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ARTICLE

The impact of pollen consumption on honey bee (*Apis mellifera*) digestive physiology and carbohydrate metabolism

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Abstract

Carbohydrate-active enzymes play an important role in the honey bee (*Apis mellifera*) due to its dietary specialization on plant-based nutrition. Secretory glycoside hydrolases (GHs) produced in worker head glands aid in the processing of floral nectar into honey and are expressed in accordance with age-based division of labor. Pollen utilization by the honey bee has been investigated in considerable detail, but little is known about the metabolic fate of indigestible carbohydrates and glycosides in pollen biomass. Here, we demonstrate that pollen consumption stimulates the hydrolysis of sugars that are toxic to the bee (xylose, arabinose, mannose). GHs produced in the head accumulate in the midgut and persist in the hindgut that harbors a core microbial community composed of approximately 10^8 bacterial cells. Pollen consumption significantly impacted total and specific bacterial abundance in the digestive tract. Bacterial isolates representing major fermentative gut phylotypes exhibited primarily membrane-bound GH activities that may function in tandem with soluble host enzymes retained in the hindgut. Additionally, we found that plant-originating β-galactosidase activity in pollen may be sufficient, in some cases, for probable physiological activity in the gut. These findings emphasize the potential relative contributions of host, bacteria, and pollen enzyme activities to carbohydrate breakdown, which may be tied to gut microbiome dynamics and associated host nutrition.

KEYWORDS

carbohydrate metabolism, digestion, honey bee (*Apis mellifera*), microbiota, nutrition

1 | INTRODUCTION

The dietary specialization of the honey bee has selected for substantive physiological changes and microbial associations over the course of evolution (Kunieda et al., 2006; Kwong & Moran, 2015; Terra & Ferreira, 1994). Foraging workers collect floral nectar as the principal carbohydrate source of the colony, while pollen provides a source of diverse carbohydrates, amino acids, lipids, and vitamins. Hive social organization is often viewed as a superorganism in which the physiology of each individual is reflected by the task they perform in the colony (Hölldobler & Wilson, 2009). Task assignment depends primarily on age but is also influenced by the nutritional demands of the colony (Crailsheim, Schneider, & Hrassnigg, 1992; Seeley, 1982). Head hypopharyngeal gland secretions contain glycoside hydrolases (GHs), a family of carbohydrate-active enzymes involved in hydrolysis of glycosidic bonds in complex sugars. The processing of floral nectar into honey involves forager bee secretions containing α-glucosidase (EC 3.2.1.20) (Nishimoto et al., 2001; Ohashi, Sawata, Takeuchi, & Natori, 1996; Takewaki, Chiba, Kimura, Matsui, & Koike, 2014). This class of GHs catalyzes the $\alpha(1\rightarrow4)$ hydrolysis of nonreducing terminals of sugar substrates such as sucrose and maltose, and other glucosides including phenyl α-D-glucose (Rye & Withers, 2000).

Pollen processing involves the packing of flower-collected pollen into wax comb with the addition of honey and worker head gland secretions. Stored pollen harbors very few bacteria and can be considered a preservation environment evolved to minimize microbial growth and prevent spoilage (Anderson et al., 2014). Moreover, honey bees prefer to consume freshly collected pollen (Carroll et al., 2017). Glucose and leucine are absorbed in the anterior two-thirds of the midgut, suggesting that host digestion concludes in this region (Crailsheim, 1988aa, 1988b). Pollen consumption and utilization have been investigated in considerable detail (Alaux, Dantec, Parrinello, & Le Conte, 2011; Brodschneider & Crailsheim, 2010; Crailsheim et al., 1992; Moritz & Crailsheim, 1987), however little is known about the metabolic fate of undigested pollen biomass in the gut or its effects on the gut microbiota.

Metagenomic and metatranscriptomic analyses of the honey bee gut identified diverse carbohydrate-processing functions including a repertoire of bacterial GH-encoding genes predicted to be involved in hydrolysis of plant polymers (Engel & Martinson, 2012; Lee, Rusch, Stewart, Mattila, & Newton, 2014). The midgut is an unstable environment for microbial establishment due to the expression and shedding of peritrophic membrane that occurs with the consumption of pollen or pollen substitutes (Douglas, 2015). However, the hindgut harbors a large microbial community of approximately 10^8–10^9 bacterial cells (Engel & Moran, 2014; Martinson, Moy, & Moran, 2012). This gut region is divided into two sections, the ileum and rectum. Each section is populated with a distinct microbial community comprising a core group of ubiquitous species clusters. Among the Gram-positive bacteria are the *Lactobacillus* Firm 5 and clade and the *Bifidobacterium asteroides* species cluster (Kwong, Engel, Koch, & Moran, 2014). Among the Gram-negative bacteria, *Gilliamella apicola* (*Gammaproteobacteria*) is a functionally diverse species cluster.

Based on metagenomic analyses, carbohydrate-active enzymes encoded by the honey bee gut microbiome were predominantly assigned to the *Lactobacillus*, *Bifidobacterium*, and *Gammaproteobacteria* phylotypes and reflect the carbohydrate content of the bee diet (Engel & Moran, 2014; Lee et al., 2014). Carbohydrate metabolism by gut microbiota involves a multitude of GHs, which may act synergistically with host or pollen digestive enzymes to release mono-, di-, and oligosaccharides (Bayer, Belaich, Shoham, & Lamed, 2004; Flint, Bayer, Rincon, Lamed, & White, 2008; Scharf, Karl, Sethi, & Boucias, 2011). Isolates of the bee symbiont *G. apicola* exhibit pectinase functionalities and can utilize sugars that are toxic to the honey bee such as pectin, mannose, xylose, and arabinose (Zheng et al., 2016). Toxic monomer hydrolysis and the overall involvement of bacterial GH activities in honey bee digestive processes remain largely undefined. Sequence-based methods have little utility to characterize gene functions because the vast majority of enzyme families have convergent substrate specificities that make it difficult to infer function based solely on sequence information (Cantarel et al., 2009). Hence, biochemical characterization of bacterial GH activities is necessary to confirm and quantify the metabolic functions encoded by the collective gut microbiome.

