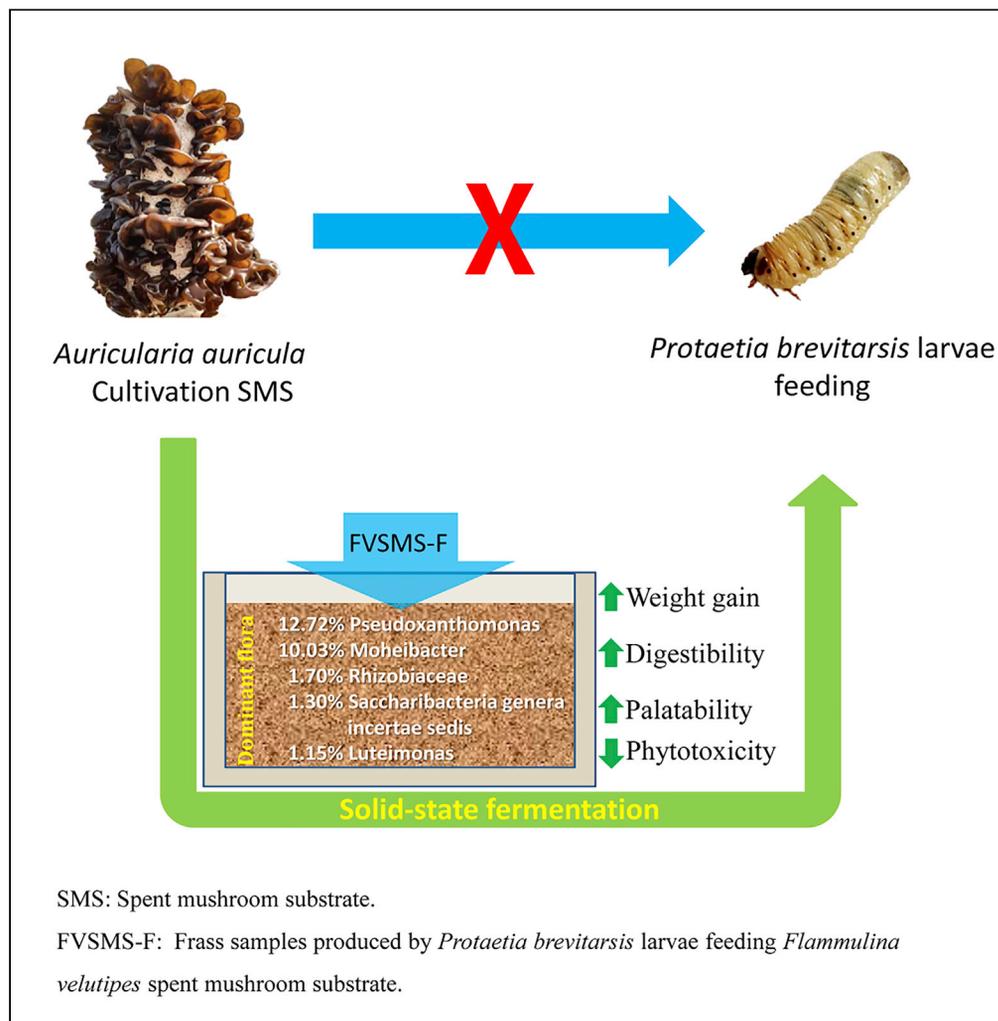


Article

Microflora for improving the *Auricularia auricula* spent mushroom substrate for *Protaetia brevitarsis* production



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Highlights

Larvae frass microflora can ferment *Auricularia auricula* spent mushroom substrate

The fermentation can improve feed intake, weight gain, and phytotoxic removal efficiency

The genera *Luteimonas*, *Moheibacter*, and *Pseudoxanthomonas* were functional bacteria



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Microflora for improving the *Auricularia auricula* spent mushroom substrate for *Protaetia brevitarsis* productionBaohai Du,^{1,2} Huina Xuan,^{1,3} Lili Geng,¹ Weihang Li,² Jie Zhang,¹ Wensheng Xiang,² Rongmei Liu,^{2,*} and Changlong Shu^{1,4,*}

SUMMARY

Mushroom cultivation is a sustainable agricultural waste utilization method, but the lack of high-value utilization of the produced spent mushroom substrate (SMS) has hindered the development of mushroom cultivation-based circular agricultural systems. Conversion and utilization of SMS via *Protaetia brevitarsis* larvae (PBL) have proven to be a high-value AASMS utilization strategy. However, *Auricularia auricula* SMS (AASMS), which contains woodchips, is less palatable and digestible for PBL. To solve this problem, in this investigation, we screened out microflora (MF) for AASMS fermentation by comparing the fermentation performance as well as the effect on PBL feed intake, weight gain, and AASMS phytotoxic compound removal efficiency. In addition, by bacterial community analysis, the genera *Luteimonas*, *Moheibacter*, and *Pseudoxanthomonas* were predicted to be functional bacteria for AASMS fermentation and contribute to palatability and digestibility improvement.

