Dynamics of the Physicochemical Characteristics, Microbiota, and Metabolic Functions of Soybean Meal and Corn Mixed Substrates during Two-Stage Solid-State Fermentation

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ABSTRACT Substantial annual economic loss in livestock production is caused by antinutritional factors in soybean meal and corn mixed substrates, which can be degraded by microbial fermentation. Although considerable efforts have been made to explain the effects of fermentation on soybean meal and corn-based feed, the dynamics of the physicochemical characteristics, microbiota, and metabolic functions of soybean meal and corn mixed substrates during solid-state fermentation remain unclear. Here, multiple physicochemical analyses combined with high-throughput sequencing were performed to reveal the dynamic changes that occur during a novel two-stage solid-state fermentation process. Generally, inoculated bacteria rapidly proliferated in the initial 12-h aerobic fermentation (P = 0.02). Notably, most nutritional changes occurred during 12 to 24 h compared to 0 to 12 h. Second-stage anaerobic fermentation increased the bacterial abundance and lactic acid content (P < 0.00). Bacillus spp., Enterococcus spp., and Pseudomonas spp. were predominantly involved in the maturation of the fermented mixed substrates (P < 0.05). Additionally, the available phosphorus exhibited the greatest interaction with the microbial community structure. Cellular processes and environmental information processing might be the main metabolic processes of the microbiota during this fermentation. An in vivo model further evaluated the growth-promoting effects of the fermented products. These results characterized the dynamic changes that occur during two-stage solid-state fermentation and provided potential references for additional interventions to further improve the effectiveness and efficiency of solid-state fermentation of feed.

IMPORTANCE Solid-state fermentation (SSF) plays pivotal roles not only in human food but also farm animal diets. Soybean meal (SBM) and corn account for approximately 70% of the global feed consumption. However, the nutritional value of conventional SBM and corn mixed substrates (MS) is limited by antinutritional factors, causing substantial economic loss in livestock production. Although emerging studies have reported that SSF can improve the nutritional value of SBM-based substrates, the dynamic changes in the physicochemical features, microbiota, and metabolic functions of MS during SSF remain poorly understood, limiting further investigation. To provide insights into the dynamics of the physicochemical characteristics and the complex microbiome during the two-stage SSF of MS, multiple physicochemical analyses combined with high-throughput sequencing were applied here. These novel insights shed light on the complex changes that occur in the nutrition and microbiome during two-stage SSF of MS and are of great value for industrial feed-based practices and metabolomic research on SSF ecosystems.
KEYWORDS microbiota, metabolic functions, nutritional value, two-stage solid-state fermentation

Soybean meal (SBM) and corn account for approximately 70% of the global feed consumption. Approximately 772.37 million tons of soybean meal and corn were used in global livestock production in 2018 (Alltech, Nicholasville, KY). However, the utility of conventional SBM and corn mixed substrates (MS) is limited by several antinutritional factors (ANFs), which inhibit the bioavailability of nutrients and reduce animal health status (1). In 2018, it was estimated that $32.9 billion was lost due to the adverse effects of ANFs on farm animals (Alltech, Nicholasville, KY). Soybean antigenic proteins, mainly glycinin and beta-conglycinin, have been shown to cause antigenic protein hypersensitivity, which is attributed to gut injury, inflammation, and diarrhea (2). Trypsin inhibitors (TIs) hinder the activity of the endogenous enzyme trypsin, thereby causing digestive disorders (3). By binding positively charged molecules, phytic acid can reduce nutrient digestibility (4). Additionally, some carbohydrate-based ANFs, such as cellulose and amylose, can interfere with nutrient digestibility and increase nutrient emission (5). Overall, ANFs inhibit the bioavailability of nutrients and cause physiological disorders.

Solid-state fermentation (SSF), an age-old biotechnology, has been widely used to promote the nutritional quality of feed by promoting nutrient utilization and decreasing ANF levels (6). In terms of feed quality improvement, SSF remains much more economical and beneficial than other methods. The global feed production by SSF approached 2 million tons in 2018 (National Engineering Research Center of Biological Feed). The nutritional quality of the fermented feed depends on the type of microorganism used for inoculation. Aspergillus spp. and Bacillus subtilis are commonly used in ANF degradation due to their capacity to produce enzymes such as protease, amylase, xylanase, pectinase, and amylase under aerobic conditions (7). Some research has demonstrated the degradation of ANFs and hydrolysis of macronutrient factors by microbes in SBM-based substrates (8, 9). Additionally, lactic acid (LA) bacteria are commonly used in fermented feed to produce organic acids, especially LA, under anaerobic conditions (10). However, few studies have combined the features of both aerobic and anaerobic SSF to optimize the quality of fermented feed. In addition, the dynamic changes in physicochemical characteristics, microbial community structure, and metabolism of MS during SSF remain unclear, impeding further research.

Therefore, SBM and corn were used as the main fermented substrates, which were inoculated with an effective combination of B. subtilis and Enterococcus faecium to achieve a novel two-stage SSF process (first stage, aerobic fermentation; second stage, anaerobic fermentation). Multiple physicochemical analysis methods combined with high-throughput sequencing were applied to provide insights into the dynamic changes in physicochemical features and complex microbiomes during the two-stage SSF of MS. Furthermore, weaned piglets were used as an in vivo model to investigate the effects of fermented MS (FMS) on growth performance, nutrient utilization, and anti-inflammatory activity. These findings provide insights into the potential changes in the physicochemical characteristics, main bacteria, and metabolism of FMS during two-stage SSF and provide valuable information for industrial feed-related practices and microbiome research on SSF.