Digestive GHs encoded by the honey bee genome are not transcribed in the alimentary tract (Lee et al., 2014). β-Glucosidase (EC 3.2.1.21) and β-galactosidase (3.2.1.23) originate in secretory head glands and have been purified from the midgut (Peng, 1980; Pontoh & Low, 2002). In addition to carbohydrate digestion, these enzymes may convert dietary β-glycosides into biologically active aglycone forms that cause systemic alterations in honey bee gene

expression (Mao, Schuler, & Berenbaum, 2013, 2015). Plant phenolic glycosides are prevalent dietary components incorporating glucose, galactose, xylose, arabinose, and mannose residues (Bravo, 1998; Pyrzynska & Biesaga, 2009). Host enzyme-inaccessible glycosidic linkages might be cleaved by hindgut bacteria and the resulting aglycone further metabolized by both host and microbiota (Filannino, Di Cagno, Addante, Pontonio, & Gobbetti, 2016).

Despite considerable interest in improving honey bee nutrition, little is known about the metabolism of recalcitrant dietary carbohydrates and glycosides that are major constituents of pollen biomass. The gut microbiota can be considered a separate organ with its own metabolism capable of processing indigestible food, producing products that benefit the host. Here, we quantified the effects of pollen consumption on honey bee carbohydrate metabolism and the establishment of fermentative bacterial phylotypes in the gut. Tissue-specific GH enzyme activities and bacterial abundances were recorded in honey bees fed experimental diets. Our findings document previously uncharacterized modulation of honey bee digestive physiology by pollen consumption as well as the potential relative contributions of host, bacterial, and pollen GH activities to carbohydrate metabolism.

2 | METHODS

2.1 | Honey bees and experimental setup

Experiments were conducted in July 2016 at the USDA—ARS Carl Hayden Honey Bee Research Center Tucson, Arizona, USA. Adult bees were sourced from brood frames of 16 healthy colonies containing late-stage pupae, from which adults emerged naturally at 35°C and 50% relative humidity. Newly emerged bees (300 individuals) no older than 3 h postemergence were collected into a single container placed into nucleus colony boxes containing the following diet treatments: stored pollen, autoclaved pollen, and no pollen (sucrose syrup only). For comparison to bacterial abundance in bees from a natural colony context, approximately 100 newly emerged individuals from the same age cohort were marked with enamel paint and placed into healthy colonies and sampled in parallel with laboratory treatment groups.

For the hive-stored pollen diet treatment, a pollen frame near the center of the brood nest from a healthy colony was removed and installed into the center of an experimental nucleus colony box. Criteria for the frame selection included the presence of sufficient freshly stored pollen that had been packed but not covered with honey, a characteristic of "older" stored pollen. For the autoclaved pollen diet treatment, mixed corbicular pollen pellets were worked into a paste by the addition of 10% H_2O w/v, autoclaved, artificially packed into an empty drawn comb frame, and installed into the respective diet treatment box. A single frame of empty drawn comb was provided to the syrup only diet group and an additional frame of empty drawn comb was provided to all of the experimental groups for added vertical orientation space. Experimental boxes were maintained at 35°C and 50% relative humidity throughout the course of the study and provided sterilized 70% sucrose solution in two 30 ml drip bottles. Bees were sampled at day 3 and day 9 onto dry ice and stored at −80°C for biochemical and molecular analyses.

2.2 | DNA extraction

Three tissue types were used for DNA extraction and subsequent determination of bacterial abundance: midgut, ileum, and rectum. For dissection, bees were held by the thorax with sterile forceps in one hand, and an abdominal sternite was grasped with a second pair of forceps to facilitate removal of the entire digestive tract from the abdomen. Three midguts, ileums, or rectums per sample were pooled and placed immediately into 2 ml bead-beating tubes containing 0.2 g of 0.1 mm silica beads and 300 μl of 1X TE buffer and immediately frozen on dry ice and stored at −20°C for DNA extraction. Prior to DNA extraction the samples were bead beaten for a total of 2 min in 30 s intervals. To each sample, 100 μl lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5% Triton X-100, 80 mg/ml lysozyme, pH 8.0) was added and the samples were incubated at 37°C for 30 min to facilitate thorough lysis of bacterial cells. DNA was then purified from the samples using a GeneJet Genomic DNA Purification Kit according to the manufactures instructions for

Gram-positive bacteria. This procedure was repeated on 12 pools of marked individuals homogenously sampled from healthy field colonies or nucleus colony boxes corresponding to each diet treatment.

2.3 | Quantitative PCR estimation of gut tissue bacterial abundance

Separate standard curves were generated for each of the characteristic bacterial phylotypes using independent serial dilutions of a plasmid standard containing a fragment of the respective bacterial 16S rRNA gene (Martinson et al., 2012). As a negative control, quantitative PCR (qPCR) was performed with each primer pair using plasmid template representative of the different bacterial phylotypes to ensure that the primers were specific to their target sequences. The qPCR results were expressed as the total number of 16S rRNA gene copies per sample by multiplying by the total DNA concentration in each sample.

2.4 | Pollen protein preparations

Pollen cores of at least 100 mg were sampled using sterile cut 1,000 μl pipette tips. Pollen cores were homogenized in 1.2 ml of B-PER (Thermo Fisher) a nondenaturing, nonionic, detergent-based protein extraction reagent. The homogenate was centrifuged for 10 min at 14,000 rcf at 4°C and the supernatant was removed, stored on ice, and used in the GH assay described below.

