

# Gut Microbiota of *Ostrinia Nubilalis* Larvae Degrade Maize Cellulose

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## Research

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# Abstract

## Background

While most insects rely on gut bacteria to digest cellulose and produce sugars or fatty acids that are then available to the host, this has been disputed in Lepidopteran larvae due to their simple gut morphology and rapid digestive throughput. The European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is a devastating pest that feeds the lignocellulose-rich tissues of maize plants. However, the potential role of ECB gut microbes in degrading maize cellulose remains unexplored. Here, we investigate the gut microbiota of ECB fed with different diets and the potential function of their gut bacteria in maize lignocellulose degradation.

## Results

The diversity and composition of gut bacterial communities varied dramatically between the ECB larva fed with artificial diets (ECB-D) and maize plants (ECB-M). Draft genomes of the bacterial isolates from ECB-D and ECB-M show that the principal degraders of cellulose mainly belonged to Firmicutes or Proteobacteria and were primarily found in the midgut. The bacterial isolates contained genes encoding various carbohydrate-active enzymes (CAZyme). Furthermore, scanning electron microscopy (SEM) revealed significant breakdown of lignocellulose in maize treated by the two bacterial isolates for nine days *in vitro*. Cellulose content in maize particles treated with BI-M were significantly lower than those treated with BI-D or the control (*Kruskal–Wallis* test:  $X^2 = 6.72$ ,  $df = 2$ ;  $P = 0.0259$ ). Metabolomic analyses reveal that maize particles treated by two bacterial isolates generate distinctive metabolomic profiles, with enrichment for different monosaccharides and amino acids.

## Conclusion

The results indicated that the diet of the host impacts the composition and the function of its gut microbiota, and that ECB exploits specific gut microbes to digest maize lignocellulose with distinctive products. Our study provides valuable microbiota resources for lignocellulose bioconversion.

## Background

Cellulose is a renewable and abundant biomass resource widely distributed in all higher plants [1, 2]. The long chains of cellulose polymers are linked together to shape into microfibrils arranged in uniform forming a crystalline structure that cause cellulose less susceptible for biodegradation [3]. Cellulose degradation is the most critical step for its utilization, but current methods (e.g. physical and chemical) employed for processing cellulose are high in cost and cause environmental pollution [4,5]. Many phytophagous insects, such as termites, beetles, and wasps, possess efficient microscale bioconversion systems of cellulose in their bodies [6-9]. Gut microbiota that produce cellulases to digest lignocellulose into sugars or fatty acids provide a major energy source to these species. These systems can contribute valuable information about utilizing plant cellulosic biomass as a sustainable energy source [10].

While the vast majority of Lepidoptera are also herbivores, their gut microbes show relatively low diversity and can be impacted by environmental factors compared to other phytophagous insects [11-13]. It has been previously demonstrated that many caterpillars carry diverse gut bacteria with essential functions for the host [14, 15], but considering their simple gut morphology and rapid digestive throughput, it remains disputed whether microbes persist in the host gut and contribute to host phytophagy [16]. For example, a recent study showed that wild leaf-feeding caterpillars lack a resident symbiotic gut microbiome [17]. Furthermore, although many caterpillars consume cellulose-rich plants (e.g. maize and rice), the composition and role of gut microbiota in their feeding and digestion remain unclear. The European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), is a devastating pest that feeds on the leaves and stalks of maize, which contain polysaccharides including cellulose, hemicellulose and lignin [18, 19]. Like other insects thriving on a cellulose-rich diet, ECB host a diverse bacterial community in their gut [14], of which some produce cellulases [20]. However, the role of gut bacteria in their digestion of cellulose has received little attention. Although no convincing evidence of maize cellulose digestion through the gut microbiota of ECB has been reported, developments in next-generation sequencing technologies provide opportunities to better characterize microbiome and its associated genomic resources in non-model organisms.

While most insects rely on gut bacteria to digest cellulose and produce sugars or fatty acids that are then available to the host, this has been disputed in Lepidopteran larvae due to their simple gut morphology and rapid digestive throughput. In this study, we survey the compositions and potential functions of gut microbes in ECB larvae fed with different diets with high-throughput sequencing of the 16S rRNA gene. We isolate and identify two cellulose-degrading bacteria strains from the gut of ECB larvae that were fed artificial diets and maize plants respectively. We also analyze draft genomes of the two bacterial isolates and identify genes encoding the carbohydrate active enzymes (CAZymes) including glycoside hydrolase (GH), polysaccharide lyase (PL), carbohydrate esterase (CE), glycosyl transferase (GT), and carbohydrate-binding module (CBM). Finally, we estimate the lignocellulose degradation efficiencies of the two bacterial isolates *in vitro* and the metabolomic profiles of maize straw after being exposed each strain. This study highlights the importance of the gut microbiota in host ecology and evolution and provides valuable microbiota resources for cellulose bioconversion.

## Results

### Gut microbiota of ECB larvae is determined by diet

The 16S rRNA gene sequencing analysis produced a total of 350225 quality-filtered sequences from 12 samples with an average of 29185 reads, and the 61 OTUs obtained were then clustered with 97% sequence identity. We subsequently classified the OTUs into 34 genera, 30 families, 24 orders, 13 classes and 9 phyla. Most of the sequences from the bacterial communities associated with the gut of larval ECB belonged to Firmicutes (50%), followed by Proteobacteria (31%), Bacteroidetes (5%), Patescibacteria (5%). The bacterial community composition was visibly different between ECB-M and ECB-D (Fig. 1a-c). At the phylum level, Firmicutes was dominant in ECB-D with 96.7% relative abundance, whereas those from

ECB-M were split among Proteobacteria (62.1%), Bacteroidetes (10.7%), Patescibacteria (8.5%), Verrucomicrobia (5.4%), and Actinobacteria (4.6%; Fig. 1a). LEfSe showed that nine OTUs were significantly different between ECB-M and ECB-D, of which eight were more abundant and one less abundant in ECB-D (Fig. 1c). The  $\beta$ -diversity of the microbiota in ECB-M, indicated by the Shannon index, was significantly higher than that of ECB-D ( $t = 35.35$ ,  $P < 0.0001$ ; Fig. 1d, Table S2). The PCoA score plot showed an obvious separation between the two groups (PERMANOVA:  $F = 239.26$ ,  $R^2 = 0.95988$ ,  $P < 0.003$ ; Fig. 1e).

Functional capacity analysis of the gut microbial communities revealed that 29 pathways enriched in each gut were similar among two groups and primarily associated with metabolism (Fig. 1f). The ECB-associated bacterial symbionts contain genes involved in carbohydrate metabolism and amino acid metabolism. Genes involved in carbohydrate metabolism were significantly different between ECB-M and ECB-D ( $t = 30.13$ ,  $P < 0.0001$ ; Fig. 1f, Table S3). The results indicated that host diet may alter the gut microbiota of ECB larvae and its associated functions.