RESULTS
Fermentation inoculum selection and process design. Our previous study rationally screened B. subtilis CW4 from different fermented foods (11). To further improve the rate of ANF degradation, the effects of microbes alone or in combination with a neutral protease on 24-h protein degradation were compared (see Fig. S1A in the supplemental material). The results showed that inoculation with $10^7$ CFU/g Saccharomyces cerevisiae, $10^7$ CFU/g E. faecium CWEF, and 300 U/g neutral protease was not effective enough for protein degradation. Furthermore, different LA bacteria were
Table 1 Analyzed nutrient content of FMS at 0, 12, 24, and 48 h

| Component                      | Mean ± SD at time (h) |
|--------------------------------|-----------------------|
|                               | 0                     | 12                     | 24                     | 48                     |
| C/N ratio                     | 10.76 ± 0.20 A        | 10.66 ± 0.15 AB        | 10.14 ± 0.15 B         | 9.60 ± 0.17 C          |
| pH                            | 6.46 ± 0.06 B         | 6.53 ± 0.08 B          | 6.76 ± 0.06 A          | 5.20 ± 0.13 C          |
| Dry matter content (%)        | 92.47 ± 0.07          | 92.42 ± 0.10           | 92.09 ± 0.04           | 91.44 ± 0.09           |
| Crude protein content (%)     | 28.37 ± 0.86 C        | 29.51 ± 0.40 BC        | 30.83 ± 0.69 AB        | 31.37 ± 0.43 A         |
| TCA-SP content (%)            | 1.58 ± 0.19 D         | 2.89 ± 0.13 C          | 6.55 ± 0.18 B          | 10.95 ± 0.05 A         |
| Free amino acid content (%)   | 0.28 ± 0.08 B         | 0.29 ± 0.02 B          | 0.77 ± 0.03 A          | 1.04 ± 0.21 A          |
| Small peptide content (%)     | 1.26 ± 0.15 D         | 2.60 ± 0.19 C          | 5.70 ± 0.23 B          | 9.90 ± 0.16 A          |
| NDF content (%)               | 13.61 ± 0.64 A        | 13.19 ± 0.13 A         | 9.47 ± 0.50 B          | 9.38 ± 0.36 B          |
| ADF content (%)               | 6.24 ± 0.30           | 6.09 ± 0.13            | 5.93 ± 0.48            | 5.98 ± 0.39            |
| Total starch content (%)      | 37.88 ± 0.85 A        | 36.85 ± 0.53 A         | 36.97 ± 0.69 A         | 30.92 ± 0.72 B         |
| Amylose content (%)           | 4.70 ± 0.26 A         | 4.45 ± 0.22 A          | 3.46 ± 0.19 B          | 2.13 ± 0.17 C          |
| Ash content (%)               | 6.93 ± 0.48           | 7.18 ± 0.73            | 7.62 ± 0.79            | 7.34 ± 0.76            |
| Ca content (%)                | 0.19 ± 0.02           | 0.20 ± 0.04            | 0.18 ± 0.01            | 0.17 ± 0.01            |
| Total P content (%)           | 0.48 ± 0.02           | 0.49 ± 0.02            | 0.51 ± 0.01            | 0.49 ± 0.03            |
| Phytate phosphorus content (%)| 0.37 ± 0.02 A         | 0.33 ± 0.03 AB         | 0.30 ± 0.01 B          | 0.23 ± 0.03 C          |
| Available phosphorus (%)      | 0.11 ± 0.01 D         | 0.16 ± 0.01 C          | 0.21 ± 0.00 B          | 0.25 ± 0.01 A          |
| Glycinin content (%)          | 70.83 ± 5.91 A        | 50.30 ± 3.23 B         | 26.15 ± 2.53 C         | 22.65 ± 4.18 C         |
| β-Conglycinin content (%)     | 65.31 ± 4.38 A        | 58.09 ± 4.76 A         | 28.63 ± 4.65 B         | 16.34 ± 3.32 C         |
| Trypsin inhibitor content (%) | 6.12 ± 1.27 A         | 3.49 ± 0.62 B          | 0.51 ± 0.11 C          | 0.24 ± 0.05 C          |

*Means with different letters in each row differ at P < 0.05.

added for the following 48 h for anaerobic fermentation to increase the levels of probiotic and microbial metabolites (Fig. S1B). Interestingly, the inclusion of 10⁷ CFU/g E. faecium and 10⁷ CFU/g Lactobacillus plantarum for two-stage fermentation did not hinder the degradation effects of CW4. Additionally, E. faecium was more effective at reducing pH than was L. plantarum. Therefore, a novel and effective inoculum combination and fermentation process (10⁷ CFU/g B. subtilis CW4, aerobic fermentation for 24 h; and 10⁷ CFU/g E. faecium CWEF, anaerobic fermentation for 48 h) was selected for further study.

**Chemical composition.** SBM and corn are widely consumed by farm animals and contain different C/N ratios. To obtain novel substrates that contain a better C/N ratio for bacterial growth, rice dried distillers grains with solubles (rDDGS) was chosen as a supplementary ingredient. SBM, corn, and rDDGS were mixed at a ratio of 2:2:1 (Table S1). The determined nutrient content of FMS at 0 h, 12 h, 24 h, and 48 h is presented in Table 1. First-stage fermentation dynamically and markedly influenced the properties of proteins in MS. The hydrolysis rates of glycinin and beta-conglycinin were 67.50% and 74.98%, respectively, after B. subtilis treatment, while little degradation was observed during the second stage of anaerobic fermentation. In the present study, the enzyme activity of neutral protease was notably improved from 12.32 ± 2.61 (SD) to 219.42 ± 8.76 U/g during B. subtilis fermentation (Fig. S1E). The initial TI percentage was 6.12% ± 1.27%. After 24 h of fermentation, the TI concentration decreased to 0.51% ± 0.11% and further decreased to 0.24% ± 0.05% during second-stage fermentation. The neutral detergent fiber (NDF) content decreased significantly from 13.61% ± 0.64% to 9.38% ± 0.36% during the processes. Correspondingly, the phytate phosphorus content decreased from 0.37% ± 0.02% to 0.23% ± 0.03%. The total starch and amylose levels were significantly reduced by 18.37% and 54.68%, respectively.

The crude protein content increased from 28.37% ± 0.86% to 31.37% ± 0.43%. Notably, the trichloroacetic acid-soluble protein (TCA-SP) concentration gradually increased from 1.58% ± 0.19% to 2.89% ± 0.13% over the 0 h- to 12-h time period. A dramatic increase was observed in the following 12 h, and the final content was 10.95% ± 0.05%. Additionally, similar trends were observed for the levels of free amino acids and small peptides (SPs), which increased by 2.71 and 6.67 times, respectively, after fermentation. In addition, the LA content and pH of the FMS reached 108.67 ± 9.39 mmol/kg and 5.21, respectively (Fig. S1D).