2.5 | Honey bee head and digestive tract protein preparations

Three tissue types were used for protein extraction and determination of enzymatic activity: head, midgut, and hindgut (ileum plus rectum). Three head, midgut, or hindgut tissues per sample were pooled and homogenized in 1.2 ml of B-PER (Thermo Fisher) protein extraction regent. The homogenate was centrifuged for 10 min at 14,000 rcf at 4°C. The supernatant was removed, stored on ice, and used in the GH assay described below. This procedure was repeated on 12 pools of marked individuals sampled from nucleus colony boxes corresponding to each diet treatment.

2.6 | Bacterial isolation and protein preparations

Bacterial strains were isolated from honey bee guts as previously described (Bottacini et al., 2012; Kwong & Moran, 2013; Zheng et al., 2016) with slight modifications. Bees were immobilized on ice, dissected, and fresh hindgut homogenates were plated on MRS agar (*Lactobacillus* Firm 5 and *B. asteroides*) or tryptic soy agar (*G. apicola*). Colonies appeared after 48–72 h of incubation at 37°C in a 5% CO_2-enriched atmosphere and were identified by sequencing the bacterial 16S rRNA gene. To generate liquid cultures, bacterial isolates were subcultured to 5 ml of respective liquid growth media and incubated at 37°C in 5% CO_2 for 72 h. Bacterial protein fractions were generated as previously described (AEM 1985 methods) with slight modifications. Cultures were centrifuged for 10 min at 14,000 rcf at 4°C and the pellet was washed by resuspension in sterile 0.05 sodium phosphate buffer. The procedure was repeated twice and pellet was resuspended in 0.2 M McIlvane buffer (0.1 M citric acid and 0.2 M K_2HPO_4, pH 5.0) and disrupted by bead beating. The homogenate was centrifuged for 10 min at 14,000 rcf at 4°C. The supernatant was decanted (soluble, intracellular fraction), and the pellet was resuspended in 1 ml of 0.2 M McIlvane buffer pH 5.0 (insoluble, membrane bound fraction). Protein concentrations were determined using a bicinchoninic acid kit (Pierce) and the fractions were stored on ice for use in the GH assay described below.

2.7 | Measurement of GH activities

GH activities were determined by measuring the liberation of ρ-nitrophenol from a panel of 4(ρ)-nitrophenol-linked sugar substrates according to previously described methods (Bravo-Ferrada et al., 2013; Ferreira, Torres, & Terra, 1998; Grimaldi, Bartowskiy, & Jiranek, 2005) with slight modifications. Assays were carried out in a total volume of 80 μl and performed in 96-well ELISA microplates. For each reaction, 40 μl of McIlvane buffer (0.1 M citric

acid and 0.2 M K_2HPO_4, pH 5.0) was used (McIlvaine TC (1921). To each well, 20 μl of protein extract was added. Enzyme substrates were initially dissolved in methanol, diluted 10-fold in assay buffer and 20 μl were added to give a final concentration of 2.5 mM for each of the following substrates: ρ-nitrophenyl-β-D-glucopyranoside (ρNP-βGlu), ρ-nitrophenyl-α-D-glucopyranoside (ρNP-αGlu), ρ-nitrophenyl-β-D-galactopyranoside (ρNP-βGal), ρ-nitrophenyl-α-D-galactopyranoside (ρNP-αGal), ρ-nitrophenyl-α-L-fucopyranoside (ρNP-αFuc), ρ-nitrophenyl-β-D-xylopyranoside (ρNP-βXyl), ρ-nitrophenyl-α-L-arabinofuranoside (ρNP-αAra), and ρ-nitrophenyl-α-D-mannopyranoside (ρNP-αMan) (Sigma). For enzyme activities against ρNP-αGlu and ρNP-βGal, gut protein homogenates were diluted 20-fold. Assays were incubated at 37°C for 20 min and enzymatic activity was terminated by the addition of 160 μl of 0.5 M Na_2CO_3. The reaction was clarified by centrifuging the 96-well plate at 2,204 rcf for 15 min and transferring 180 μl of the supernatant to a new 96-well plate and the absorbance (400 nm) determined using a microplate spectrophotometer (Synergy HT®, BioTek) set to automatic path-length correction. Controls without protein extract or without substrate were included for each assay. The reactions were performed in technical duplicates with replicate values typically within 5% of one another. A 4-nitrophenol standard curve was generated using 0–700 μM final concentrations and used to determine the amount of 4-nitrophenol released in each reaction (Figure S1) and run on each plate. For the purposes of this study, the terms α- or β-glycosidase refer to hydrolysis of the corresponding 4-nitrophenyl α- and β-glycosides.

2.8 | Statistical analysis

Specific GH activities in native tissue homogenates were compared among diet treatment groups using two-way ANOVA with a Bonferroni correction for multiple comparisons. Total and phylotype-specific bacterial abundances in digestive tract sections were compared among colony bees and laboratory diet treatment groups using two-way ANOVA with a Bonferroni correction for multiple comparisons.