INTRODUCTION

Mushroom agriculture can convert low-quality waste streams to high-quality food, and is considered to be a sustainable agricultural waste utilization method. *A. auricula* is an edible fungus that is used as a traditional food and medicine in Asia. *A. auricula* has low caloric and fat levels and can provide all essential amino acids as well as dietary fiber for humans. In addition, it also has anticancer, anti-inflammatory, hypoglycaemic, hypolipidaemic, and anticoagulant properties. Therefore, *A. auricula* production is widespread in Asia, especially in China, which is responsible for 90% of the total production. In 2019, China's *A. auricula* production reached 7 million tons, being carried out mainly in Heilongjiang, Jilin, Henan, and Hubei provinces, and played an important role in the circular economy.

The spent mushroom substrate (SMS) is usually considered a waste product, especially *A. auricula* SMS, which mainly contains woodchips and is difficult to utilize for a high-value purpose with conventional approaches (Leong et al., 2022). Our previous investigation showed that *Protaetia brevitarsis* (Scarabaeidae) larvae (PBLs) can feed on *A. auricula* SMS (AASMS) (Wang et al., 2019; Wei et al., 2020), leading to the production of a higher-value product (mature larvae). The harvested PBLs are edible and contain high-quality protein and lipid components (Ghosh et al., 2017; Kim et al., 2017), and they are also used as a traditional medicine in China and Korea. The compounds or extracts from PBLs with these health-related properties have been characterized by using experimental animal models or cell culture (Lee et al., 2013, 2017a, 2021; Im et al., 2018; Ahn et al., 2019; Ganguly et al., 2020; Hwang et al., 2020; Krishnan et al., 2020; Seo et al., 2021). In addition, the PBL-produced frass is also a high-value product; it is a high-quality organic fertilizer with high humic acid (HA) levels and is beneficial for plant growth (Li et al., 2019).

However, a previous investigation showed that the PBL feeding efficiency on AASMS from different sources varies greatly, and PBLs even refuse to feed on some AASMS samples. In addition, the phytotoxicity of AASMS-produced frass is still high, and the HA content is lower than that of other SMS (Wei et al., 2020). Considering that PBLs show efficient feeding and transformation of SMS from most other mushroom species (data not shown), the reason for PBLs poor feeding performance and transformation effect on AASMS might be that the metabolites and mycelia produced during *A. auricula* growth have poor palatability or are not easy to digest. Microorganisms can secrete a large number of hydrolases to decompose

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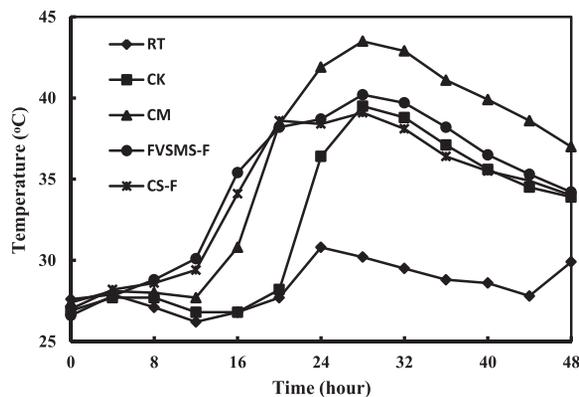


Figure 1. Temperature-heating profile (THP) of AASMS foam box small pile fermentation
RT, room temperature profile; CK, THP of AASMS fermented with natural MF; CM, THP of AASMS fermented with chicken manure MF; FVSMS-F, THP of AASMS fermented with the MF of PBL frass produced by feeding FVSMS; CS-F, THP of AASMS fermented with the MF of PBL frass produced by feeding corn straw.

biomass and can synthesize a variety of biomolecules at the same time. In animal farming, microbes have been applied in solid-state fermentation (SSF) of feed to improve the nutritional characteristics, digestibility, palatability, and safety of feed (Yang et al., 2021). Therefore, in this investigation, we screened and evaluated microbial flora (MF) that could break down biomolecules in AASMS that cause poor palatability and indigestibility. This study is of great significance for further high-value utilization of AASMS by PBLs.

RESULTS AND DISCUSSION

Screening microflora to produce *Auricularia auricula* spent mushroom substrate solid-state fermentation feed

PBLs are important biomass decomposers, and their feeding habit makes PBLs a potential insect resource that can convert decaying plant residues or other organic wastes to high-value insect proteins, fats or other compounds, while the digested biomass is converted to organic fertilizers with high HA content (Li et al., 2019; Wang et al., 2019). In recent years, PBLs have been used as an important tool in the circular economy, and many SMSs have been evaluated as feed to raise PBLs (Wei et al., 2020; Ham et al., 2021). Our recent data showed that AASMS was not as good as other SMSs when applied as PBL feed, which was mainly reflected by the antifeedant activity and the persistent phytotoxicity of AASMS (Wei et al., 2020). Therefore, addressing the challenge of feeding PBLs with AASMS is of great significance for the further development of circular agriculture.

Antifeedant activity and phytotoxicity have been reported to be correlated with some biomolecules (Klein Gebbinck et al., 2002; Azim et al., 2018; Yang et al., 2021), and we hypothesized that the challenge in feeding PBLs with AASMS was caused by unpalatable or indigestible biomolecules. In the field of animal nutrition, microorganisms are widely applied in solid-state fermentation (SSF) of feed to change the nutritional characteristics, digestibility, palatability, and safety of feed. During the SSF process, macromolecular substances and anti-nutritional factors are converted to more efficient and nontoxic nutrients by the metabolic activities of microorganisms (Yang et al., 2021). Thus, in this investigation, we attempted to screen MF that can be used to produce AASMS SSF feed for PBLs.