**Electrophoresis and microscopic observation.** In the present study, the protein profile of the FMS was distributed in the range of 20 to 100 kDa (Fig. 1B). For...
unfermented MS, the proteins were in the range of 20 to 100 kDa. The molecular weights of the main protein fractions in the unfermented MS were approximately 80, 50, and 37 kDa. After 24 h of fermentation, the large proteins in the FMS were hydrolyzed into molecules with markedly smaller masses (<30 kDa), resulting in a decrease in the levels of large proteins (>35 kDa). The protein profile showed marginal enrichment during the anaerobic stage of fermentation compared to the aerobic stage of fermentation. Interestingly, the protein profile of FMS at 12 h was similar to that of the MS, although the chemical analysis results were different.

A total of 728 protein spots were detected in the MS sample by two-dimensional electrophoresis (2DE) (Fig. 1Ca). These spots were localized in the isoelectric point (pI) range of 3 to 10. The protein profiles changed dramatically during 12 to 24 h of MS fermentation (Fig. 1Cb and c). These results demonstrated that a considerable number of the proteins were degraded to SPs during SSF. Thirteen major proteins from the MS protein profile were selected to identify the degradation of antigenic proteins during

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FIG 1  Experimental design, electrophoresis, and SEM image of FMS. (A) Experimental design. (B and C) SDS-PAGE (B) and two-dimensional (2D) electrophoretograms (C) of FMS at fermentation times of 0 h, 12 h, 24 h, and 48 h. (B) SEM images of MS and FMS after 72 h of fermentation at ×100 (a and b), ×1,500 (c to f), and ×5,000 (g to j) fold magnification. d, days; MW, molecular weight.
fermentation (Fig. 1Ca) and were found to be reduced in the FMS protein profile (Fig. 1Cc). The data listed in Table 2 include the protein spot number(s), protein identity, molecular weight, pI value, MASCOT score(s), and source. Beta-conglycinin and glycmin were found in the 2DE profile of MS, and the levels of these molecules decreased in FMS. The alpha (spots 1, 14, 15, and 16), alpha’ (spot 2), and beta’ (spots 5, 19, 20 and 21) subunits of beta-conglycinin and each acidic chain and basic chain of glycmin were well resolved, detected, and identified. Spot 1 at 0 h was degraded into spots 14, 15, and 16, suggesting a decrease in the level of the alpha subunit of beta-conglycinin. Similarly, the beta subunit of beta-conglycinin (spot 5) and glycmin G4 (spot 8) decomposed into spots 19, 20, 21, and 17. Overall, the spots in Fig. 1Cc are much smaller than those in Fig. 1Ca. Upon further examination, granule-bound starch synthase 1 (spots 4), trypsin inhibitor A (spot 10), class I heat shock protein (spot 11), nucleoside diphosphate kinase 1 (spot 12), and basic 7S globulin (spot 13) were also well resolved, detected, and identified. Notably, TI A (spot 10) disappeared after 48 h.

Scanning electron microscopy (SEM) was applied to investigate the physical structures of MS and FMS. Figure 1D shows the surface images of MS and FMS at magnification factors of 1,100, 1,500, and 5,000. After 48 h of fermentation, more fragmental structures were detected. At the same magnification factor, SBM and corn in MS had relatively large, compact, and smooth-faced structures, while SBM and corn in FMS had smaller cracked structures and large holes.

Changes in the bacterial community. Overall, 258,144 high-quality sequences were collated. Additionally, the general 16S rRNA operational taxonomic unit (OTU) numbers reached 1,390 based on 97% sequence similarity (Table 3). Combined with Good’s coverage index (99.6% ± 0.00%, data not shown), the results suggested that the samples exhibited abundant OTU coverage and that the sequencing depth was sufficient for analysis of the actual structure of the bacterial community during SSF. Figure 2A shows that the number of OTUs decreased after the addition of B. subtilis during initial 24 h. In contrast, the number of OTUs increased after following 24 h of anaerobic fermentation. A Venn plot (Fig. 2B) shows the common and unique OTUs in the groups. Fifteen OTUs as core genera were shared by all of the groups. The

### TABLE 2 Spot numbers for 2D maps

| Spot no. | Protein                        | Mol wt (kDa) | pI   | MASCOT score(s) | Source     |
|----------|--------------------------------|--------------|------|-----------------|------------|
| 1, 14, 15, 16 | α subunit of β-conglycinin | 70.25        | 5.07 | 641, 310, 299, 211 | Soybean    |
| 2        | α’ subunit of β-conglycinin   | 74.28        | 5.47 | 219             | Soybean    |
| 3        | Sucrose-binding protein       | 60.48        | 6.42 | 317             | Soybean    |
| 4        | Granule-bound starch synthase 1 | 65.92      | 6.59 | 574, 686, 694   | Corn       |
| 5, 19, 20, 21 | β’ subunit of β-conglycinin | 50.52        | 5.88 | 520, 478, 348, 461 | Soybean    |
| 6        | Glycinin G5                   | 57.92        | 5.6  | 521             | Soybean    |
| 7        | Glycinin G3                   | 54.36        | 5.46 | 166             | Soybean    |
| 8, 17    | Glycinin G4                   | 63.55        | 5.29 | 401, 257        | Soybean    |
| 9        | Glycinin G2                   | 54.36        | 5.46 | 99, 306, 364, 274 | Soybean    |
| 18       | Glycinin G1                   | 55.67        | 5.89 | 340             | Soybean    |
| 10, 22, 23, 24 | Trypsin inhibitor A    | 23.99        | 4.99 | 351             | Soybean    |
| 11       | 17.5-kDa class I heat shock protein | 17.53  | 5.33 | 79              | Soybean    |
| 12       | Nucleoside diphosphate kinase 1 | 16.43     | 5.93 | 250             | Soybean    |
| 13       | Basic 7S globulin             | 46.36        | 8.68 | 172             | Soybean    |