3 | RESULTS

3.1 | GH activities in pollen

Three feeding treatments were used to experimentally manipulate honey bee pollen consumption and quantify changes in carbohydrate digestion and gut microbiota establishment. Two diets contained pollen but varied with regard to the type of pollen provided (hive-stored pollen or autoclaved pollen) and one group was provided only sucrose syrup (no pollen). To determine if the pollen used in feeding treatments was enzymatically active, GH activities were tested against a panel a panel of 4-nitrophenol (ρNP)-linked sugar substrates recapitulating prevalent plant oligosaccharides and glycosides. β- and α-galactosidase were the predominant enzyme activities in hive-stored pollen cored from experimental diet treatment frames (Figure 1). Enzyme activities examined in different bee-collected (corbicular) pollen types sampled from incoming foragers were hardly detectable with the exception of β-galactosidase activity in a single pollen type (1.55 μM 4-nitrolphenol/min (mg pollen)$^{-1}$), which exceeded the average activity determined in stored pollen (0.72 μM 4-nitrophenol/min (mg pollen)$^{-1}$) (Figure S2A). No detectable enzyme activity was found in mixed corbicular pollen that was autoclaved for use as a feeding treatment.

Exocellulase activity (a microbial-derived activity) against the model substrate ρNP-βCel was not detected in any of the stored pollen samples under the conditions tested.

3.2 | Effects of pollen consumption on honey bee GH activities

Relative diet consumption was measured as a function of total hindgut content after 9 days of ad libitum access to diet treatments. Diet consumption after 9 days differed significantly between the groups ($P < 0.001$, Figure S3). The average

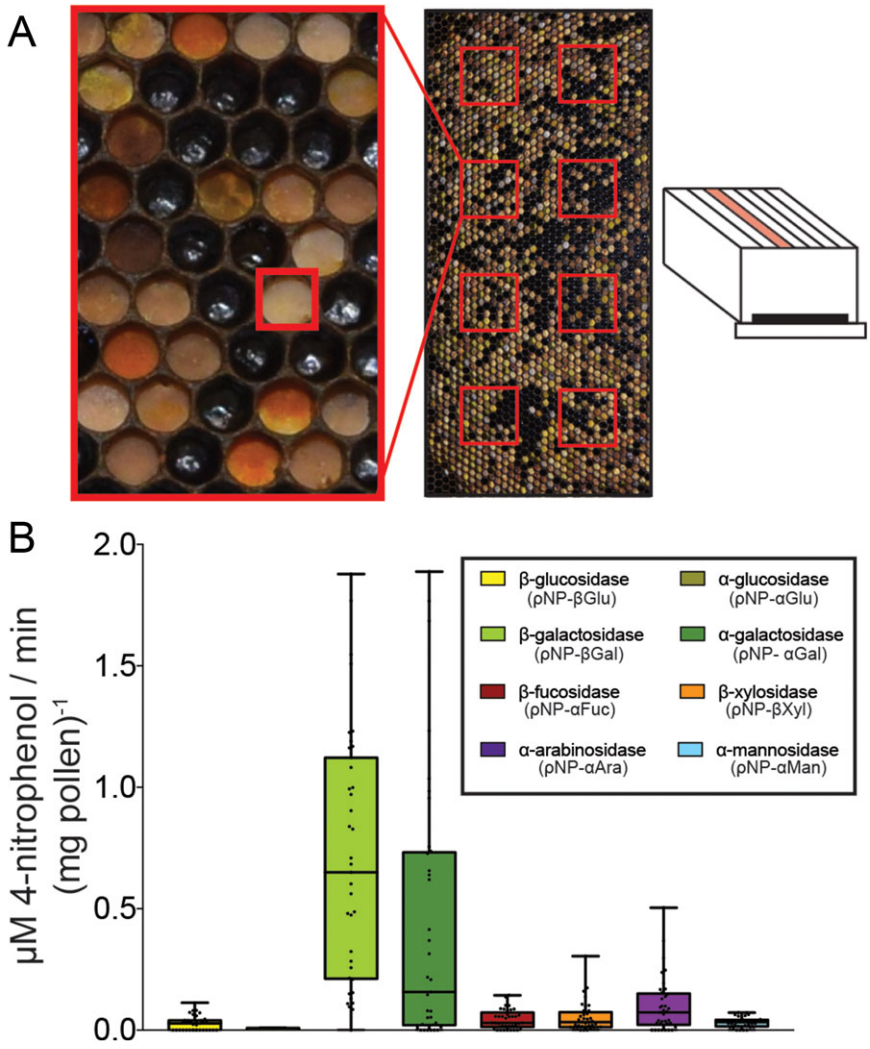

FIGURE 1 Glycoside hydrolase (GH) activities detected in honey bee stored pollen used as an experimental diet treatment. (A) Schematic showing a stored pollen frame obtained from a field colony. Prior to initiating diet treatments, pollen samples were spacially cored for biochemical analyses and then installed into a laboratory nucleus colony box containing newly emerged bees. (B) Specific GH activities detected in cores of stored pollen using a panel of α- or β-linked 4-nitrophenol glycosides and expressed per milligram of pollen ($n = 38$). Box and whiskers plots show all values with the lower and upper edge of each box denoting the 25th to 75th percentiles and the median as a horizontal bar

hindgut content was lowest in the no pollen diet group (0.21 mg/bee). Consumption of stored pollen (4.89 mg/bee) was significantly higher than that of autoclaved pollen (3.12 mg/bee).