Foam box small pile fermentation was employed for microorganism screening and small-scale feed SSF. During screening, CM, which is commonly used for compost (Zhang et al., 2021), was employed as a positive control to detect heating in foam box small pile fermentation. The temperature-heating profile (THP) of the CM treatment (Figure 1) showed that the AASMS-supplemented CM could be efficiently fermented, and the microbial metabolic process-produced heat increased the temperature earlier than the CK treatment, which was natural fermentation without artificial inoculation with foreign microorganisms. Taking this as a control, we compared the fermentation effect of MF from PBL frass supplements. The resulting THP showed that both AASMS-supplemented *Flammulina velutipes* SMS frass (FVSMS-F, frass produced from *F. velutipes* SMS) and corn straw frass (CS-F, frass produced from corn straw) produced heat faster than the control, and the temperature increased earlier. This result indicated that the MF contained in

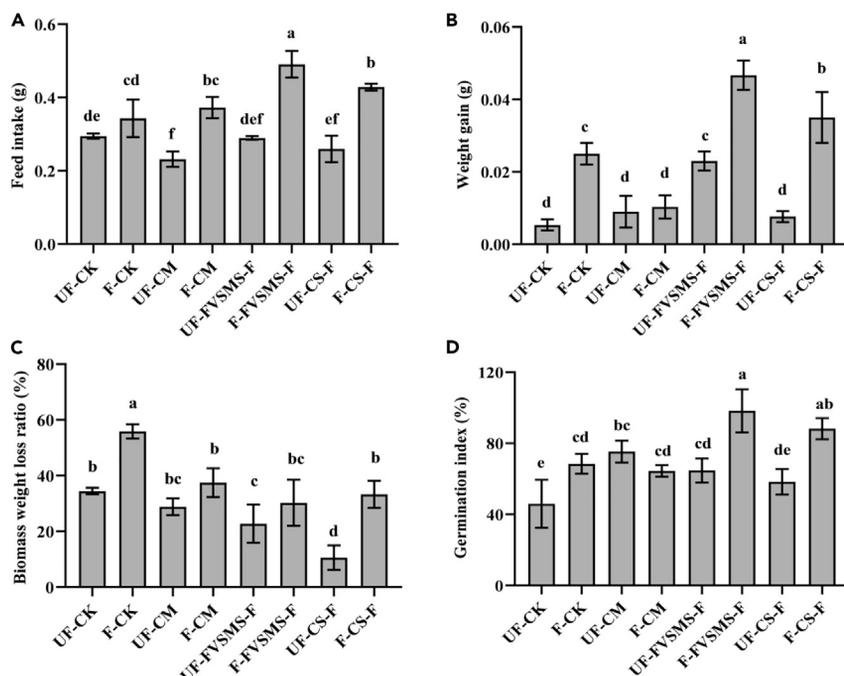


Figure 2. AASMS palatability and digestibility and removal of phytotoxicity from samples

(A) Daily feed intake per gram of PBL.

(B) Daily weight gain per gram of PBL.

(C) Daily biomass weight loss ratio per gram of PBL.

(D) The germination index of AASMS and frass. UF-CK and F-CK, unfermented and fermented AASMS with natural MF; UF-CM and F-CM, unfermented and fermented AASMS with MF from chicken manure; UF-FVSMS-F and F-FVSMS-F, unfermented and fermented AASMS with MF from PBL frass produced by feeding *Flammulina velutipes* SMS; UF-CS-F and F-CS-F, unfermented and fermented AASMS with MF from PBL frass produced by feeding corn straw.

FVSMS-F and CS-F can decompose AASMS biomass better than that from CM and may have greater potential to break down unpalatable and indigestible biomolecules in AASMS, thereby improving palatability and digestibility.

After foam box small pile fermentation, the effect of SSF on palatability and digestibility was assessed by feeding experiments, and the PBL feed intake data were used to characterize palatability. As shown in Figure 2, after SSF, all treatments inoculated with artificial MF showed significantly increased feed intake (Figure 2A), indicating an improvement in palatability. Among the treatments with improved palatability, the samples fermented with FVSMS-F and CS-F MF showed better palatability, and the feed intake for these treatments was significantly higher than that for the CM treatment. The data showed that there was no significant increase in feed intake compared with the unfermented treatments, and the addition of CM led to reduced palatability. Thus, the data suggested that the increases in PBL feed intake were not owing to the presence of preferred flavors in the supplements but rather owing to the microorganisms present in the supplements that degraded the biomolecules that reduce the palatability of AASMS during SSF (Yang et al., 2021). Animals have evolved symbiotic MF that help them digest food, e.g., the symbiotic MF inhabiting the termite gut allows them to digest cellulose (Brune and Dietrich 2015), and the frass or feces contain a similar MF as the digestive tract (Xuan et al., 2022) that can efficiently break down the biomolecules in feed. In ecosystems, as a decomposer, PBLs can consume decaying biomass containing a wide variety of biomolecules and microbial metabolites, suggesting that PBLs have evolved a symbiotic digestive tract MF that can break down those molecules. In this investigation, the MF from PBL frass was much better at improving the palatability of AASMS than that from CM, probably because the MF in FVSMS-F and CS-F could decompose molecules responsible for the poor palatability of AASMS.