### TABLE 3 Characteristics of amplicon libraries

| Characteristic | Data for samples at time (h)\(^a\) | Total no. |
|----------------|-----------------------------------|-----------|
| No. of sequences | 69,151 ± 4,299 61,811 ± 627 64,525 ± 5,021 62,657 ± 8,350 | 258,144 |
| No. of OTUs | 411 ± 13 A 327 ± 13 B 319 ± 16 B 333 ± 17 B | 1,390 |
| Chao1 index | 676.73 ± 15.96 A 720.83 ± 57.93 A 701.71 ± 45.62 B 639.51 ± 45.99 A | |
| Shannon index | 1.90 ± 0.21 B 0.58 ± 0.03 C 0.53 ± 0.07 C 2.42 ± 0.13 A | |
| Simpson index | 0.50 ± 0.09 B 0.10 ± 0.01 C 0.09 ± 0.02 C 0.70 ± 0.02 A | |

\(^a\)Means with different letters in each row differ at \(P < 0.05\).
FIG 2 Microbial diversity and community structure during two-stage SSF (n = 4). (A) Observed OTU line chart. (B) Venn diagram representing the common and unique OTUs found at each fermentation time point. (C) 3D principal-component (PC) analyses of samples conducted based on unweighted UniFrac distances. (D and E) Phylum-level (D) and genus-level (E) compositions of the bacterial community in FMS. (F) LEfSe histogram showing the LDA scores (>3.5) computed for features at the OTU level. Letters indicate the taxonomy of the bacteria: p, phylum; c, class; o, order; f, family; g, genus.
three-dimensional (3D) principal-component analysis (PCA) plot (Fig. 2C) shows that samples at 0 h, 12 h, and 48 h were well resolved and obviously distinct, while the OTUs of the samples at 12 h and 24 h exhibited fewer differences than did those of the other samples.

In general, more than seven bacterial phyla were found in all the samples (Fig. 2D). In the MS samples, *Cyanobacteria* and *Proteobacteria* accounted for 99.96% ± 0.10% of the sequences. However, as fermentation progressed, *Firmicutes* rapidly became the primary members of the community, accounting for approximately 98% of the sequences.

With regard to the changes in bacterial community structure, the results at the genus level were similar to those at the phylum level (Fig. 2E). Unfermented materials contained various native bacteria, including pathogens such as *Enterobacter* spp. As the overall fermentation progressed, the predominant bacteria changed from *Cyanobacteria* and *Proteobacteria* to *Bacillus* spp., *Enterococcus* spp., and *Pseudomonas* spp. This result was consistent with the culture results for some detected microbes (Fig. S1C).

Furthermore, the linear discriminant analysis (LDA) effect size (LEfSe) results showed significantly different taxonomy among different fermentation time points (Fig. 2F). After 24 h of aerobic fermentation, the abundances of the genera *Bacillus* and *Aerococcus* increased significantly. After 24 h of anaerobic fermentation, *Enterococcus* spp., *Pseudomonas* spp., and *Lactobacillales* were predominant. The results of LEfSe were further verified by multiple-test correction (Fig. S2).

**Bacterial metabolism of fermented mixed substrates.** The microbial metabolic functions shown in Fig. 3 were obtained based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. A majority of the predicted protein sequences ranged from 55.27% ± 1.35% to 0.72% ± 0.20% at four time points among six different metabolic functions (Fig. 3A), which represented different pathways (Fig. 3B). Notably, carbohydrate metabolism, amino acid metabolism, and membrane transport accounted for more than 30% of the enriched pathways throughout the fermentation period. Furthermore, the sequences related to amino acid metabolism, excretory system, and the transport, catabolism, and metabolism of other amino acids were significantly enriched during first-stage fermentation (*P* < 0.00) (Fig. S3A). The sequences at 48 h, which were related to carbohydrate metabolism, membrane transport, and metabolism, were highly distinct from the pathways in the sample at 0 h and 24 h (*P* < 0.00).

At level 3 of microbial gene functions, some differences in efficiency were observed during SSF (Fig. 3C). The abundances of a majority of the genes assigned to amino acid metabolism (alanine, aspartate, and glutamate metabolism and arginine and proline metabolism) and carbohydrate metabolism (amino sugar and nucleotide sugar metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, and starch and sucrose metabolism) increased gradually during fermentation (*P* < 0.05). Similarly, the genes associated with transporters, ATP-binding cassette (ABC) transporters, and transcription factors were markedly enriched from 5.13%, 3.12%, and 0.86% to 8.95%, 3.69%, and 2.37%, respectively (*P* < 0.05). In contrast, the abundances of most genes related to cellular processes and signaling and energy metabolism decreased with fermentation. Interestingly, the abundances of genes involved in translation and nucleotide metabolism decreased during the aerobic fermentation period, while a considerable increase was observed following anaerobic fermentation. As expected, the gene functions related to *B. subtilis* (sporulation and other iron-coupled transporters) were improved after the addition of *B. subtilis* and reduced during second-stage fermentation. The detailed differences in gene functions are shown in Fig. S3B. The results of PICRUSt prediction at level 3 were verified by multiple-test correction (Fig. S4).

**Relationship of the bacterial community with physicochemical features and metabolic functions.** Conetwork analysis was applied to assess the relationship between physicochemical characteristics and microbial communities. The C/N ratio, available phosphorus (AP) content, SP content, pH, LA content, glycinin content, and NDF
FIG 3  Dynamics of bacterial functional profiles during MS fermentation processes analyzed by PICRUSt ($n = 4$). (A) Level 1 metabolic pathways. (B) Level 2 KEGG ortholog functional predictions. (C) Level 3 KEGG ortholog functional predictions of the relative abundances of the top 30 metabolic functions.
content were selected as physicochemical characteristics. During aerobic fermentation, most genera were positively related to each other (Fig. 4A). In contrast, \textit{Bacillus} spp. exhibited a negative association with most other genera. \textit{Bacillus} spp. were positively related to AP content, whereas most genera, such as \textit{Agrobacterium} spp., \textit{Erwinia} spp., \textit{Paenibacillus} spp., and \textit{Lactobacillus} spp., were negatively related to AP content. Similar patterns were found for SP and glycinin. As expected, the genera that had a negative relationship with SP and a positive relationship with glycinin were inhibited by \textit{Bacillus} spp. Under anaerobic conditions, \textit{Bacillus} spp. and \textit{Enterococcus} spp. became the predominant genera (Fig. 4B). \textit{Enterococcus} spp. had a positive relationship with LA. In contrast, \textit{Bacillus} spp. were negatively related to SP and LA. As shown in Fig. 4C, during the 0- to 24-h period, \textit{Bacillus} spp. were markedly positively related to environmental information processing and cellular processes. \textit{Leuconostoc} spp. and \textit{Lactobacillus} spp. were significantly enriched in the genetic information processing and organic systems, while \textit{Bacillus} spp. were negatively associated with these pathways. Under anaerobic
conditions, *Bacillus* spp. still had a positive relationship with cellular processes, while *Enterococcus* spp. were notably positively related to environmental information processing (Fig. 4D).