### **Cellulose degrading bacteria in the larva gut of ECB**

The two cellulolytic strains (BI-M and BI-D) produced clear zones around the colonies after Congo red staining (Fig. S1). The genomic features of the two bacterial isolates are as shown in Table S4. Sequencing of the two isolates showed that *Klebsiella* (13.37%), *Streptococcus* (13.1%), and *Enterococcus* (5.19%) were the dominant genera in BI-M (Fig. 2a, Table S5), while *Bacillus* (26.53%), *Enterococcus* (18.42%), and *Enterobacter* (22.57%) were high in proportion in BI-D (Fig. 2b, Table S5). These bacterial genera were mainly located in the midgut of ECB larva (Fig. 3).

A total of 781 CAZyme genes were detected from BI-M bacteria, which can be broken down into 344 GHs, 212 GTs, 75 CBMs, 109 CEs, 13 PLs and 28 AAs. In comparison, 599 genes, including 235 GHs, 159 GTs, 63 CBMs, 110 CEs, 10 PLs and 22 AAs were found in BI-D (Fig. 2c, Table S6). Within the most abundant CAZyme category (GHs), 48 families were identified in BI-M, mainly GH1 ( $\beta$ -glucosidase), GH2 ( $\beta$ -galactosidase,  $\beta$ -mannosidase, and  $\beta$ -glucuronidase), and GH13 ( $\alpha$ -amylase, and  $\alpha$ -glucosidase), whereas BI-D harboured 49 GHs, including GH18 (chitinase, and endo- $\beta$ -N-acetylglucosaminidase) (Fig. 2c; Table 1). These results suggest that bacterial isolates from the gut of larval ECB fed with artificial diets and maize exhibit variation in their GH repertoires.

### **Degradation maize cellulose by the microbiota *in vitro***

SEM analysis showed that the surfaces of the untreated maize particles were smooth (Fig. 4a), whereas the structure of maize particles treated with both BI-D and BI-M was destroyed (Fig. 4b, c). Cellulose content in maize particles treated with BI-M were significantly lower than those treated with BI-D or the control (*Kruskal–Wallis* test:  $\chi^2 = 6.72$ ,  $df = 2$ ;  $P = 0.0259$ ; Fig. 4e). Meanwhile, lignin, cellulose and hemicellulose were all reduced on maize particles treated with BI-D and BI-M compared to untreated ones (Fig. 4d-f). The cellulolytic activity of endoglucanase,  $\beta$ -glucosidase, and exoglucanase in the cultures of maize particles treated with BI-D and BI-M were higher than those in untreated maize particles, albeit not

significantly (Fig. 4g-i). Taken together, the results suggested that the bacterial isolates BI-D and BI-M exhibited the ability of maize cellulose degradation *in vitro* to different extents.

### Metabolomic profiles of maize cellulose *in vitro*

Untargeted metabolome analyses document that maize particles treated by two bacterial isolates generate distinctive metabolomic profiles, with enrichment for specific monosaccharides and amino acids (Fig. 5). The principal component analysis (PCA) shows the difference in metabolic profiles of maize treated by the bacterial isolates (Fig. 5a, b). The major axes of the PCoA explain 58.6% of the variance for positive ionization mode in metabolomic profiles (PC1= 37.7% and PC2=20.9%; Fig. 5a), and 84.0% of the variance (PC1= 70.3% and PC2=13.7%) for negative ionization mode (Fig. 5b).

For both ionization modes, a comparison of relative metabolite levels with a one-way ANOVA showed that 38.9% (210 of 540) of the monitored metabolites varied significantly ( $P < 0.05$ ) among three samples (Table S7). The levels of D-fructose, cellobiose, D-maltose, L-rhamnofuranose and isomaltose in maize particles treated with both BI-D and BI-M were significantly higher than those in untreated maize particles (D-fructose:  $F = 10.146$ ,  $P < 0.01$ ; cellobiose:  $F = 19.358$ ,  $P < 0.001$ , D-maltose:  $F = 9.95$ ,  $P < 0.01$ ; L-rhamnofuranose:  $F = 7.931$ ,  $P < 0.01$ , and isomaltose:  $F = 10.702$ ,  $P < 0.01$ ; Fig. 5c, d; Table S7). Additionally, there were significant differences in levels of several amino acids among the metabolic profiles of maize particles treated or untreated by bacterial isolates, including ornithine ( $F = 23.38$ ,  $P < 0.001$ ), L-glutamic acid ( $F = 13.455$ ,  $P < 0.001$ ), L-lysine ( $F = 5.21$ ,  $P < 0.05$ ), L-isoleucine ( $F = 9.4291$ ,  $P < 0.01$ ), L-threonine ( $F = 17.93$ ,  $P < 0.001$ ), L-histidine ( $F = 40.595$ ,  $P < 0.0001$ ), and L-tryptophan ( $F = 43.127$ ,  $P < 0.0001$ ) (Fig. 5c, d; Table S7).

Several small molecular aromatic metabolites, such as phenol, 2-phenylethanol, coniferyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxyphenylacetaldehyde, p-anisic acid, vanillin, phenylacetic acid, phenylacetaldehyde, 2-aminobenzoic acid, mannitol, 4-nitrophenol, 4-hydroxyphenylpyruvic acid, syringic acid, sinapic acid, sinapyl alcohol, D-glucuronic acid, vanillic acid, D-arabitol, 3-hydroxybenzoic acid, D-xylitol, and L-lactic acid, were detected in the samples treated by both bacterial isolates (Fig. 5c, d; Table S7).

**Table 1. A partial list of CAZymes identified in the bacterial isolates from the ECB larvae fed with an artificial diet (BI-D) or maize plants (BI-M).**