Then, the biomass weight loss ratio (BWLR) during digestion was calculated for further AASMS digestibility analysis. The data showed that all the fermented treatments (including the CK treatment) had a higher

BWLR than the corresponding unfermented treatments (Figure 2C). It is believed that the increase in temperature during SSF may enhance the activity of the MF as well as the produced enzymes, which makes the substances in AASMS easier to hydrolyze in the PBL digestive tract. At the same time, the weight gain was determined to investigate whether the digested AASMS could be converted to nutrients and be absorbed by PBLs. The results showed that SSF significantly improved the weight gain in the FVSMS-F, CS-F, and CK treatments but not the CM treatment (Figure 2B). This result indicated that the enhanced biomass decomposition in the F-CM treatment did not lead to the production of more absorbable nutrients for PBLs. During SSF, in the F-FVSMS-F, F-CS-F and F-CK treatments, MF with lignocellulose, mushroom mycelium and metabolite decomposition abilities exhibited selective proliferation, helping PBLs digest AASMS and produce more absorbable nutrients. However, owing to chicken digestibility issues, CM contains much more starch and cross protein (Sell-Kubiak et al., 2017), and the CM MF also selectively proliferates to decompose these ingredients during SSF, so the CM MF does not help PBLs digest AASMS but may compete with PBLs for nutrients. Therefore, the added CM only led to a BWLR improvement but was not beneficial for PBL weight gain. In addition, compared with CS-F, FVSMS-F MF was more beneficial for PBL weight gain, and it could even significantly improve PBL weight gain without SSF. It is possible that both the FVSMS and AASMS contain similar components, such as mushroom mycelia and metabolites. PBL enriches an MF that can decompose these components during the feeding and digestion of the FVSMS, and the FVSMS-F MF was more efficient in decomposing the AASMS biomass than CS-F.

Phytotoxicity is the adverse effect of an organic substance on plant growth and seed germination, and PBL digestion has been reported to effectively reduce phytotoxicity (Li et al., 2019). Previously obtained data have shown that AASMS not only has poor palatability for PBLs but also cannot be easily removed by PBLs via digestion (Wei et al., 2020). The above data indicated that FVSMS-F and CS-F MF with SSF can improve the breakdown of biomolecules in AASMS, suggesting that they also have the potential to help PBLs digest phytotoxic molecules in AASMS and reduce phytotoxicity. Therefore, we assessed the GI (Zhao et al., 2013) to evaluate the phytotoxicity of the produced frass. The GI is a sensitive parameter for evaluating phytotoxicity; when composting, a GI greater than 80% indicates that the compost is completely mature and phytotoxin free (Zucconi 1981). In this investigation, the data indicated that the fermentation of AASMS with FVSMS-F and CS-F MF could effectively remove phytotoxins with a GI greater than 80% (Figure 2D). In particular, the frass from the F-FVSMS-F treatment had the greatest GI (98.23%). Thus, based on a combination of the feed intake, BWLR, weight gain, and GI data, we hypothesize that the FVSMS-F MF is the best MF for producing AASMS SSF feed for PBLs.

Evaluation of the screened microflora for unsterilized coarse *Auricularia auricula* spent mushroom substrate solid-state fermentation

In the actual production process, it is difficult to pulverize AASMS into fine particles (40-mesh) and sterilize them before SSF. Therefore, we performed SSF of unsterilized AASMS with relatively coarse particles (10-mesh) using the screened FVSMS-F MF. Compared with the control treatment (unfermented, no MF added), SSF with FVSMS-F MF could significantly improve the palatability of AASMS, and the daily feed intake increased from 0.413 g/g larvae ($n = 3$, $SD = 0.022$) to 0.526 g/g larvae ($n = 3$, $SD = 0.052$). At the same time, the growth of PBLs fed fermented AASMS also improved significantly. Compared with the control, the daily weight gain of PBL increased from 0.015 g/g larvae ($n = 3$, $SD = 0.003$) to 0.051 g/g larvae ($n = 3$, $SD = 0.003$). These results show that both fine particle-sterilized AASMS and coarse unsterilized AASMS can be used for SSF to improve palatability and nutrient absorption.

Then, the phytotoxicities of the produced frass were assessed. Similar to reported data (Wei et al., 2020), PBL digestion reduced the phytotoxicity in the control treatment (unfermented, no artificial MF added). When AASMS was transformed to frass, the GI increased from 45.79% ($n = 3$, $SD = 2.04\%$) to 56.04% ($n = 3$, $SD = 3.71\%$). When AASMS was fermented with natural MF (fermented, no artificial MF added), after PBL conversion, the GI of the resulting frass was further increased to 63.43% ($n = 3$, $SD = 4.44\%$). When AASMS was fermented with FVSMS-F MF, the GI of the produced frass further increased and reached 72.83% ($n = 3$, $SD = 3.04\%$), but this was not as high as that of the fine particle sterilization treatment (Figure 2D). This result indicated that particle size and sterilization can affect the phytotoxic compound removal efficiency. The lower phytotoxic compound removal efficiency in the coarse unsterilized AASMS treatment was most likely caused by particle size. During SSF, inoculated bacteria always act on particle surface biomolecules. For the same amount of biomass, the particle surface area decreases as the particle size increases, and the number of accessible biomolecules decreases. Therefore, the frass produced from the

larger particle treatments may contain more phytotoxic molecules, resulting in a lower GI. However, the particle size will not affect the palatability of SSF feed. The palatability is mainly impacted by the molecules on the surface of the particles, and in this investigation, the molecules on the particle surface may have been decomposed by the MF used for inoculation, regardless of particle size. Thus, the PBLs had similar feed intakes but different phytotoxic compound removal efficiencies in the two different particle size treatments.