**Gene-level assessment.** Real-time quantitative PCR was further employed to assess the accuracy of 16S rRNA and PICRUSt analysis. The results of the gene-level assessment are provided in Fig. 5. Gene levels were tested in three core genera (*Bacillus* spp., *Enterococcus* spp., and *Pseudomonas* spp.) and three feed-native bacterial genera (*Methylobacterium* spp., *Cyanobacteria* spp., and *Ralstonia* spp.) during fermentation. The results of gene-level assessment were very similar to the results of the 16S rRNA analysis. In addition, some functional genes that are involved in important metabolic pathways related to fermentation were analyzed. Endoglucanase and endo-1,4-beta-xylanase contribute greatly to breaking down fiber content. MgsA is related to LA generation by LA bacteria during second-stage fermentation (12). These three genes belong to carbohydrate metabolism pathways. Subtilisin plays a critical role in protein degradation during fermentation and is an extracellular protease that is associated with membrane transport. In general, the gene-level assessment was consistent with the PICRUSt prediction.

**In vivo evaluation of fermented mixed substrates.** Supplementation with FMS significantly increased the average daily weight gain of piglets (Table S4). In addition, piglets fed FMS exhibited markedly lower diarrhea incidence than did the control group. In addition, higher villus height and villus height/crypt depth ratio were found in the FMS inclusion group than in the control group. Pigs in the MS group showed higher crude protein (CP) and total phosphorus (TP) digestibility than did those in the control group. Moreover, piglets fed FMS showed lower mRNA expression of the inflammatory cytokines interleukin 4 (IL-4) and IL-6 in the jejunum than did piglets in the control group (Fig. S5A). The phosphorylation of the key factors p38, IκB-α, and p65 in the jejunum was also reduced in the FMS group (Fig. S5B).

**Process design for two-stage solid-state fermentation of mixed substrates.** Figure S6 shows the process flowsheet for two-stage SSF to manufacture FMS using corn, soybean, and rDDGS as the substrates. The flowsheet is divided into three sections, preparation, fermentation, and finishing. The preparation section includes microbial culture and mixing. A bed-type SSF bioreactor was suitable for the two-stage solid-state fermentation. Substrate drying, pulverizing, and packaging are included in the finishing section for industrial production.

**DISCUSSION**

In the present study, a novel two-stage SSF method was used to effectively and efficiently alter the physicochemical features, microbiota, and metabolic features of MS. The ANF concentration and pH in the FMS decreased over time, whereas the levels of SP and AP increased. *Bacillus* spp., *Enterococcus* spp., and *Pseudomonas* spp. became the dominant genera and dynamically affected various metabolic pathways during two-stage SSF. Network analysis revealed the relationships of the microbial community with physicochemical features and metabolic functions. Weaned piglets were used as an in vivo model to further test the FMS in terms of growth performance, nutrient digestibility, and anti-inflammatory properties.

The glycinin, beta-conglycinin, and TI in MS were dramatically degraded during first-stage fermentation. Many proteases were secreted by *B. subtilis* during aerobic fermentation, such as aminopeptidases, serine endopeptidases, metalloproteinases, and neutral proteases (Fig. S1E), which are able to decompose proteins, including TI (7). Thus, the hydrolytic effects of the proteases secreted by *B. subtilis* resulted in a decrease in antigenic protein and TI levels in FMS. Interestingly, further decomposition of antigenic proteins and TI occurred in second-stage fermentation, perhaps due to the low pH and structural changes caused by *Lactobacillus* spp. (8). The increase in xylanase and cellulase activities might be attributed to the decrease in NDF content (Fig. S1F and G). Other enzymes from *B. subtilis*, such as amylase and phytase, can cause degradation of ANFs (13). Glycinin and beta-conglycinin are two major antigenic proteins that...
FIG 5 Gene-level assessment of bacteria and metabolic genes \((n = 4)\). The blue line chart represents the gene level. The bar chart represents the 16S level. (A) Bacillus spp. (B) Enterococcus spp. (C) Pseudomonas spp. (D) Cyanobacterium spp. (E) Methylobacterium spp. (F) Ralstonia spp. (G) Endoglucanase. (H) Endo-1,4-xylanase. (I) mgsA. (J) Subtilisin.
account for approximately 75% of the total soybean proteins and are responsible for allergic reactions (14, 15). TI may hinder digestive functions (2). Additionally, high levels of NDF, phytase, and amylose may interfere with nutrition utilization (16). Therefore, the lower antigenic protein, TI, NDF, phytase, and amylose contents indicate that FMS may have higher nutrient digestibility and lower nutrient emissions than does nonfermented MS.

The CP and SP contents of FMS tended to be higher than those of unfermented MS ($P < 0.05$), which is consistent with previous experimental research (17–19). The increase in CP content may be attributed to the loss of dry matter during fermentation (20). A rapid increase in SP content was observed in the study ($P < 0.05$). Shi et al. reported that the increase in SP content may be attributed to the digestion of large proteins during fermentation (21). An increased amount of SPs in FMS in the present study might also be due to the digestion of large peptides, especially antigenic proteins in FMS, by proteases from the probiotics. SPs are considered to have antioxidant properties (22), immunoregulatory functions (23), and desirable digestibility (11). Thus, the increased SP content indicated the functional properties of FMS. Additionally, the high LA content and low pH of FMS are essential for pathogen inhibition, feed preservation, and feed intake enhancement (10).