GHs	Number of proteins		Known activities ( <a href="http://www.cazy.org">http://www.cazy.org</a> .)
	BI-M	BI-D	
GH1	22	39	$\beta$ -glucosidase, $\beta$ -galactosidase, $\beta$ -mannosidase, others
GH2	6	6	$\beta$ -galactosidase, $\beta$ -mannosidase, $\beta$ -glucuronidase, others
GH3	6	8	$\beta$ -glucosidase, 1,4- $\beta$ -xylosidase, 1,3- $\beta$ -glucosidase, 1,4- $\beta$ -glucosidase, others
GH4	9	16	$\alpha$ -glucosidase; $\alpha$ -galactosidase; $\alpha$ -glucuronidase, others
GH5	3	2	chitosanase, $\beta$ -mannosidase, cellulase, 1,3- $\beta$ -glucosidase, others
GH8	2	5	chitosanase, cellulase, licheninase, endo-1,4- $\beta$ -xylanase, others
GH13	38	49	$\alpha$ -amylase, $\alpha$ -glucosidase, pullulanase, cyclomaltodextrinase, others
GH15	0	1	glucoamylase, glucodextranase, $\alpha,\alpha$ -trehalase
GH16	0	3	endo-1,3- $\beta$ -glucanase, licheninase, xyloglucanase, others
GH18	10	3	chitinase, endo- $\beta$ -N-acetylglucosaminidase, others
GH19	1	1	chitinase
GH20	3	1	$\beta$ -hexosaminidase, $\beta$ -1,6-N-acetylglucosaminidase, others
GH23	12	20	lysozyme, peptidoglycan lyase
GH24	3	17	lysozyme
GH25	6	10	lysozyme
GH28	1	4	polygalacturonase, exo-polygalacturonase, others
GH31	5	5	$\alpha$ -glucosidase, $\alpha$ -1,3-glucosidase, $\alpha$ -xylosidase, $\alpha$ -glucan lyase, others
GH32	4	13	invertase, endo-inulinase, endo-levanase, others
GH33	2	0	sialidase, trans-sialidase, 2-keto-3-deoxynononic acid sialidase
GH35	2	1	$\beta$ -galactosidase, exo- $\beta$ -glucosaminidase
GH36	2	7	$\alpha$ -galactosidase, $\alpha$ -N-acetylgalactosaminidase, others
GH37	2	5	$\alpha,\alpha$ -trehalase
GH38	6	5	$\alpha$ -mannosidase, $\alpha$ -1,3-1,6-mannosidase, others
GH39	0	2	$\alpha$ -L-iduronidase, $\beta$ -xylosidase
GH42	0	1	$\beta$ -galactosidase
GH43	4	13	$\beta$ -xylosidase, $\beta$ -1,3-xylosidase, xylanase, 1,3- $\beta$ -galactosidase, others

GH51	3	4	$\alpha$ -L-arabinofuranosidase, endoglucanase
GH53	0	3	endo- $\beta$ -1,4-galactanase
GH63	1	1	$\alpha$ -1,3-glucosidase, $\alpha$ -glucosidase
GH65	7	6	$\alpha$ , $\alpha$ -trehalase, maltose phosphorylase, trehalose phosphorylase, others
GH70	0	2	dextranucrase, alternansucrase, others
GH73	15	20	peptidoglycan hydrolase
GH74	3	0	endoglucanase, xyloglucanase
GH77	2	6	amylomaltase, 4- $\alpha$ -glucanotransferase
GH78	2	6	$\alpha$ -L-rhamnosidase
GH80	1	0	chitosanase
GH85	1	0	endo- $\beta$ -N-acetylglucosaminidase
GH88	3	2	d-4,5 unsaturated $\beta$ -glucuronyl hydrolase
GH92	2	3	$\alpha$ -1,2-mannosidase, $\alpha$ -1,3-mannosidase
GH94	1	2	cellobiose phosphorylase, cellodextrin phosphorylase, others
GH101	1	0	endo- $\alpha$ -N-acetylgalactosaminidase
GH102	1	2	peptidoglycan lytic transglycosylase
GH103	1	2	peptidoglycan lytic transglycosylase
GH104	0	4	peptidoglycan lytic transglycosylase
GH105	2	4	unsaturated rhamnogalacturonyl hydrolase
GH108	0	1	N-acetylmuramidase
GH109	26	29	$\alpha$ -N-acetylgalactosaminidase
GH112	1	0	lacto-N-biose phosphorylase, D-galactosyl-1,4-L-rhamnose phosphorylase
GH113	1	0	$\beta$ -mannanase
GH114	1	1	endo- $\alpha$ -1,4-polygalactosaminidase
GH125	3	2	exo- $\alpha$ -1,6-mannosidase
GH126	2	1	amylase
GH127	0	3	L-arabinofuranosidase, 3-C-carboxy-5-deoxy-L-xylose hydrolase
GH129	1	0	N-acetylgalactosaminidase
GH136	1	0	lacto-N-biosidase

GH153	1	2	$\beta$ -1,6-D-glucosamine hydrolase
GH154	2	1	$\beta$ -glucuronidase
GH158	1	0	endo- $\beta$ -1,3-glucanase

Abbreviation: GH, glycoside hydrolase.

## Discussion

In this study, we document the bacterial communities in the gut of *O. nubilalis* larvae. Draft genome and metabolome analyses show that two bacteria isolates from hosts feeding on different diets have different capabilities to digest maize lignocellulose *in vitro*, and release different downstream products. SEM provides direct evidence for degradation of maize cellulose by the ECB gut bacteria isolates *in vitro* (Fig. 6).

### Diet shapes gut microbiota of the *O. nubilalis* larva

Diet is the primary determinant of bacterial community structures in the gut of many herbivorous lepidopteran larvae [21-23]. Our findings that Firmicutes are the dominant phylum in ECB fed on an artificial diet were similar to that of a previous study [14], suggesting that the dominant phyla are relatively stable in the guts of hosts on the same diet. We also show that the dominant phyla in the gut microbiota of ECB fed on maize are distinct from bacteria in ECB reared on pepper tissues [14]. These observations indicate that diet influences the proliferation and stability of gut microbiota in *O. nubilalis* larvae. In addition, the diversity of gut microbiota in ECB fed on maize was significantly higher than that in ECB fed an artificial diet, and include taxa widespread in other environments, such as *Pseudomonas* and *Bacillus* [24]. These bacteria might have been actively or passively acquired from microbial communities inhabiting plant surfaces. This indicates that these bacteria can establish in the host gut and are not merely transient associates. Overall, our findings raise the possibility that diet may exert a strong selection pressure and shape the composition of microbiota harboured in the insect intestinal tract. Although it has been speculated that microbes cannot persist in the gut of herbivorous Lepidopteran larvae or contribute to feeding and digestion [17], here we show that *O. nubilalis*-associated bacteria serve various functions for their host. Thus, it is likely that the degree of reliance on microbes of *O. nubilalis* is underappreciated.

### The diversity of cellulolytic bacteria in the gut of *O. nubilalis* larvae

Most dominant genera in both bacteria isolates obtained from the guts of ECB larvae have been shown to exhibit cellulolytic properties in other host species. For example, *Bacillus* in the gut of *Holotrichia parallela* [25], the wood-feeding termite [26], and *Cyrtotrachelus buqueti* [27] all exhibit the ability to degrade hemicellulose or cellulose. The genus *Enterococcus* showed cellulolytic activity in the gut of *Harpalus pensylvanicus* [28]. *Enterobacter* was isolated from the gut of termites (*Anacanthotermes vagans*) and had an ability to degrade lignin and lignocellulosic materials [29]. Several *Streptococcus* species are also

cellulolytic [30, 31]. However, previous studies of Lepidopteran larvae have typically observed cellulolytic bacteria from different taxa. For example, the strain *Klebsiella* sp. MD21 has been found responsible for cellulose digestion in the gut of the cotton bollworm (*Helicoverpa armigera*) [32]. In particular, a previous study also focusing on *O. nubilalis* suggests that *Micrococcus* and *Acinetobacter* are the main groups with cellulolytic properties [20], contrasting our findings. This can be interpreted as host genotype specificity.