In nature, HAs are important components of soil organic matter that mainly originate from plant residues and microbial cells via a chemical and biological degradation process (Hayes et al., 1997), have a strong ion exchange capacity and adsorption capacity, and are widely used in the fields of environmental protection and agricultural production. HAs can stimulate the activity of microorganisms and related enzymes in the soil and affect the nitrogen cycle by influencing the distribution, bioavailability, and ultimate fate of organic nitrogen (Dong et al., 2009), thus improving the growth environment of microorganisms, promoting plant growth and enhancing soil fertility (Guo et al., 2019). Therefore, the HA content can be used as an important indicator for evaluating organic fertilizers. In this study, we also evaluated the effect of the screened MF on the production of HA. When unfermented AASMS was transformed to frass by PBLs, the HA content increased from 7.53% (n = 3, SD = 1.37%) to 15.38% (n = 3, SD = 1.99%). When AASMS was fermented with the screened FVSMS-F MF, the HA content in the frass further increased to 20.65% (n = 3, SD = 1.13%). The improvement in HA production was likely caused by the FVSMS-F MF because the control treatment of AASMS fermented with natural MF (no artificial MF added) could not increase the HA content (14.27%, n = 3, SD = 2.05%).

Microbial community analysis

FVSMS-F MF fermentation improved the palatability of AASMS for PBLs, solving the key problem in AASMS feed utilization. To identify the possible functional bacteria present in FVSMS-F MF for AASMS fermentation, we profiled the bacterial communities associated with the samples by PacBio sequencing of full-length 16S rRNA genes. After sequencing and quality control, approximately 26,000 reads of each sample were used for analysis, and the reads were submitted to the Sequence Read Archive (SRA) of GenBank (Table 1). After grouping high-quality tags at 97% sequence identity, a total of 2557 amplicon sequence variants (ASVs) were produced. It should be noted that, except for sample B2, the unfermented AASMS samples had fewer effective tags than the other treatments. Sample B was the unfermented AASMS sample and contained a large amount of mycelia that contained mitochondria, leading to the presence of a large number of mitochondrial 16S rRNA gene reads in the sequencing data. These mitochondrial tags were abandoned when subsequent bacterial community analysis was performed. The Shannon₂ index-based rarefaction curve showed that all samples tended to be flat when the 2500 effective tags of each sample were used, indicating that the sequencing depth was sufficient to represent sample diversity (Figure 3A). Subsequent beta diversity analysis showed that fermentation significantly changed the AASMS bacterial communities, suggesting the potential to discover functional bacteria by comparing the communities (Figure 3B).

The annotation showed that only 228 of 2557 ASVs could be assigned to species, which indicated that most of the bacteria in the MF were new species. Therefore, we directly used ASV data to predict the possible functional bacteria before taxonomic analysis. After abundance determination, there were 175 ASVs with significantly higher abundances in fermented AASMS than in unfermented AASMS, and the 175 ASVs constituted 60.50% of the abundance of the bacterial communities in fermented AASMS. The data suggested that the bacteria corresponding to the 175 ASVs were able to utilize AASMS and proliferate during fermentation and become dominant in the fermented samples. To identify the possible functional bacteria in FVSMS-F MF, we focused on the ASVs that were present in the FVSMS-F samples. Finally, we screened 54 out of the 175 ASVs that were confirmed to be present in the FVSMS-F samples but not present (47 ASVs) or extremely rare (seven ASVs) in the unfermented AASMS samples. The 54 ASVs were dominant and accumulated an abundance of 36.00% in fermented AASMS samples, indicating that these bacteria were able to utilize AASMS and proliferate during fermentation, possibly including the functional bacteria in FVSMS-F MF.

Xanthomonadaceae was noteworthy because it was the predominant family in the fermented AASMS samples, especially the Xanthomonadaceae bacteria with ASV abundances higher than 1.00% (Figure 3C). ASV5 (mean = 5.62%, SD = 0.94%), ASV6 (mean = 5.47%, SD = 3.72%), and ASV29 (mean = 1.63%, SD = 0.76%) were assigned to *Pseudoxanthomonas*, and the corresponding bacteria most likely play an