The protein profile obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the proteins were degraded in a time-dependent manner, which was consistent with results from previous studies on SBM fermentation by fungi or bacteria (24, 25). Interestingly, the protein profile at 12 h was markedly different from that at 24 h, which suggested that the degradation of proteins mostly occurred during the 12- to 24-h period. To further identify the proteins in FMS, 2DE was applied. The results demonstrated that a considerable number of proteins, especially glycinin, beta-conglycinin, and TI, were greatly degraded to SPs during SSF using B. subtilis and E. faecium. Overall, these SDS-PAGE and 2DE results further confirmed the changes in protein profiles and were generally consistent with the chemical analysis of MS during the fermentation process.

In contrast to MS, FMS exhibited small, cracked structures and large holes. The change in the surface structure of MS after fermentation may be associated with extracellular enzymes (especially protease, amylase, and carbohydrase) secreted during the process. The cracked and porous structure may provide increased access to enzymes for nutrient hydrolysis and may make the substrates considerably easier to utilize (26), suggesting that FMS had higher digestibility than MS. Additionally, Tang et al. reported that smaller protein aggregates may result in a higher solubility (27). Zhao et al. found that soybean proteins with loose networks and diffuse structures have higher emulsification activity and solubility (28). Thus, the physicochemical properties of MS may also have been affected by the changed microstructure in this study.

High-throughput sequencing was first applied to analyze the changes in microbial community structure and metabolic functions during the fermentation process. The decrease in OTU number during first-stage fermentation suggested that B. subtilis proliferated, inhibited other bacteria, and became the dominant bacterium. In contrast, the OTU number increased during anaerobic fermentation, which may be associated with inoculation with E. faecium and the growth of some other LA bacteria. The main phyla (Cyanobacteria, Proteobacteria, and Firmicutes) found in the present study were also obtained in some other studies related to SSF (29, 30). Bacillus spp., Enterococcus spp., and Pseudomonas spp. were identified as the core genera during two-stage SSF. Members of the heat-tolerant genus Bacillus are effective at degrading proteins and cellulose during SSF due to their strong hydrolytic abilities (29). Enterococcus is a mesophilic genus whose members generate LA (30). Pseudomonas spp. have been investigated as biocontrol bacteria to promote fermentation quality in the maturation period (31). Thus, these dominant genera indicated a selected community categorized by typical large-molecule catabolism characteristics and the capacity to produce LA, achieved by the addition of the two inocula. The evolution of bacterial structure during the process demonstrated that the artificially added inocula
not only increased the number of added bacteria but also boosted some other functional bacteria that could develop a form of symbiosis with the inocula.

The results of KEGG level 1 to 3 gene function analysis were generally consistent. As fermentation progressed, the abundances of both carbohydrate and amino acid metabolism genes gradually increased. Metabolism of cellulose and hemicellulose can produce many compounds that support bacterial growth (32). Amino acids are also an energy and carbon source for bacteria (33). These results indicated that the degradation of large carbohydrate and proteins resulted in increased levels of saccharides, SPs, and amino acids, which could be utilized by the microbiota in FMS. The gene abundances of transporters, ABC transporters, and transcription factors increased during fermentation. These metabolic functions were associated with compound production and membrane transport, suggesting the mechanism of enzyme synthesis by and activity of the core bacteria in FMS. In contrast, cellular processes and signaling and energy metabolism were inhibited by the core genera, indicating that these genes may be involved in native bacterial gene functions of MS. Although the addition of B. subtilis inhibited the growth of other MS-native microbes, it did not decrease the abundances of enzyme families, which were maintained at approximately 10%. Enzyme families are important for complex biopolymer catabolism (34). This evidence revealed the strong enzyme secretion ability of B. subtilis. Additionally, the differences in the abundances of genes involved in translation and nucleotide metabolism between the aerobic and anaerobic stages suggested the different metabolic roles of Bacillus spp. and Enterococcus spp.

AP is critical for cell growth and development (35). During the first 24 h of fermentation, most native genera in MF had a negative relationship with AP, suggesting that they may utilize AP for growth. Bacillus spp. were positively related to AP, which may be due to the higher rate of phytate phosphorus degradation than utilization by B. subtilis. In addition, Bacillus spp. were negatively related to most of the native microbes, revealing that AP strongly affected the growth of these bacteria and that Bacillus competed with other genera. Bacillus spp. participated in the transformation of large proteins and glycinin to SPs and were thus positively related to SP and negatively related to glycinin. Meanwhile, Bacillus spp. increased the pH of the fermentation system; thus, they were positively related to pH. In the second anaerobic fermentation stage, Enterococcus spp. were positively related to LA, indicating that the major function of Enterococcus spp. was LA generation. Intriguingly, Bacillus spp. were negatively related to SP, while the amount of SP continued to increase. This phenomenon suggested that although the abundance of Bacillus spp. decreased during the second stage, their metabolic activities, such as those performed via enzymes and bacteriocin, might continue to increase the amount of SPs and inhibit the growth of Facklamia spp. and Enterococcaceae.

Various bacteria were correlated with different metabolic pathways, revealing that multiple metabolic pathways were active during the two stages of the SSF. Two inoculated bacteria were enriched in environmental information processing and cellular processes throughout fermentation. These metabolic functions allowed the bacteria to grow, proliferate, and respond to the environment (36). The results demonstrated the superior adaptation of the inocula in response to fermentation. Additionally, under aerobic conditions, some feed-native genera were positively related to genetic information processing and organic systems, whereas members of Bacillus spp. were negatively involved with these metabolic pathways. This evidence suggests that Bacillus spp. might inhibit feed-native bacteria by suppressing genetic information processing and organic systems.

Both 16S rRNA analysis and PICRUSt predictions are dependent on the database, which may cause deviation; thus, real-time quantitative PCR was applied to further assess the accuracy of these results. The results of the bacterial assessment were highly consistent with the 16S rRNA analysis. Although the functional gene expression results at some time points were discrepant, the general metabolic assessments were similar.
to the PICRUSt prediction. The results of quantitative PCR (qPCR) further verified the bacterial communities and functional genes present during fermentation.