Additionally, FISH analysis of the bacteria isolates showed that bacteria with cellulolytic capabilities were mainly located in the midgut of *O. nubilalis*, unlike other insect species where they are typically found in the hindgut [33, 34]. Knowing that differences in gut structure can lead to major functional differences in insects [35-37], our results suggest that the midgut of ECB is where digestion of maize cellulose occurs, although its occurrence *in vivo* is still undetermined.

### **The mechanism of cellulose degradation by bacteria isolates**

Lignocellulose degradation requires a wide spectrum of enzymes [38, 39]. Cellulases including endoglucanases, exoglycanases, and  $\beta$ -glucosidases, may participate in cellulose digestion. Likewise, xylanases and ligninases are enzymes mainly involved in the hydrolysis of hemicellulose and lignin respectively [40]. The gut bacteria of insects produce a diverse repertoire of CAZymes [41]. One family particularly abundant in the genome of bacteria isolates obtained in this study is GH, characterized by hydrolases that act on the glycosidic bonds [42]. Specifically, GH1 and GH43 have been shown to be involved in lignocellulose degradation [43]. Thus, GHs might be responsible for the degradation of maize cellulose *in vitro*. Additionally, CEs, PLs, and AAs also contribute to the decomposition of lignocellulose. For example, CE3, CE7, CE10, AA4 and AA7 were identified in two bacteria isolates reported to play a role in the degradation of lignocellulose [44, 45, 27]. These cellulolytic enzymes can also act synergistically and increase the overall efficiency of substrate utilization [46-48].

Additionally, digestive efficiency *in vitro* differs among bacteria. It can also depend on the metabolic priorities of the bacteria, cellulase activity, and environmental factors such as temperature and pH [10, 35]. In our results, the bacteria isolates exhibited relatively low digestive efficiency of maize *in vitro*; it is unclear which of the above hypotheses can explain this phenomenon. The structure and underlying mechanisms of gut bacteria associated CAZymes and the extent of these roles awaits further investigation.

### **Variation in the metabolome among degradation products of maize**

Products of lignocellulose degradation include many monosaccharides, aromatic compounds and others [49, 10]. Here, we find that reduced sugars such as D-fructose, cellobiose, D-maltose, L-rhamnofuranose and, isomaltose accumulated in maize particles treated by the two bacteria isolates compared to the control. In contrast, the contents of glucose and fructose decreased. Therefore, it is possible that the bacteria absorbed some of the released sugars.

Additionally, gut bacteria often provide amino acids to their host [50, 51]. In this study, we find that the levels of many amino acids (e.g. ornithine, L-glutamic acid, L-lysine, L-isoleucine, L-threonine, L-histidine and L-tryptophan) increase in the metabolic profiles of maize particles treated with bacteria compared to those that were untreated, suggesting that gut bacteria can produce not only sugars but also amino acids *in vitro*. However, due to differences in physicochemical conditions of the gut compartments such as pH, redox potential, and substrates, it remains to be experimentally validated whether gut bacteria can provide amino acids to benefit itself or its host *in vivo*.

### **Application potential of gut microbiota of ECB in cellulose bioconversion**

The potential application of insect gut microbiota in cellulose degradation can alleviate negative environmental impacts in current methods [52, 53]. However, industrialization of cellulolytic microbiota remains a challenge due to its low efficiency and high cost [7]. Here, we establish that bacterial isolates from ECB are capable of degrading maize cellulose with relatively low efficiency. This potential could be further extended through genetic engineering for efficiency.

## **Conclusions**

This study shows that diet shapes the gut microbial composition in *O. nubilalis*. The two bacteria isolates, mainly belonging to Firmicutes or Proteobacteria, exhibit the ability to degrade maize cellulose *in vitro*. These results offer valuable microbiota resources for lignocellulose bioconversion and have significant potential in industrial applications. Future research will analyze the enzymatic properties of novel bacteria or genes to clarify the mechanisms of cellulose digestion in insects. A thorough understanding of how cellulosic biomass is digested by gut microbiomes would not only solidify the importance of the gut microbiota in the ecology and evolution of insects, but also provide valuable insight towards industrial application of microbiota for cellulose conversion.

## **Methods**

### **Insect collection and maintenance**

Specimens of *O. nubilalis* were collected from maize plants (*Zea mays* L.) in Nanjing, China (N32°02', E118°52') in 2019. Larvae were reared at 25 ± 1°C under 70 % relative humidity and 15 h light: 9 h dark conditions. The larvae were split into two groups and fed separate diets: an artificial diet (designated as ECB-D), and maize plants (*Z. mays* strain Nannong3; designated as ECB-M).

### **Analysis of the ECB intestinal microbiota**

**DNA extraction, library construction and sequencing.** The guts of individual ECB were dissected in sterilized phosphate-buffered saline (PBS) and surface sterilized using 75% ethanol and sterile dH<sub>2</sub>O. Total DNA was extracted using the QIAGEN DNeasy Kit (Germany) according to the manufacturer's protocol. Amplicons were obtained using the universal primers 341F (5'-CCTAYGGGRBGCASCAG-3') and

806R (5'-GGACTACNNGGGTATCTAAT-3'), extracted from 2% agarose gels, and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, U.S.) and quantified using QuantiFluor™ -ST (Promega, U.S.). Purified PCR products were quantified with Qubit®3.0 (Life Invitrogen). Amplicons from DNA samples and the negative control were sequenced with an Illumina HiSeq 2500 platform (Shanghai BIOZERON Co., Ltd).

**Microbiota analysis.** Raw reads were filtered for quality, assembled, and clustered using UCHIME. Sequences were assigned to operational taxonomic units (OTUs) at 97% sequence similarity using UPARSE (version 7.1; <http://drive5.com/uparse/>). Taxonomic assignment of representative sequences was done using the RDP Classifier (<http://rdp.cme.msu.edu/>) against the silva (SSU132)16S rRNA database with a confidence threshold of 70%. To remove potential sequencing artefacts, taxa with < 0.1% abundance summed across all samples were removed. Alpha and beta diversity, as well as differential abundance were calculated in MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca/>; Dhariwal [54]). The metabolic potential of the microbiomes was predicted with PICRUSt. LEfSe was used to screen for significant differences in the inferred metabolic pathways.

### **Cellulose-degrading bacteria isolated and identified**

**Isolation and identification of cellulolytic bacteria.** The 50 guts dissected from ECB larvae were blended, homogenized, and filtered using 10 µm syringe filters. A 1 ml quantity of each bacterial suspension was immediately plated on Luria-Bertani (LB) media with 5 g/L carboxymethyl cellulose (CMC) agar and incubated at 30°C and 225 rpm for cellulolytic bacteria screening. Congo red dye was used to screen for cellulose-degrading bacteria, as described by Teather and Wood [55]. Two bacterial isolates were obtained, one from the gut microbiota of ECB larva fed with an artificial diet (designated as BI-D), and another from those fed with maize plants (BI-M) (Fig. S1).