The saccharolytic potential of the head, midgut, and hindgut were measured using native tissue homogenates and the aforementioned model enzyme substrates. Pollen consumption markedly stimulated head enzyme activities against all of the substrates tested with the exception of ρNP-αGal ($P < 0.001$, Figure 2). Bees-fed pollen accumulated increased levels of soluble head protein indicating diversion of that nutrition to protein synthesis in the hypopharyngeal glands ($P < 0.001$, Figure S4). When head enzyme activities were expressed per milligram of soluble protein, pollen-diet effects remained significant whereas differences between the two pollen diets did not reach significance (Figure S5). Head β-glucosidase activity was positively correlated with the hydrolysis of ρNP-βXyl, ρNP-βFuc, and ρNP-αAra and exhibited highly similar pH-activity responses against those substrates (Figure S6).Head-specific hydrolysis

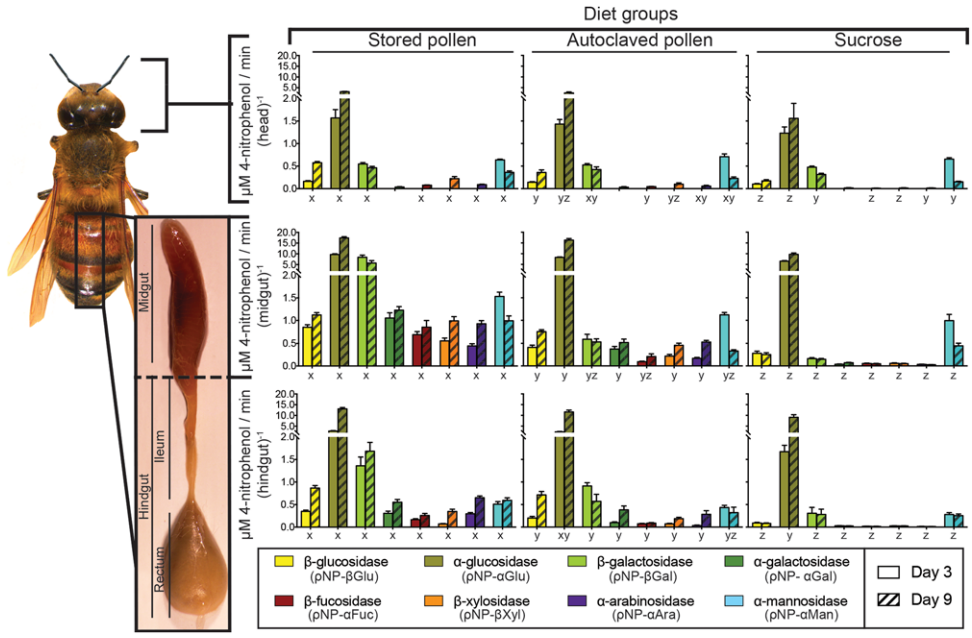

FIGURE 2 Effects of diet treatments on glycoside hydrolase activities in honey bee tissue homogenates at 3 (solid bars, $n = 12$) and 9 days (striped bars, $n = 12$). Error bars represent standard error (SE). Within each tissue type and enzyme activity (color), different letters indicate a significant difference between diet groups ($P < 0.05$, two-way ANOVA and Bonferroni's correction for multiple comparisons). Significant interactions between diet and age factors were determined for multiple enzyme activities in different tissue types and are summarized in Figures S7–S9

of all substrates increased with age except ρNP-αMan, which decreased with age ($P < 0.001$). Significant interactions between diet and age factors were found for head enzyme activities against substrates ρNP-βGlu, ρNP-βFuc, ρNP-βXyl, ρNP-αAra, ρNP-αMan (Figure S7).

Pollen consumption significantly increased midgut and hindgut GH enzyme activities against all of the substrates tested ($P < 0.001$, Figure 2B). The stored pollen diet consistently induced the highest levels of gut enzyme activities. Notably, in bees that consumed stored pollen, β-galactosidase levels expressed per midgut (Figure 2B) exceeded those expressed per milligram pollen (Figure 1). In comparison to the autoclaved pollen diet, consumption of stored pollen led to a 14.2- and 10.7-fold increase in midgut β-galactosidase levels on day 3 and day 9, respectively.

Midgut and hindgut GH activities against substrates ρNP-βGlu, ρNP-αGlu, ρNP-αGal, ρNP-βXyl, and ρNP-αAra increased significantly with age ($P < 0.001$). Midgut-specific hydrolysis of ρNP-αMan decreased with age ($P < 0.001$). Hindgut-specific hydrolysis of ρNP-αGal and ρNP-βFuc increased with age ($P < 0.001$ and $P = 0.048$, respectively). Significant interactions between diet and age factors were found for midgut and hindgut enzyme activities against ρNP-βGlu, ρNP-βXyl, and ρNP-αAra, as well as midgut-specific hydrolysis of ρNP-αGlu and hindgut-specific hydrolysis of ρNP-αGal (Figures S8 and S9).

Exocellulase activity against ρNP-βCel was not detected in head, midgut, or hindgut soluble protein homogenates under the conditions tested.

3.3 | Effects of pollen consumption and colony context on bacterial phylotype abundance in the digestive tract

The hindgut was subdivided into the ileum and rectum to measure the effects of pollen consumption on gut section specific bacterial phylotype abundance using qPCR profiling of bacterial 16S rRNA gene copies. For comparison to the natural hive environment, marked bees from healthy colonies were evaluated in parallel with laboratory groups

