4 h.



Scheme 1. Purification scheme to enrich LA-like factors from JTT. Most lipids were removed in the initial delipidation steps, whereas LPSs stayed in the aqueous layer because of the large polysaccharides. The crude LPS sample was hydrolyzed to LAs. The resulting LAs were extracted to the organic layer to obtain an 'LA-enriched' sample (in the red box).

LAs from polysaccharides; LAs are then extracted to the 'LA-enriched' organic layer. Thus, if LPS variants exist in JTT, their LA fragments would be seen in the 'LA-enriched' organic layer (the red box in Scheme 1).

JTT was put through this scheme to obtain the 'LA-enriched' organic layer, which was further fractionated by silica gel chromatography. TLC analysis of the fractions showed many spots with the R_f values consistent with LAs (Fig. 2a): LAs typically appear in the R_f range between 0.2 and 0.6 in this condition ($CHCl_3$/MeOH/H_2O/NH_4OH, 40:25:4:2 (v/v/v/v)).[30] When these fractions were tested for immunostimulatory activity, many of them potently induced ICAM-1 in monocytes (Fig. 2b). Taken together, these results showed that our purification protocol enriched LA-like factors from JTT.

Direct measurements of LA-like factors by mass spectrometry: The results presented above indirectly supported the presence of LPS variants in JTT. But molecular evidence was lacking. Thus we examined the purified LA-like factors with high-resolution electrospray ionization mass spectrometry (HRESIMS). LAs typically have the structures of phosphoryl diglucosamine with multiple acyl chains, as exemplified in Figure S1; their molecular weights range from 1500 to 2500 Da; their formulas typically contain 2–4 nitrogens and a large number (>80) of carbons.

HRESIMS of the LA-like factors revealed many ions in the typical molecular weight range of LA. Among them were ions corresponding to molecular formulas of $C_{83}H_{145}N_2O_{23}P$ (Exact Mass: 1568.9976) and $C_{120}H_{217}N_4O_{38}P$ (Exact Mass: 2353.4908) (Cpd **1** and Cpd **2** in Fig. 3, respectively); the observed ions are summarized in Table S2 in Supplementary data. These formulas were consistent with the general structural backbone of LA (Fig. S1). However, they did not have exact matches to known LAs in the SciFinder database. This led us to believe that the purified factors could be structural variants of LA. To our knowledge the current study is the first to obtain direct molecular evidence of LPS variants in immune-boosting herbs.

Plant-colonizing proteobacteria were detected in Angelica sinensis: Our results indicated immune-boosting bacteria contribute to the activity of JTT. Although bacterial communities in some plants have been characterized,[3–9] little is known about the bacteria in JTT. As the first step to characterize bacterial communities in JTT, we conducted a preliminary metagenomic analysis of *Angelica sinensis*, which is one of the most potent immunostimulatory herbs in JTT (Fig. S2). To characterize the bacterial diversity and its variability, this study examined three samples of *A. sinensis* (dried root).

DNA was extracted from the three samples and subjected to PCR amplification of the 16S ribosomal RNA (16S rRNA) gene, which is a widely used phylogenetic marker of microbial taxa.[31] The study examined three PCR primer pairs, namely, P1(fM1,

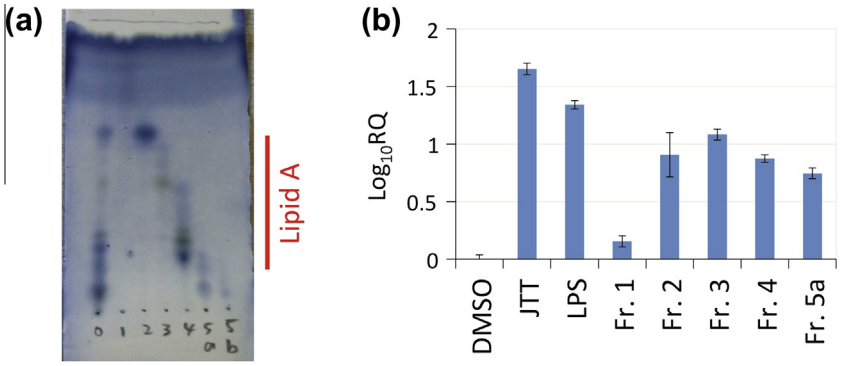


Figure 2. Characterization of LA-like factors in JTT. (a) TLC profiles of LA-like factors. In the condition used ($CHCl_3$/MeOH/H_2O/NH_4OH, 40:25:4:2, v/v/v/v), LAs typically show up in the R_f region highlighted by the red bar (R_f 0.2–0.6). (b) Immunostimulation by LA-like factors as determined by qRT-PCR of ICAM-1 in THP-1 monocytes. JTT (100 μg/mL); LPS (100 ng/mL); LA-like factors (5 μg/mL).