Weaned piglets were used as an in vivo model to evaluate the nutrient utilization and anti-inflammatory effects of FMS. FMS was beneficial for piglet growth performance, which may be due to the high digestibility of FMS and probiotic roles of the inocula. The increased CP and TP digestibility of FMS suggested the ease of utilization of the structures of FMS and the low N and P emissions. The improved villus height-to-crypt depth ratio indicated that FMS improved piglet gut morphology. Intestinal mucosal immune responses to glycinin were enhanced by high levels of IL-4 and IL-6 (37). The MAPK/NF-κB pathway is critical for regulating inflammatory genes (38). The reduced levels of IL-4 and IL-6 and phosphorylation of p38, IκB-α, and p65 in the jejunum further confirmed that FMS reduced the immune disorder that might be caused by antigenic proteins.

In summary, this study provides a novel method for improving the nutritional quality of MS and provides a basis for demonstrating that the inoculated microbes dynamically change the physicochemical features, microbiota, and metabolic functions during two-stage SSF, which could serve as a valuable resource for industrial feed-based practices and metabolomic research on SSF systems. Further studies should focus on the use of additional enzymes during first-stage fermentation and inoculation with other LA bacteria during second-stage fermentation to further reduce the ANF content of FMS and produce various types of organic acids.

MATERIALS AND METHODS

Microorganisms and enzymes. B. subtilis CW4 (NCBI accession no. MH885533) was obtained from a traditional fermented food (pickled vegetables) and was selected by using a soybean antigenic protein screening plate. E. faecium CWEF (NCBI accession no. MN038173) was isolated from the gut of a healthy pig. Both B. subtilis and E. faecium are government-authorized probiotics in China.

S. cerevisiae (CGMCC 2.3973), L. plantarum (CGMCC 1.16089), Lactobacillus casei (CGMCC 1.8727), and neutral protease from Bacillus spp. (P3111; Sigma-Aldrich Corp., St. Louis, MO, USA) were purchased to compare their effects on protein degradation and LA production during different fermentation processes.

Preparation of fermented mixed substrates. For inoculation, B. subtilis and E. faecium were cultured in Luria broth and de Man, Rogosa, and Sharpe liquid medium at 37°C for 10 h and 18 h, respectively. The fermented substrates were finely mixed in a corn/SBM/rDDGS ratio of 2:2:1 (total, 200 g). Then, the MS was placed in a 500-ml Erlenmeyer flask and supplied with water to achieve a 35% moisture concentration. Next, a sterile membrane was added to the Erlenmeyer flask.

The moist MS was inoculated with B. subtilis (10^7 CFU/g) and fermented at 37°C for 24 h (first-stage aerobic fermentation). Then, the sterile membrane was removed, and each Erlenmeyer flask was supplied with E. faecium (10^7 CFU/g). The mouth of the Erlenmeyer flask was sealed with a rubber plug for the second-stage anaerobic fermentation at 37°C. All samples were set up in quadruplicate. Moist samples (approximately 100 g) at 0 h, 12 h, 24 h, and 48 h were collected to determine the numbers of microorganisms and microbial metabolites and for 16S rRNA gene sequencing, and the remaining samples were dried at 60°C for 24 h, cooled, ground, and subjected to physicochemical analysis, SDS-PAGE, and 2DE.

Microorganisms and metabolites. The pH and microbiological counts at different fermentation times were analyzed as described by Wang et al. (39). Xylanase and cellulase activities were analyzed by the dinitrosalicyclic acid method reported by Wongputtisin et al. (40). The activity of neutral protease was detected as mentioned by Ueda et al. (41).

Chemical analysis. Samples at 0 h, 12 h, 24 h, and 48 h were obtained for analysis of the dry matter, CP, NDF, acid detergent fiber (ADF), total starch, amylose, ash, calcium (Ca), TP, and AP contents using AOAC international guidelines (42). Total organic carbon (TOC) and nitrogen were measured on a Vario EL cube (Elementar Americas, Inc., Hanau, Germany). The TCA-SP, free amino acid, and SP contents of the sample were analyzed as reported by Ovisipour et al. (43). The phytate phosphorus content was measured according to the method described by Thompson and Erdman (44). The concentrations of antigenic proteins in MS and FMS were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Jiangsu Meibiao Biological Technology Co., Ltd., Jiangsu, People’s Republic of China), according to the manufacturer’s protocol.

Microscopic inspection. Changes in the physical properties of the substrates before and after fermentation were examined by SEM according to the protocol of the Electronic Microscopy Center of Zhejiang University. The microstructures of MS and FMS were observed using a field-emission scanning electron microscope (KYKY-EM3200, China) at ×100, ×1,500, and ×5,000 magnifications.

Electrophoresis. The protein profile of MS was obtained as described by Zhang et al. (45). Samples (25 g) at 0 h, 12 h, 24 h, and 48 h were soaked overnight in 50 ml of 50 mM acetate buffer (pH 5.0) containing 5 μg/ml protease inhibitor (Roche, Switzerland) at 4°C. The suspension was centrifuged and filtered (Millipore, USA). The soluble protein samples were concentrated by ultrafiltration (cutoff, 10 kDa;
For SDS-PAGE, 12% polyacrylamide separating gels were used for electrophoresis. Approximately 5 µg of protein sample was placed in each well, and the sample was separated at 55 mV for 200 min. The gel was stained with Coomassie brilliant blue (CBB) R-250 (Bio-Rad, USA) for 60 min and destained with eluent.

For 2DE, 400 µg of protein was placed onto analytical and preparative gels. Isoelectric focusing (IEF) was conducted using the Ettan IPGphor IEF system (GE Amersham) and pH 3- to 10-immobilized pH gradient (IPG) strips (13 cm, nonlinear; GE Healthcare). The IPG strips were rehydrated in rehydration buffer containing the protein samples. Equilibration buffer was used to equilibrate the gel strips. Then, the strips were subjected to 2DE after transfer onto 12.5% SDS-polyacrylamide gels. Protein spot identification was conducted as reported in reference 9.

**DNA extraction, Illumina MiSeq sequencing, and metabolic function prediction.** Total DNA was extracted from 16 samples using the E.Z.N.A. soil DNA kit (Omega Bio-Tek, Norcross, GA, USA). A NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and 1% agarose gel electrophoresis were used to analyze DNA content and quality.