Table 1. Summary of microbial community analysis data

Samples	SRA Acc. No.	Clean Reads	Effective Tag	ASV
A-1	SRR18710308	26,012	7029	226
A-2	SRR18710307	26,566	6444	246
A-3	SRR18710296	26,756	6852	150
A-4	SRR18710292	26,469	5299	169
A-5	SRR18710291	26,340	8015	264
A-6	SRR18710290	26,038	7822	328
B-1	SRR18710289	26,516	4847	84
B-2	SRR18710288	26,697	13,915	692
B-3	SRR18710287	26,590	4706	196
B-4	SRR18710286	26,850	5683	156
B-5	SRR18710306	26,692	3401	99
B-6	SRR18710305	26,481	2801	131
C-1	SRR18710304	26,189	16,280	768
C-2	SRR18710303	26,546	8477	401
C-3	SRR18710302	26,684	20,678	1036
C-4	SRR18710301	26,846	12,317	647
C-5	SRR18710300	26,132	9733	367
D-1	SRR18710299	26,820	13,694	195
D-2	SRR18710298	26,528	14,072	282
D-3	SRR18710297	26,449	12,313	269
D-4	SRR18710295	26,128	16,436	517
D-5	SRR18710294	26,646	12,464	279
D-6	SRR18710293	26,105	11,733	325

Note: SRA Acc. No. is the Sequence Read Archive accession number in GenBank for the reads; Effective Tag is the number of high-quality reads produced after quality control and filtering of chimeric sequences used in the final ASV table.

important role in breaking down biomolecules in AASMS that cause poor palatability, as many *Pseudoxanthomonas* species have been reported to have a strong ability to decompose a variety of organic molecules, including pesticides, aromatics, diesel oil and plastics (Young et al., 2007; Nopcharoenkul et al., 2013; Meng et al., 2015; Giri et al., 2016; Lee et al., 2017b; Lin et al., 2019; Sun et al., 2021; Yue et al., 2021). ASV2 (mean = 1.15%, SD = 0.40%) was assigned to *Luteimonas*, and *Luteimonas* species may also contribute to the decomposition of AASMS, as many *Luteimonas* species have been reported to decompose lignocellulose (Han et al., 2018; Lin et al., 2020). *Luteimonas* species not only rapidly proliferated during fermentation but were also more abundant in frass samples (Figure 3C). These data suggest that the gut environment is more favorable for *Luteimonas* species; therefore, *Luteimonas* species may contribute more to AASMS digestibility improvement.

At the ASV level, ASV3 (mean = 10.03%, SD = 2.29%), which was assigned to the genus *Moheibacter*, was the most abundant (Figure 3C). Previous research has shown that *Moheibacter* species usually colonize environments rich in organic matter (Zhang et al., 2014; Schauss et al., 2016; Liu et al., 2021; Yu et al., 2022); these data suggest that *Moheibacter* species can decompose and utilize organic matter. In this study, *Moheibacter* species were present in PBL frass samples, indicating that the species can colonize the PBL digestive tract and help PBLs digest organic matter. When the frass was inoculated into AASMS, *Moheibacter* rapidly proliferated during fermentation and became the most abundant genus, indicating that *Moheibacter* species have a strong ability to decompose and utilize AASMS, and *Moheibacter* is likely one of the main functional genera responsible for improving AASMS palatability and digestibility. In addition, ASV25 and ASV35, which are assigned to the family Rhizobiaceae and *Saccharibacteria* genera incertae sedis, are also worth studying further. Although there are few reports on the taxa involved in organic matter decomposition, in this study, the ASV25 and ASV35 abundances increased significantly during the fermentation process, and these became the dominant flora (Figure 3C), suggesting that the corresponding

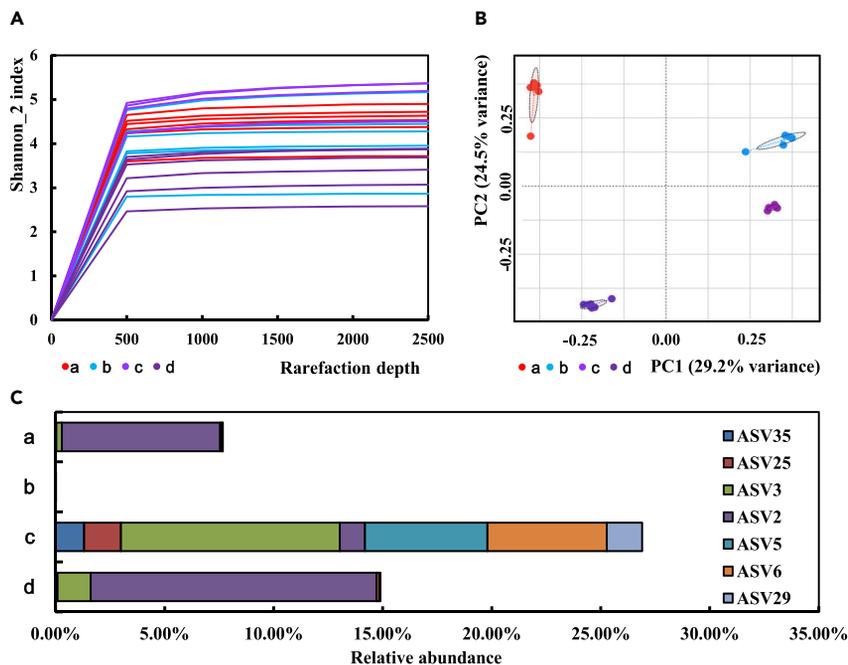


Figure 3. Diversity analysis of the bacterial community

(A) Shannon₂ index-based rarefaction curve.

(B) Principal coordinate analysis of the bacterial communities.