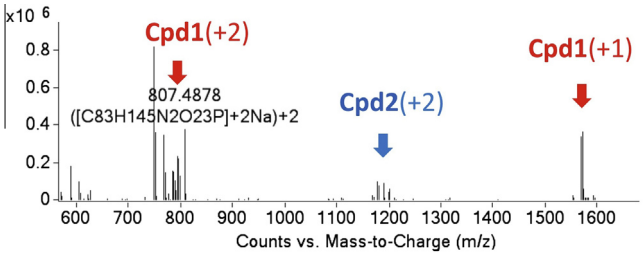


Figure 3. HRESIMS analysis of LA-like factors (Fraction 3 in Fig. 2). Ions corresponding to two LA-like factors (Cpd **1** and Cpd **2**) were observed in Fraction 3.

rC5),[32] P2(926f/1392r),[4] and P3(1114f/1392r),[4] all of which have been used to amplify prokaryotic 16S rRNA in the presence of plant DNA. The three primer pairs gave PCR amplicons with the expected sizes (Fig. S3). The amplicons were sequenced by Illumina MiSeq, which gave high quality reads (72.1% of reads had the quality score of 30 or higher; see Fig. S4). The sequencing showed that two primer pairs (P1 and P2) mostly amplified chloroplast and mitochondria DNA in *A. sinensis*. However, P3 revealed a complex bacterial community in *A. sinensis* (Phylum: 21, Class: 59, Order: 108, Family: 219, Genus: 519) (Fig. 4a). The bacterial communities varied substantially from sample to sample (Fig. 4b). But, the genus *Rahnella* was the most abundant in all three samples (Fig. 4c). *Rahnella* is a genus of Gram-negative bacteria widely distributed in water and plants.[33–35] Interestingly, the abundance (%) of *Rahnella* in *A. sinensis* samples appeared to correlate with their immunostimulatory activity (Fig. 4c).

The current study supports the emerging theory of the bacterial contribution in immune-boosting herbs.[10–12] In this theory, the herbs do not necessarily produce immunostimulants; instead they enrich beneficial bacteria with immune-boosting activity, which we call 'herbal probiotics'. Humans may have been benefiting from herbal probiotics, albeit unknowingly, throughout the history of herbal medicine. Although some immune-boosting herbs are known to produce immunostimulants, such as QS21 by *Quillaja saponaria*,[28,29] the active factors of many herbs are poorly characterized. If they are LPS variants from plant-colonizing bacteria, they are difficult to identify because they exist in minuscule quantities and are buried in the large amounts of plant-derived compounds, such as glycolipids and carbohydrates. In addition, LPS variants in plants are heterogeneous mixtures from various plant-colonizing bacteria. Unless they are specifically looked for, as exemplified in the current study, it is difficult to even notice their presence in herbs. Thus, it is possible that the bacterial contribution is more prevalent than currently recognized.

Our preliminary 16S metagenomic study provided a glimpse into the complex bacterial community in *A. sinensis*. Many were Gram-negative bacteria, which was consistent with our observation of the LA-like immunostimulatory factors in JTT. The bacterial community varied from sample to sample; so did the immunostimulatory activity. Interestingly, the genus *Rahnella*, which was abundant in all three samples, appeared to be associated with

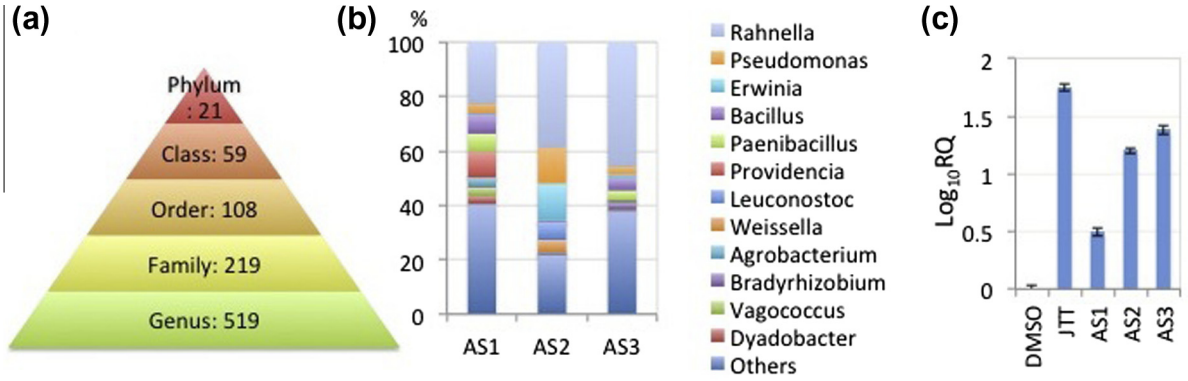


Figure 4. Metagenomic analysis of *Angelica sinensis*. (a) The diversity of bacterial community in *A. sinensis* samples. For each sample, the shown result is the average of three independent replicates. (b) Bacterial genera identified in the three *A. sinensis* samples. *Rahnella* was the predominant genus in all three *A. sinensis* samples. (c) Immunostimulatory activity of three *A. sinensis* samples (AS1, AS2, and AS3). The activity was measured by qRT-PCR of ICAM-1 in THP-1 cells. THP-1 cells were treated with samples (5 μg/mL) for 4 h.

the activity. *Rahnella* is widely distributed in water, soil, and the rhizosphere of plants.[36] Many strains in this genus are known as rhizobacteria with plant growth-promoting activities.[37–39] The current study raised a possibility that some *Rahnella* species are enriched in *A. sinensis* and contribute to its immunostimulatory activity. Further studies are underway to characterize the bacteria in *A. sinensis* and other herbs in JTT.

In conclusion, the current study presented, for the first time, mass spectrometric evidence of LPS variants in immune-boosting herbs. In addition, the study presented the first metagenomic analysis of *A. sinensis*. These results support the notion that plant-colonizing bacteria contribute to the activity of JTT, although further studies are needed to determine the immunological properties of associated bacterial populations. The current study sets the stage to uncover previously overlooked roles of 'herbal probiotics' in JTT and possibly other immune-boosting herbs.

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Supplementary data

Supplementary data (additional figures, tables, and experimental procedures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.12.036.

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