**Genomic DNA extraction and genome sequencing.** Total genomic DNA was extracted using the Bacteria DNA Kit (OMEGA), and quality control was subsequently carried out on the purified DNA samples. Genomic DNA was quantified using the TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA), and DNA libraries were constructed under standard procedures. The qualified Illumina pair-end library would be used for Illumina NovaSeq 6000 sequencing (Shanghai BIOZERON Co., Ltd, Shanghai, China). Raw reads were trimmed and quality controlled by Trimmomatic (version 0.36 <http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic>). Filtered data were used for downstream analyses. We used *ab initio* prediction methods to obtain gene models for bacterial isolates. Gene models were identified using GeneMark.

**CAZyme assignment of genome sequences.** Protein sequences were analyzed using the dbCAN CAZyme annotation algorithm (<http://bcb.unl.edu/dbCAN2/blast.php>) with default parameters to determine the carbohydrate active enzymes (CAZymes) in the two bacterial isolates (BI-D and BI-M),

**Fluorescence *in situ* hybridization (FISH).** To investigate the localization of the dominant genera in the two bacteria isolates within the digestive tract of ECB larvae, FISH was performed with a genus-specific

probes (Table S1). Gut tissue samples were washed twice with PBS. Then, samples were fixed in 1 mL of 4% paraformaldehyde for 30 min, followed by two washes in 1 mL of PBS for 2 min. Hybridization was performed for 12 h at 42 °C using 1 mL of hybridization buffer (20 mM Tris-HCl pH 7.4, 0.02% sodium dodecyl sulphate (SDS), 0.9 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 60% formamide) and 0.002 mL of the probe. After hybridization, samples were washed twice at 46 °C for 30 min in 1 mL of hybridization wash buffer. Finally, samples were viewed under a ZEISS LSM 700 confocal microscope (Carl Zeiss, Germany). Figures were processed with PHOTOSHOP 4.0 software (Adobe Systems Inc., San Jose, CA, USA). A FISH reaction without a probe was performed as a negative control.

### **Maize degradation by bacterial isolates *in vitro***

**Experimental design.** Experiments were carried out on six independent biological replicates of each bacterial isolate. Maize straw was powdered and milled through a 0.5 mm screen. The two bacteria isolates (5 mL) were separately cultured in 100 mL of liquid medium at a PH of 8.0, with a temperature of 37°C and 200 rpm for 9 days, respectively. The culture medium comprised of maize straw 5 g/L, yeast extract 0.5 g/L, malt extract 0.5 g/L, tryptone 0.5 g/L, NaCl 0.5 g/L, KH<sub>2</sub>PO<sub>4</sub> 0.2 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.13 g/L and CaCl<sub>2</sub> 0.5 g/L. The negative control did not contain any bacteria isolates. The culture was centrifuged at 13,000 rpm for 10 min. The supernatant was sterilized and stored at -20°C for determination of cellulolytic activity and metabolomic analysis. Endoglucanase, β-glucosidase and exoglucanase activity were determined using the Solarbio assay kit (Solarbio, Beijing, China). The maize particles were collected and observed with scanning electron microscopy (SEM). The obtained deposit was dried and weighed to determine levels of cellulose, hemicellulose and lignin content as described by Van Soest [56].

**Scanning electron microscopy.** SEM was conducted as described by Karnovsky [57]. Maize particles were fixed overnight at 4 °C in 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) and 0.1 M sodium phosphate buffer (pH 7.2). Fixed samples were washed three times in 0.1 M sodium phosphate buffer (pH 7.2) and were stepwise dehydrated with 30%, 50%, 70%, 80%, 90% and 100% concentrations of ethanol, followed by a final treatment using 100% acetone. Then, specimens were critical point dried and coated with gold particles in the Technics Hummer VI Sputter Coat Unit (Anatech, USA). After gold-sputtering, the samples were observed under SEM (SU-8010, JEOL Ltd., Japan) at an acceleration voltage of 5.0 kV.

**Metabolomic analysis.** One mL of the supernatant from each sample was quickly sampled after centrifugation and stored at -80°C. Six biological replicates of each isolate were shipped on dry ice to Shanghai BIOZERON Co., Ltd (Shanghai, China) for metabolomic analysis. The metabolites were extracted in 80% methanol by vortexing for 10 min, centrifuged, and then the supernatant was filtered using a 0.22 μm membrane. One μL of each sample was loaded and analyzed using liquid chromatography-mass spectrometry (LC-MS). Non-targeted metabolite profiling was carried out on a Thermo Ultimate 3000 system equipped with an ACQUITY UPLC® HSS T3 (150×2.1 mm, 1.8 μm, Waters).

Statistical differences between samples were investigated with a one-way ANOVA, followed by Tukey's HSD post hoc test and FDR correction using MetaboAnalyst [58].

## Declarations

### Acknowledgements

Not applicable.

### Author contributions

JFL and TS conceived and designed the experiments; JFL and SFC performed the experiments; JFL and XXT analyzed the data; JFL and TS wrote the manuscript. All authors revised the manuscript and approved it for publication.