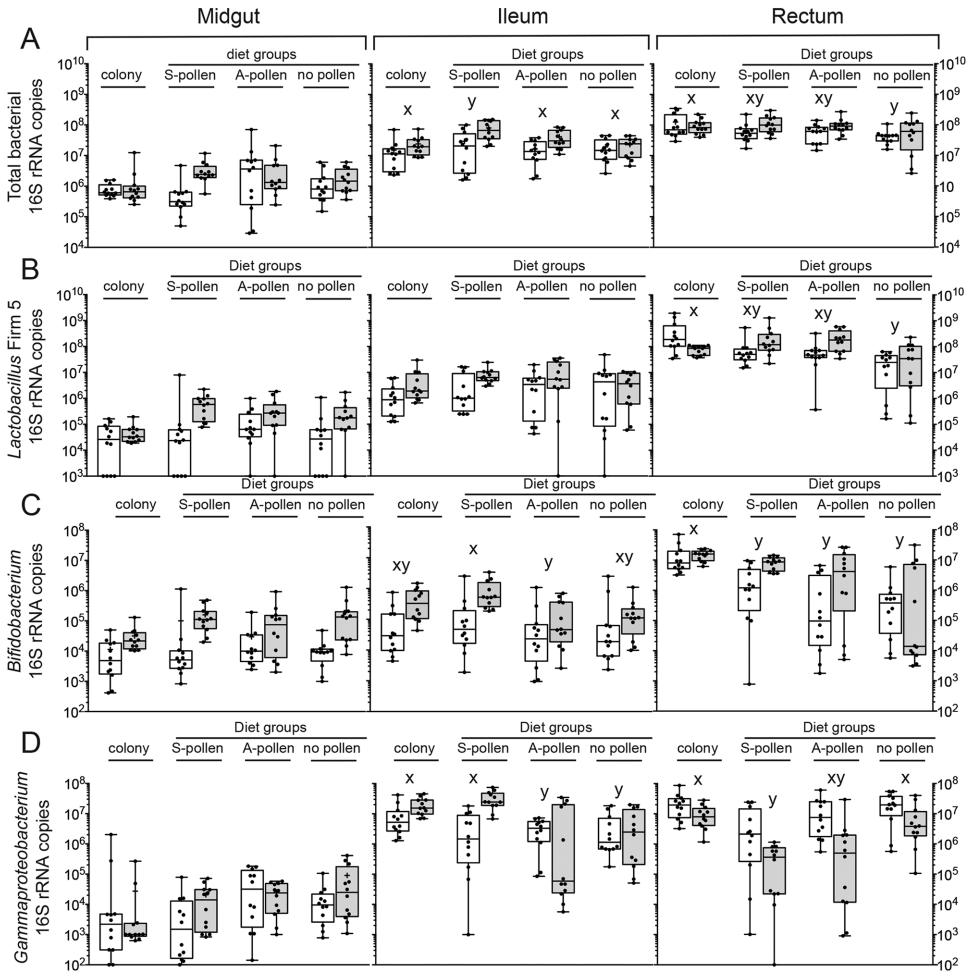

FIGURE 3 Effects of diet and colony contact on bacterial phylotype abundance in honey bee digestive tract sections at 3 (solid bars, $n = 12$) and 9 days (gray bars, $n = 12$). Experimental groups consisted of marked bees from the same cohort homogenously sampled from field colonies (colony) or laboratory nucleus colony boxes fed bee-stored pollen (S-pollen), autoclaved pollen (A-pollen), or no pollen. Box and whiskers plots show all values, with the lower and upper edge of each box denoting the 25th to 75th percentiles and the median as a horizontal bar. Within each phylotype and gut section, letters indicate a significant difference between diet groups ($P < 0.05$, two-way ANOVA and Bonferroni's correction for multiple comparisons). Significant interactions between treatment and age factors were found for multiple phylotypes in different gut sections and are summarized in Figure S10

from the same cohort that were fed experimental diet treatments. A significant treatment effect was found for the accumulation of total bacterial 16S rRNA gene copies in the ileum ($P < 0.001$, Figure 3A) and the laboratory group fed stored pollen had the highest accumulation of bacterial 16S gene copies in this gut region. Total bacterial abundance in the ileum increased with age ($P < 0.001$) and a significant treatment–age interaction was determined ($P = 0.013$).

Under typical conditions *Lactobacillus* Firm 5 occurs sporadically throughout the midgut and ileum but its preferred niche is the rectum (Figure 3B): Treatment had a significant effect on the accumulation of 16S rRNA gene copies in the rectum ($P = 0.042$) and the no pollen laboratory group featured the lowest accumulation of 16S gene copies in this gut region. No age effect was determined, however we found a significant interaction between treatment and age ($P = 0.002$)

The niche of *Bifidobacterium* in the early adult gut is largely restricted to the rectum and not found in any real abundance in the midgut or ileum (Figure 3C): The accumulation of midgut 16S rRNA *Bifidobacterium* gene copies increased

with age ($P = 0.016$). Treatment had a significant effect on ileum 16S gene copies ($P < 0.035$), which was attributable to increased abundance in stored pollen-fed bees compared to autoclaved pollen-fed bees. Ileum 16S abundance increased with age ($P = 0.037$). Treatment had a significant effect on 16S gene copies in the rectum ($P < 0.001$) and colony bees had the highest abundance of 16S gene copies in this gut region. Rectum 16S abundance increased with age, except in the no pollen diet group, which decreased with age ($P = 0.042$).

Gammaproteobacterium most often occur in the ileum and midgut and in lower abundance in the rectum (Figure 3D): Significant treatment and age effects as well as an interaction between treatment and age were determined for *Gammaproteobacterium* 16S rRNA gene copy abundance in the ileum ($P < 0.001$). The stored pollen diet led to the highest accumulation of 16S gene copies in the ileum. A significant effect of treatment ($P = 0.003$) and age ($P < 0.001$) was determined for 16S gene copy accumulation in the rectum. Bees-fed stored pollen featured the lowest accumulation of 16S gene copies of *Gammaproteobacterium* in the rectum.

3.4 | Characterization of bacterial GH activities in axenic cultures

The gut microbiota can be considered a separate organ with its own collective metabolism. Bacterial isolates representing the *Lactobacillus* Firm 5, *B. asteroides*, and *G. apicola* species clusters were cultured from the honey bee hindgut, identified by BLAST (NCBI) analysis of their 16S genes, and their repertoire of GH enzyme activities were tested in vitro. Enzyme activities were determined for the membrane bound (insoluble) and intracellular (soluble) protein fractions in order to assess their localization. Insoluble protein fractions featured the highest levels of enzymatic activities and the hydrolytic capabilities of the representative isolates were variable (Figure 4). Two unique isolates belonging to the *Gillamella apicola* species cluster exhibited markedly different enzyme profiles. Exocellulase activity as determined by the hydrolysis of $_{\rho}$NP-βCel was detected in isolates belonging to the *B. asteroides and G. apicola* species clusters.