(C) Relative abundances of ASVs corresponding to major functional microorganisms (relative abundances higher than 1.00% in sample group c). Sample group a, PBL frass samples produced by feeding FVSMS; sample group b, unfermented AASMS samples; sample group c, AASMS samples fermented with MF from frass samples produced by feeding FVSMS; sample group d, PBL frass samples produced by feeding sample group c.

ASV25 and ASV35 species are able to decompose the molecules in AASMS and can also be used for AASMS fermentation.

Overall, by bacterial community analysis, some bacterial species in MF were predicted to be functional bacteria for AASMS fermentation, contributing to improved palatability and digestibility. A limitation of this work is that key functional bacteria in MF were not isolated and assessed. Considering that the quality of natural flora is not easy to control and the yield is not easy to guarantee, we propose that synthesizing artificial MF by mixing the key functional bacteria in isolation and then separately fermenting is a better strategy to produce quality-controlled applicable MF. It should be noted that the endogenous MF from insect frass should be safer than other sources. During PBL transformation, even if the MF is not artificially added, the microorganisms in the frass will still be cloned in the organic matter (such as SMS) (Xuan et al., 2022), and the application of MF to SSF just changes the amount of time that the MF acts on AASMS.

In conclusion, with the aim of improving the palatability and digestibility of AASMS for PBLs, this investigation screened out an MF by comparing the fermentation performance as well as the effect on PBL feed intake, weight gain, and AASMS phytotoxic compound removal efficiency and evaluated the flora under simulated production conditions. In addition, by bacterial community analysis, the genera *Luteimonas*, *Moheibacter*, and *Pseudoxanthomonas* in FASMS-F MF were predicted to be functional bacteria for AASMS fermentation and to contribute to improvements in palatability and digestibility. Further work should include the isolation and evaluation of key bacteria in the MF, as well as studies on the production of a stable and effective MF for AASMS fermentation.

Limitations of the study

The isolation and functionally study of the key microorganisms from the MF have not been conducted at this stage.

The identification of the molecules in AASMS that cause bad palatability, digestibility, and frass phytotoxicity has not been conducted at this stage owing to the difficulty to extract and separate molecular components from AASMS samples.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHORS' CONTRIBUTIONS

CS and RL conceived and designed the experiments. BD conducted the experiments, analyzed and compiled data. HX and BD analyzed humic acid content in the PBL frass. CL and LG provided the insect and collected samples. BD, WX, RL, and CS wrote the article. JZ and CS oversaw these experiments and data analysis, JZ, WX, and CS edited the article. All authors read and approved the final article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Sodium hydroxide	Sinopharm Group Chemical Reagent, China	O0575
Hydrochloric acid	Sinopharm Group Chemical Reagent, China	10,011,008
Sulfuric acid	Sinopharm Group Chemical Reagent, China	10,021,618
Potassium dichromate	Sigma-Aldrich	207,802
Ferrous sulfate	Sigma-Aldrich	F8633
1,10-Phenanthroline Monohydrate	Sigma-Aldrich	P9375
Agarose	Tiagen	RT101
2×Trans-Start FastPfu Fly PCR Super-Mix	Trans-Gen Biotech, China	AS231-01
QIAquick Gel Extraction Kit	Qiagen	28,704
SMRTbell Template Prep Kit	PacBio	100-259-100
Experimental models: Organisms/strains		
Rapeseed	Fujian Xiaofu Seed Co. LTD	NA
<i>Protaetia brevitarsis</i>	Gongzhuling, Jilin Province	NA
Oligonucleotides		
27F,1492R	This paper	NA
Softwares and algorithms		
QIIME 2	Bolyen et al. (2019)	NA
USEARCH	Edgar 2010	NA
RDP 16S Training set v18 database	Cole et al. (2014)	NA
Other		
Qubit 2.0 Fluorometer	Thermo Scientific	Q32866
FEMTO Pulse system	Agilent	M5330AA
Gel imaging system	Bio-Rad	12,009,077

RESOURCE AVAILABILITY

Lead contact

All requests for additional information and resources should be directed to the Lead contact, Changlong Shu (shuchanglong@caas.cn).

Materials availability

This study did not generate new unique reagents.

Data availability

The datasets generated during the current study are available in the NCBI SRA repository under accession numbers provided in [Table 1](#).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects and rearing conditions

A *P. brevitarsis* population originating from Gongzhuling, Jilin, was used in this experiment ([Wang et al., 2019](#); [Wei et al., 2020](#)). The third instar PBL feeding experiment was carried out in a plastic box incubated at a relative humidity of 70%, a temperature of approximately 25°C, and a day/night ratio of 16 h:8 h. The AASMSs were obtained from Heilongjiang Huateng Biotechnology Co., Ltd., located in Jiamusi,

Heilongjiang. Chicken manure (CM) was collected from a chicken farm located in Cangzhou, Hebei. Corn straw and *F. velutipes* SMS (FVSMS) collected from Langfang, Hebei, were used to feed the PBLs and produce the corresponding frass, respectively. All the feed was crushed and passed through a 10-mesh or 40-mesh sieve before feeding to the PBLs.