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### Data Availability

Illumina sequence data of gut microbiota of ECB are available on the NCBI Sequence Read Archive (SRA) under accession number [SRP265703](#); Draft genome sequences are available on the NCBI Sequence Read Archive (SRA) under accession number PRJNA636935.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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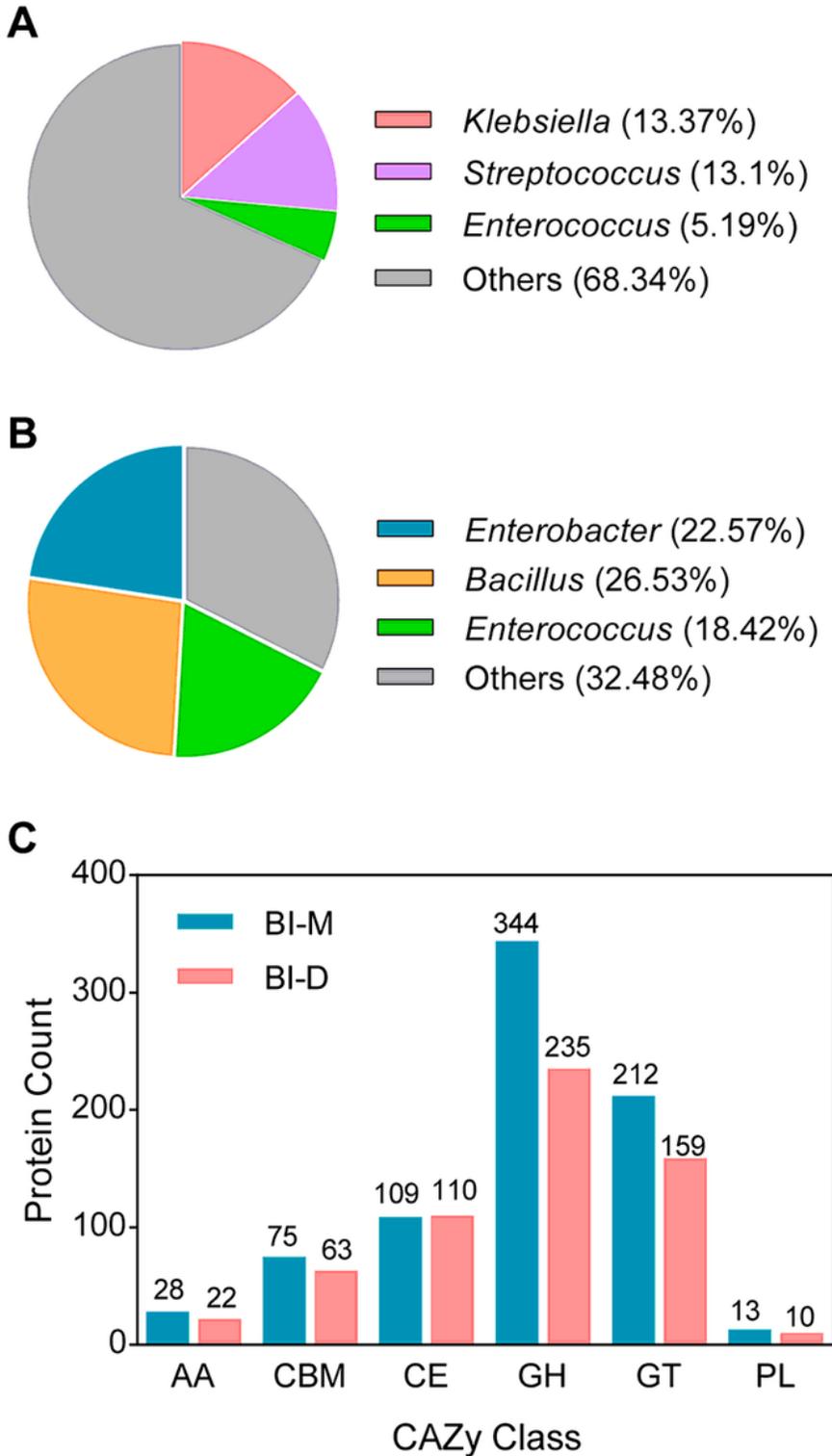
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## Figures



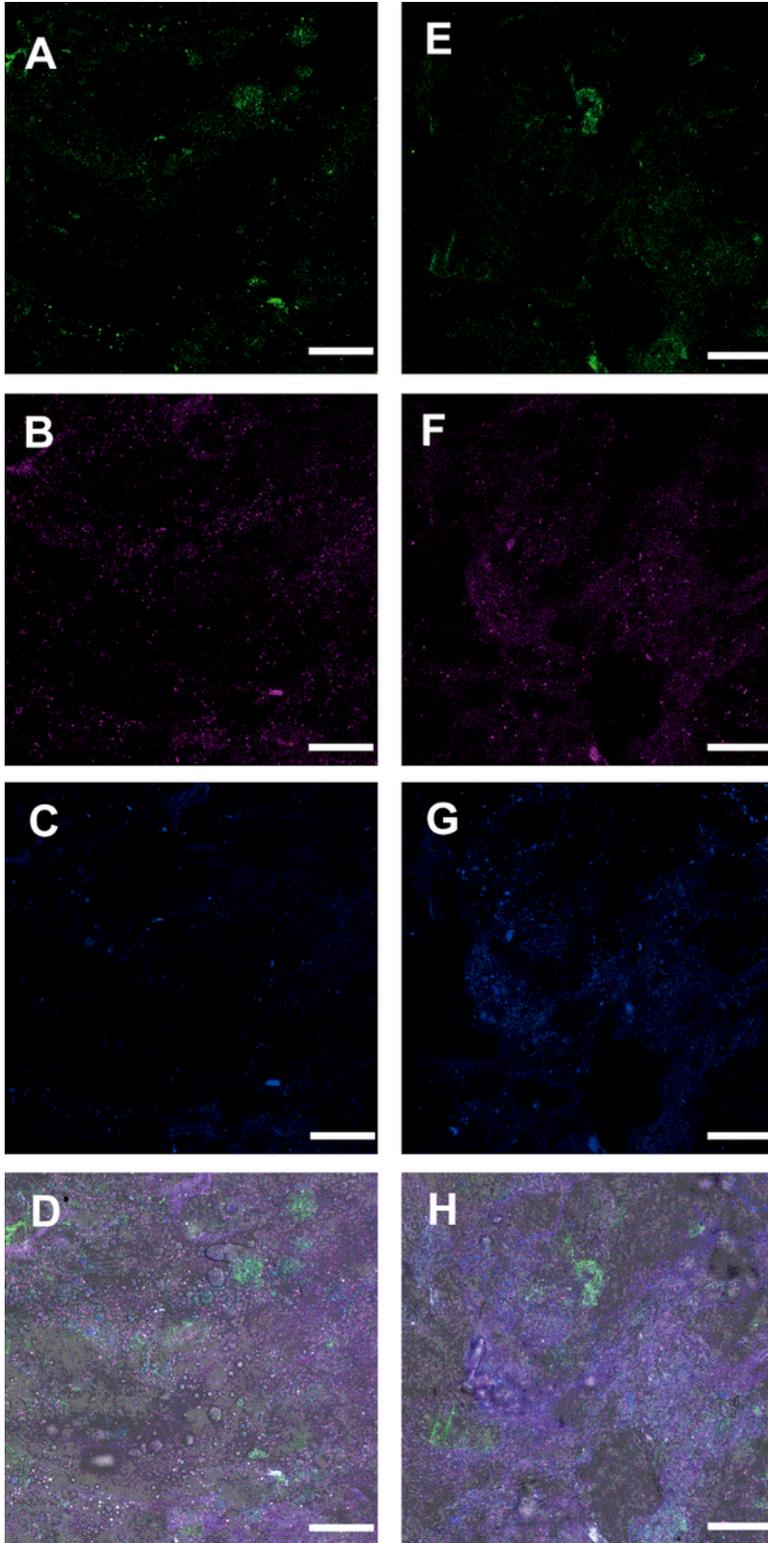
Heatmap showing the main function of microbiota present in the larval gut of ECB-D and ECB-M with different abundance.