4 | DISCUSSION

The experiments presented here emphasize the nature and extent of GH activities in the honey bee as well as the effects of pollen consumption on digestive physiology. These results provide novel insights into nonstarch carbohydrate metabolism, which may be tied to microbiome dynamics and host nutrition. Digestive enzyme production typically occurs in the insect midgut, and in many cases also the salivary system (Ferreira et al., 1998; Terra & Ferreira, 1994). Located in the head, hypopharyngeal glands are a highly derived feature of honey bees characteristic of the genus *Apis*. These specialized glands function as a secretory organ involved in colony nutrition processing and distribution among other varied functions. Head-specific expression of honey bee α-glucosidases peak in the forager life stage and facilitate the conversion of floral nectar into honey (Costa & da Cruz-Landim, 2005; Nishimoto et al., 2001; Ohashi et al., 1996; Takewaki et al., 2014). However, the metabolic fate of nonstarch dietary carbohydrates and their effects on the gut microbiota have not been investigated in detail.

Transcriptional regulation of numerous metabolic functions in response to dietary manipulation has been documented in the honey bee (Alaux et al., 2011; Wheeler & Robinson, 2014). Elucidation of dietary mechanisms regulating individual physiology will clarify how assimilation of pollen nutrition is maintained in honey bees. Here, we provide novel evidence for a positive regulatory effect of pollen consumption on head GH activities. β-Glucosidase levels in the head were positively correlated with β-xylosidase, β-fucosidase, and α-arabinosidase levels and shared highly similar pH-activity responses. These results indicate that the honey bee β-glucosidase cleaves additional terminal monosaccharides including xylose, fucose, and arabinose, findings that are consistent with evolutionary trends of insect β-glucosidase converging on a single enzyme capable of hydrolyzing a variety of dietary glycosides (Ferreira et al., 1998). Peptide mass analysis indicated that the β-glucosidase isolated from the honey bee head was the same as those isolated from the crop and midgut (Pontoh & Low, 2002). Our findings are consistent with the production of β-glucosidase in the hypopharnygeal glands, where it is secreted into the mouth during feeding to be later shared with other individuals or transferred to the midgut.

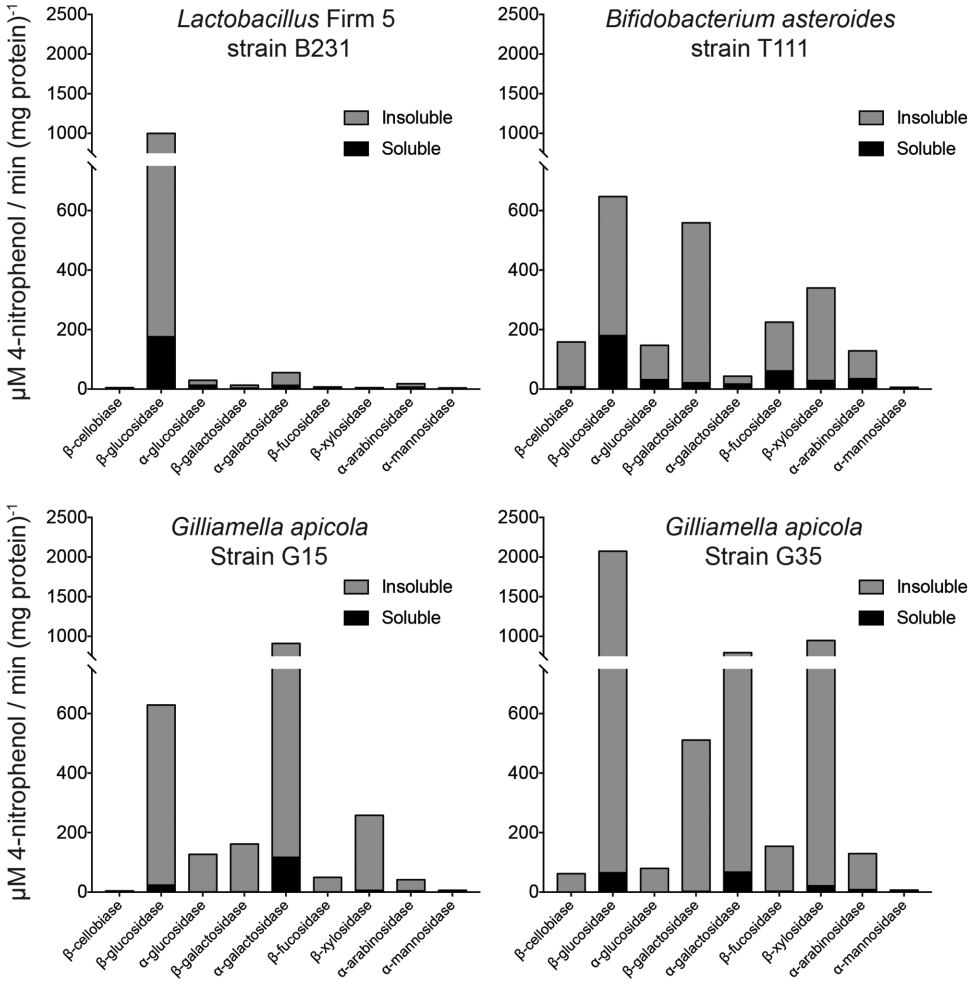

FIGURE 4 Glycoside hydrolase activities in axenic cultures of characteristic bacteria isolated from honey bee hindgut homogenates. Stacked bars indicate enzyme activities in membrane bound (insoluble) and intracellular (soluble) protein fractions