METHOD DETAILS

AASMS small pile fermentation

Foam box small pile fermentation was employed to test the fermentation ability of each MF. Before fermentation, the AASMS was crushed, sterilized, dried and passed through a 40-mesh sieve, and one-tenth the amount (by weight) of frass or CM containing MF was dispersed and suspended in sterile water. Then, the AASMS was mixed with the frass or CM, and the moisture content was adjusted to 60%. Each mixture was put into a foam box (38 × 22 × 16 cm) and compacted with a thermometer sensor placed at the bottom to record the temperature.

Determination of insect feeding rate and weight gain

First, we determined the moisture content (MC). For each sample, the wet weight (WW) was determined. After drying at 80°C until the weight did not change, the dry weight (DW) was determined. Then, the MC (%) was calculated as $(WW-DW)/WW$. To determine the insect feeding rate, according to the MC, we calculated the weight of the fermented sample with a DW of 250g and adjusted the humidity to 60%. Then, 50 third instar PBLs were placed into the sample and fed for 2 days at room temperature (approximately 25°C). Then, the frass and remaining AASMS were separated and weighed. All the samples were collected and kept at -40°C for subsequent experiments. Before and after the feeding experiment, the PBL weights were determined to calculate weight gain.

Phytotoxicity assessment

Seed germination experiments using water extracts and rapeseed seeds were employed to assess the phytotoxicity. To perform the phytotoxicity assessment, all the samples were dried and crushed through a 100-mesh sieve. The crushed samples were suspended in distilled water at a ratio of 1:10 (m/v) and shaken for 2 h. Then, the water extracts were obtained by centrifugation at 12,000 rpm for 15 min (Wei et al., 2020). The seed germination experiment was carried out in a sterile Petri dish (9 cm in diameter) with a filter paper bed (with four layers of filter paper). The filter paper beds were soaked in 10 mL of extract or distilled water, and 15 rapeseed seeds were evenly placed and then incubated in the dark at 26°C for 3 days. The germination index (GI) for each sample was calculated using the following formula (Li et al., 2019).

$$GI = (ANT \times ALT) / (ANC \times ALC) \times 100\%$$

Where ANT is the average number of germinated seeds in the experimental group, ALT is the average root length of the experimental group, ANC is the average number of germinated seeds in the control group, and ALC is the average root length of the control group.

Humic acid (HA) separation and quantification

To perform the assessment, all the samples were dried and crushed through a 100-mesh sieve. After weighing, the samples were suspended in 0.1 M NaOH (Sinopharm Group Chemical Reagent, China) at a weight to volume ratio of 1:10 and shaken at 220 rpm and 37°C for 24 h. Then, the suspensions were centrifuged at 8000 rpm for 5 min to obtain the supernatant containing HA. Then, the extracted supernatant was acidified with 3 M HCl (Sinopharm Group Chemical Reagent, China) to pH 1.0 and allowed to stand at room temperature for 24 h to precipitate HA. After washing with sterile water, the precipitate was redissolved in 0.1 M NaOH and quantified by a modified Walkley & Black chromic acid wet oxidation method according to the Chinese National Standard (NY525-2012).

Bacterial community analysis

Total genomic DNA was extracted from AASMS and frass using the method described by (Du et al., 2022) and DNA concentration and purity were assessed with 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water. The specific primers 27F (5'-AGAGTTTGATCCTGGCT CAG-3') and 1492R (5'-GNTACCTTGTTACGACTT-3') with the barcode were used for PCR amplification of the distinct regions of 16S rRNA genes. PCR reactions were carried out with Trans-Start FastPfu DNA

Polymerase (Trans-Gen Biotech, China), each primer at 0.2 μ M, and about 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 90 s. Finally, an additional elongation was performed at 72°C for 5 min. Then, 5 μ L of PCR products were mixed with the same volume of 2X loading buffer (containing SYBR Green I) and electrophoresis was performed on 2% agarose gel. The resulting gel was subsequently analyzed by a gel imaging system (Bio-Rad, USA) and the PCR product content was estimated based on the fluorescence intensity. After PCR products were mixed in equidense ratios, the PCR product mixture was purified with a QIAquick Gel Extraction Kit (Qiagen, Germany). Finally, the 16S rRNA gene sequencing libraries were generated using a SMRTbell Template Prep Kit (PacBio, USA) following the manufacturer's recommendations. The generated library was quality controlled and quantitated on the Qubit 2.0 Fluorometer (Thermo Scientific, USA) and FEMTO Pulse system (Agilent, USA), respectively. Lastly, the qualified library was sequenced on the PacBio Sequel IIe systems (PacBio, USA).

Then, the DADA2 plugin of QIIME 2 ([Bolyen et al., 2019](#)) was used for error correction, quality control, filtering of chimeric sequences and creation of an amplicon sequence variant (ASV) table. ASVs were annotated by the USEARCH SINTAX algorithm (version 11.2.64) with the RDP 16S Training set v18 database ([Cole et al., 2014](#)). The confidence for classification was set to 0.8 as recommended, and ASVs annotated as chloroplast or mitochondria were abandoned. USEARCH (version 11.2.64) was used to calculate alpha diversity metrics and beta diversity between samples.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification was performed as described in the relevant [method details](#) sections above. Statistical differences among these group means was determined by one-way ANOVA test. The least significant difference (LSD) method was used for multiple comparisons. Values followed by the same letter are not statistically different according to the independent t-test at $P < 0.05$.