**Figure 2**

Draft genomes of two bacterial isolates from the gut of ECB larvae. (A) and (B) represent the relative abundance of the dominant bacteria from the gut of ECB larvae fed with an artificial diet (BI-D) and

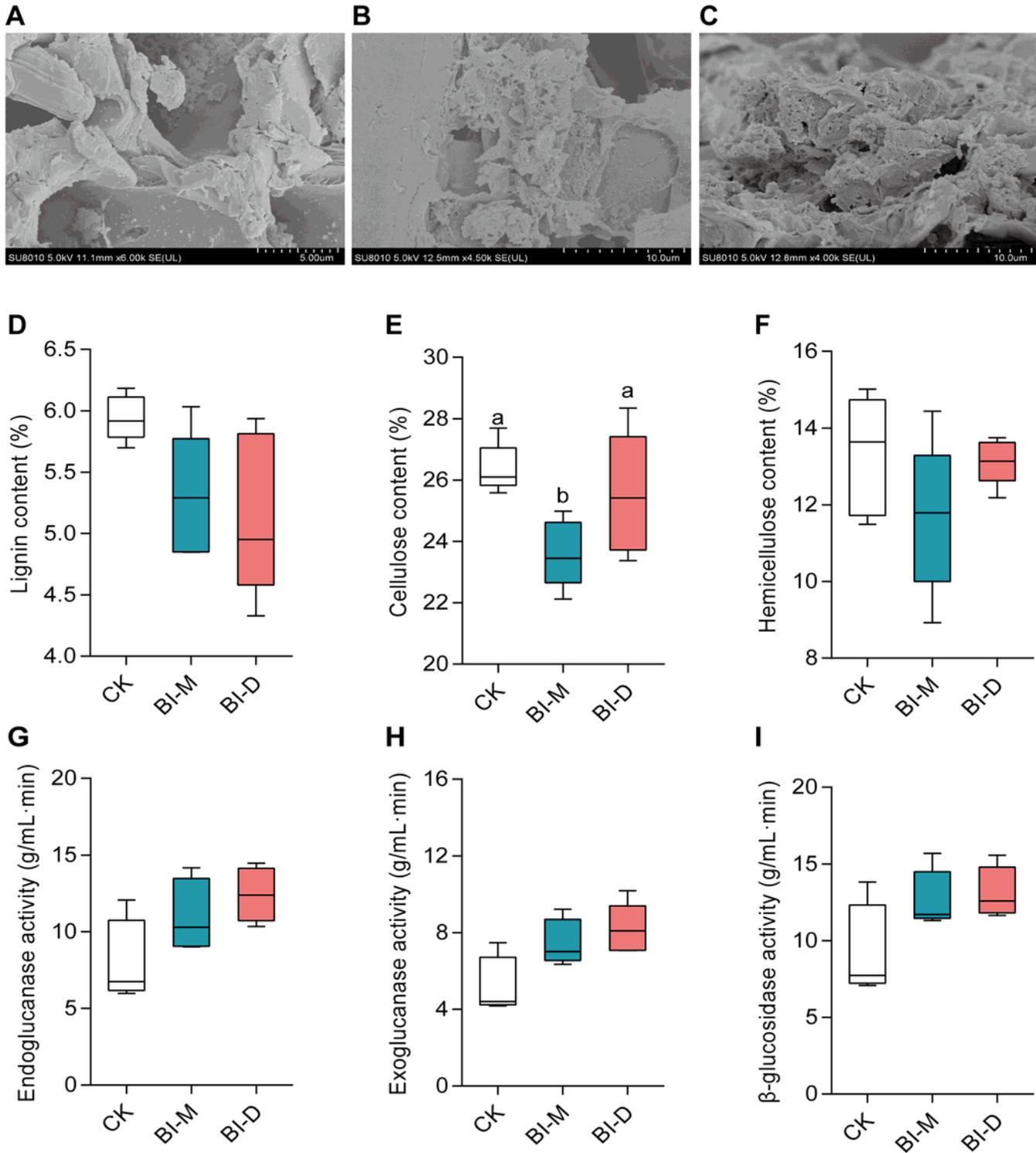
maize plants (BI-M) respectively. (C) The number of CAZyme genes defined in the draft genomes of BI-D and BI-M.



**Figure 3**

Fluorescence in situ hybridization analysis. FISH analysis of the localization of (A) *Streptococcus* (green), (B) *Klebsiella* (purple), (C) *Enterococcus* (blue), and (D) three bacteria in the larval midgut of ECB-M, and

(E) *Bacillus* (green), (F) *Enterobacter* (purple), (G) *Enterococcus* (blue), and (H) three bacteria in the larval midgut of ECB-D.



**Figure 4**

In vitro maize cellulose degradation by bacterial isolates BI-D and BI-M. Scanning electron micrographs of (A) untreated maize particles, (B) maize particles treated by BI-D for nine days, and (C) maize particles treated by BI-M for nine days. The content of (D) lignin, (E) cellulose and (F) hemicellulose in maize

particles after treated for nine days. The cellulose-associated enzyme activity of (G) endoglucanase, (H) exoglucanase, and (I)  $\beta$ -glucosidase in the culture medium of maize particles treated with BI-D and BI-M for nine days. Horizontal lines in the boxes represent group medians, and whiskers represent the 10th–90th percentiles. Superscripts (a, b) indicate significant differences between different groups ( $P < 0.05$ ).