The V3-V4 gene regions of the bacterial 16S rRNA gene were amplified with primers 338F (5'-ACT CCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR was conducted as follows: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s for annealing at 55°C, and 45 s for elongation at 72°C; and a final extension at 72°C for 10 min. The AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA) and Quantifluor-ST instrument (Promega, USA) were used to further extract, purify, and quantify the PCR products. The MiSeq platform (Shanghai Majorbio Biopharm Technology Co., Ltd.) was used to describe the bacterial community based on the gene segment from the V3-V4 portion of the 16S rRNA gene. Subsequently, raw Illumina FASTQ files were demultiplexed, quality filtered, and analyzed using Quantitative Insights into Microbial Ecology (QIIME v1.9.1). Raw fastq files were quality filtered by Trichromatic and merged by FLASH. OTUs were clustered with 97% similarity cutoff using UPARSE (version 7.1).

The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Greengenes 16S rRNA database using a confidence threshold of 70%. The assembled MiSeq sequences were submitted to the NCBI’s Sequence Read Archive (SRA software (Media Cybernetics, Rockville, MD). The assembled MiSeq sequences were submitted to the NCBI’s Sequence Read Archive (SRA software (Media Cybernetics, Rockville, MD). The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Greengenes 16S rRNA database using a confidence threshold of 70%. The assembled MiSeq sequences were submitted to the NCBI’s Sequence Read Archive (SRA software (Media Cybernetics, Rockville, MD). The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Greengenes 16S rRNA database using a confidence threshold of 70%. The assembled MiSeq sequences were submitted to the NCBI’s Sequence Read Archive (SRA software (Media Cybernetics, Rockville, MD). The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Greengenes 16S rRNA database using a confidence threshold of 70%. The assembled MiSeq sequences were submitted to the NCBI’s Sequence Read Archive (SRA software (Media Cybernetics, Rockville, MD). The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Greengenes 16S rRNA database using a confidence threshold of 70%.

The main differentially abundant genera were selected by the LEfSe method (https://huttenhower.sph.harvard.edu/galaxy/). To predict metabolic genes during the process, PICRUSt (https://huttenhower.sph.harvard.edu/galaxy/) was applied to obtain a functional profile from the 16S rRNA data. Prior to metagenome prediction, the OTUs of 16S rRNA sequences were analyzed using PICRUSt. PICRUSt and KEGG were used to obtain functions for the genes that were predicted to be present in the samples and to assign the genes into metabolic pathways.

Network analysis was conducted using Cytoscape, and the nonparametric Spearman correlation coefficient was greater than 0.8.

**Quantitative analysis of commensal bacterial and functional genes.** The extracted DNA was used to quantify the bacterial content and metabolic functions of the FMS system. DNA was extracted, and quantitative PCR for 16S rRNA genes and functional genes was performed with SYBR green master mix (Roche, Mannheim, Germany) using a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA); the results were normalized to the total bacterial DNA content. The gene-specific primers for the qPCR are listed in Table S2.

**Animal management, nutrient digestibility, and intestinal morphology assessment.** The in vivo experimental design is presented in Fig. 1A.b. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Briefly, a 21-day experiment was conducted with 144 weaned pigs (Duroc × Large white × Yorkshire) with body weights of 9.00 ± 0.65 kg, which were randomly allocated to the control group or the 10% FMS supplementation group. Each group contained 6 pens, and each pen had 12 piglets. Piglets were fed four times a day, and they had free access to water and feed. The ingredients and nutrient content of the diets are presented in Table S3. Nutrient digestibility was calculated as described by Wang et al. (46). The middle jejunum of the piglets was harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections of 5-µm thickness were deparaffinized in xylene and stained with hematoxylin and eosin (H&E). Images were obtained using a DM3000 microscope (Leica Microsystems, Wetzlar, Germany). The villous height and crypt depth of the jejunum were measured using Image-Pro software (Media Cybernetics, Rockville, MD).

**Real-time quantitative PCR and Western blot analysis.** Quantitative PCR of cytokines was conducted as described above. For Western blotting, total protein extracts of scraped jejunal mucosa or cells were harvested using the Total protein extraction kit (KeyGen BioTECH, Nanjing, China). Equivalent amounts of protein were separated by SDS-PAGE and electrophoblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA), followed by blocking with 5% fat-free milk. Then, membranes were incubated overnight at 4°C with primary antibodies, including p-p38, p-IκB, p-p65, and β-actin antibodies (Abcam, China). After washing with Tris-buffered saline with Tween 20 (TBST), membranes were incubated with secondary antibodies for 1 h at room temperature. The protein bands
were visualized with an electrochemiluminescence (ECL) assay kit (Servicebio, Wuhan, China), and the band intensity was quantified using the ImageJ software.

**Statistical and bioinformatics analyses.** The data were analyzed using the SAS software (version 9.2; SAS, Inc., Chicago, IL, USA). Statistical differences between experimental groups were evaluated by Student’s t tests and one-way analysis of variance (ANOVA) with Duncan’s multiple-range test or at least a significant difference test. All data are expressed as the mean and standard deviation (SD). The interquartile range method, followed by quantile normalization within replicates, was performed for data filtering and normalization during the assay. P values of ≤0.05 represent a significant difference. For network analyses, the nonparametric Spearman correlation coefficient and significance between bacteria and physicochemical characteristics or bacteria and metabolic functions were calculated using the coplot package of R (R Core Team, 2014). The nonparametric Spearman correlation coefficient, which was greater than 0.8, and significance, which was smaller than 0.05, were selected for further network analysis. The heatmap package of R (R Core Team, 2014) was applied to generate heat maps of genera and L3 predicted microbial gene functions. Bar plots were generated in GraphPad Prism 7 (San Diego, CA, USA). Multiple-testing corrections of distinguished species and predicted metabolic functions during fermentation were employed using Welch's test and the Benjamini-Hochberg false-discovery rate (FDR) method for statistical analysis of metagenomic profiles (STAMP version 2.1.3).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 2.6 MB.
**FIG S2**, TIF file, 2.5 MB.
**FIG S3**, TIF file, 2.9 MB.
**FIG S4**, TIF file, 2.6 MB.
**FIG S5**, TIF file, 0.7 MB.
**FIG S6**, TIF file, 1.6 MB.
**TABLE S1**, DOCX file, 0.1 MB.
**TABLE S2**, DOCX file, 0.1 MB.
**TABLE S3**, DOCX file, 0.1 MB.
**TABLE S4**, DOCX file, 0.1 MB.

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