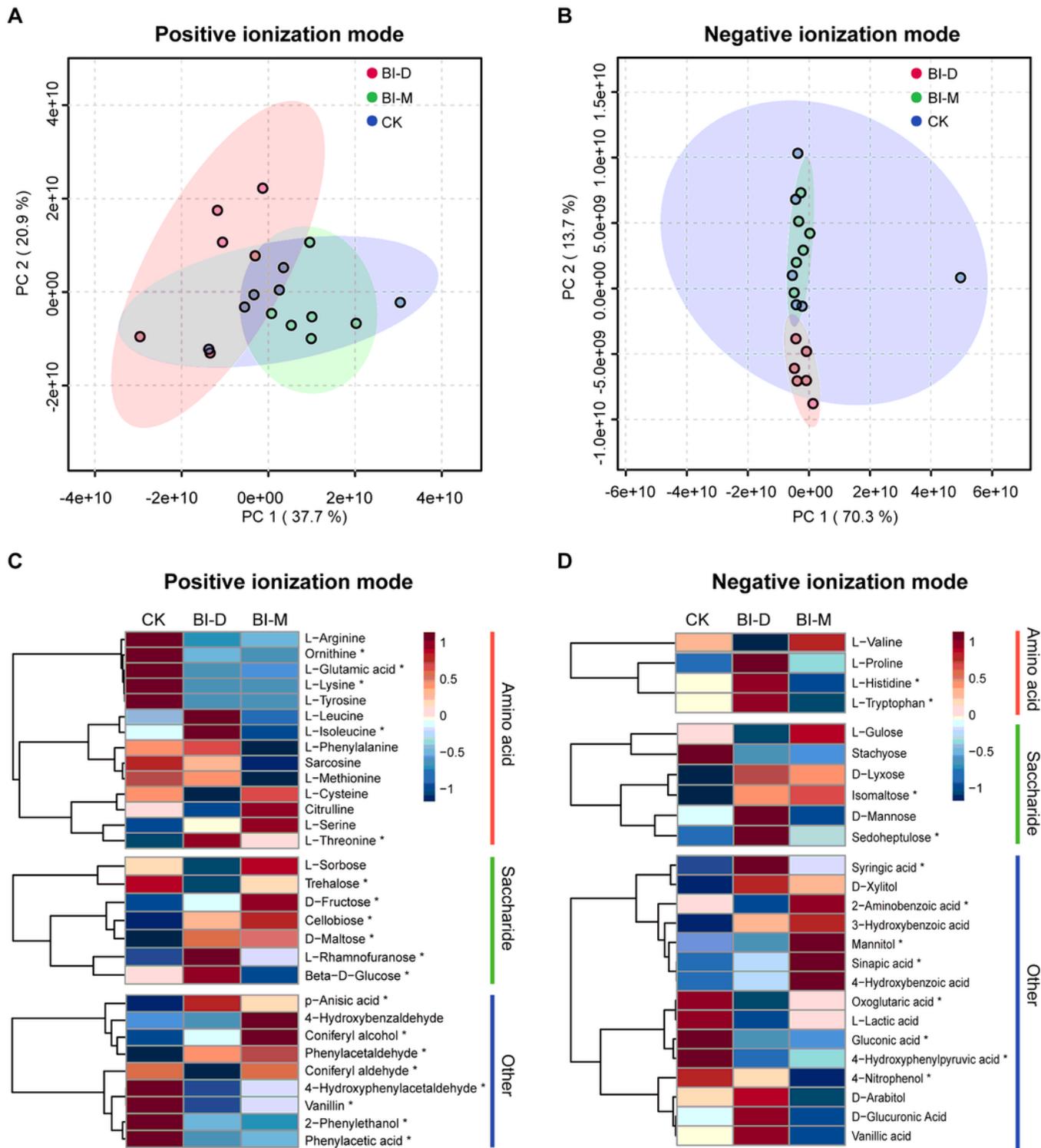
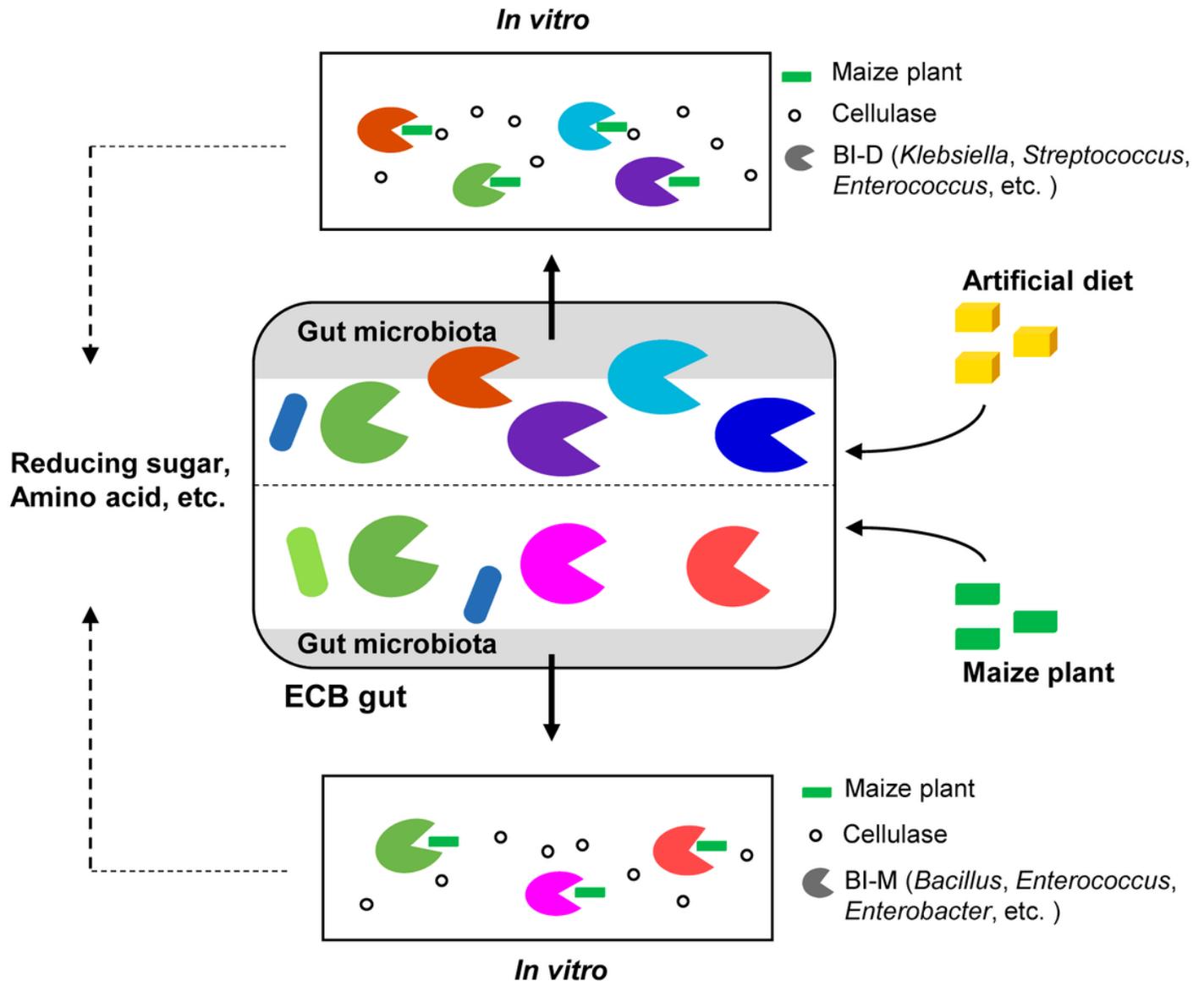


Figure 5

Metabolomic analysis of *in vitro* degradation of maize particles by two bacterial isolates. PCA score plot of metabolic profiles in (A) positive and (B) negative ionization modes. Colored circles represent the metabolic profiles of individual samples. Ellipses indicate 95% confidence region for each group. Relative metabolite levels of maize cellulolytic degradation by two bacterial isolates in (C) positive and (D) negative ionization modes. The color scale shows levels for each metabolite relative to the average abundance. Asterisks indicate significant differences ( $P < 0.05$ ) between each group. Summary statistics are provided in Table S7.



**Figure 6**

Graphical summary of the main results. Diet shapes the gut bacterial community of ECB larvae. Two bacterial isolates from the guts of ECB larvae exhibited the ability to degrade maize cellulose to varying degrees *in vitro* and produced distinctive metabolomic profiles, including reduced sugars and amino acids.

## Supplementary Files

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