Pollen consumption markedly increased the saccharolytic potential of the digestive tract. High levels of GH activities in the midgut compared to the head showed that head-expressed enzymes are partially resistant to the protease activities of the midgut and can accumulate with food consumption. The coincidence in time and space of bacterial colonization and pollen consumption creates the potential for crosstalk between host and microbial metabolism as significant levels of soluble enzymatic activity are retained upon passage of food into the hindgut. The honey bee GH repertoire is capable of hydrolyzing sugars such as xylose, arabinose, and mannose; dietary components that are toxic to bees but are also metabolized by the gut microbiota (Zheng et al., 2016). It is possible that synergism between host and microbial saccharolytic processes might reclaim energy from otherwise indigestible or toxic carbohydrates in the form of bacterial fermentation products such as short chain fatty acids. Such a function may be significant during overwintering when undigested pollen remains in the rectum for many months.

By deduction, we suggest that active enzymes occurring in vital pollen grains are released into the digestive tract upon consumption, contributing to differences in gut enzyme profiles between the pollen-fed diet groups. Grogan and Hunt (1979) reported that protease enzyme levels occurring in some pollens were sufficient for probable physiological activity in the honey bee midgut. We identified β-galactosidase as the predominant enzyme activity in stored pollen. Our findings indicate that β-galactosidase activity in pollen has similar cleavage specificities to the honey bee and

occurs at dietary levels that may be sufficient to contribute to the cumulative hydrolysis of galactose, a quantitatively meaningful sugar ingested by phytophagous insects (Turunen, 1992). Quantitative differences in gut enzyme profiles between the diet treatment groups could be attributed to 36% reduced consumption of the autoclaved pollen diet compared to the stored pollen diet. However, significant discrepancies in midgut β-galactosidase levels between the two pollen diet groups could be attributed to dietary β-galactosidase intake. In bees that received the stored pollen diet, β-galactosidase levels expressed per midgut exceeded those expressed per milligram pollen. Bees-fed stored pollen featured midgut β-galactosidase levels up to 14-fold higher than bees-fed enzymatically inactive autoclaved pollen. β-Galactosidase expression occurs in mature pollen grains and is involved in pollen wall expansion and pollen tube physiology (Fry, 1995; Taiz, 1984). Mechanisms underlying plant endogenous cell wall modification may act combinatorially with exogenous enzymes to saccharify plant biomass as well as generate oligosaccharides with prebiotic properties (Flint et al., 2008; Macfarlane, Steed, & Macfarlane, 2007; Tavares, De Souza, & Buckeridge, 2015). It is possible that similar biochemical conditions emerge upon consumption of vital pollen grains where endogenous plant cell wall modifying enzymes act synergistically with honey bee digestive enzymes.

Cellulase activity has long been suggested but not functionally demonstrated as a feature of microbial metabolism in stored pollen (Anderson, Sheehan, Eckholm, Mott, & DeGrandi-Hoffman, 2011; Kunieda et al., 2006). However, hydrolysis of ρNP-βCel was not detected in stored pollen under the conditions tested. ρNP-βCel is a model substrate for cellulase activity via endohydrolysis of the $\beta(1\rightarrow4)$ bond linking cellobiose to 4-nitrophenol. These findings are in agreement with previous work indicating that hive-stored pollen is a preservation environment evolved to inhibit microbial growth and metabolism (Anderson et al., 2014).

Changes in dietary carbohydrate intake can have major effects on the abundance and fermentative outputs of gut bacteria, in turn affecting community establishment and metabolic crosstalk with the host (Flint et al., 2008; Macfarlane et al., 2007; Tungland & Meyer, 2002). Laboratory diet treatments and colony context impacted total and phylotype-specific bacterial abundance in the hindgut. This gut region likely supports microbial growth via host-provided nutrition, the buildup of recalcitrant dietary components and nitrogenous waste. The stored pollen diet increased total bacterial abundance in the ileum, whereas the absence of dietary pollen combined with laboratory isolation decreased total bacterial abundance in the rectum. Consumption of autoclaved pollen led to gut phylotype profiles that more closely resembled bees that did not consume pollen. This could be at least partially attributable to 36% reduced consumption of autoclaved pollen compared to stored pollen. Furthermore, autoclaving pollen likely altered the pH, digestibility, or preservation conditions of "natural" bee-stored pollen (Anderson et al., 2014). Atypical changes in niche-specific bacterial abundance has been linked to the consumption of altered pollen diets including gut dysbiosis and impaired host development in honey bees (Anderson & Ricigliano, 2017; Maes, Rodrigues, Oliver, Mott, & Anderson, 2016).

Among the laboratory diet groups, hindgut phylotype profiles of bees that consumed stored pollen more closely resembled the abundance and stability determined in colony bees from the same cohort. However, colony bees featured increased abundance and stability of *Gammaproteobacterium* and *Bifidobacterium* in the rectum compared to the laboratory group fed stored pollen. This result indicates that the colony context might impact bacterial abundance and establishment either through increased strain exposure or "free range" consumption of hive food resources. It has been proposed that social contact facilitates microbiota acquisition in the honey bee due to the high frequency of trophollactic (food or fluid transfer) interactions among networks of individuals within a colony (Kwong & Moran, 2015). However, experiments designed to specifically test the effects of social contact did not reveal significant changes in the microbiota of young bees exposed to older workers (Anderson, Rodrigues, Mott, Maes, & Corby-Harris, 2016). Taken together with our findings, the effects of the colony context on phylotype abundance could be attributable to pollen consumption under "normal" hive conditions. These findings collectively indicate that diet, age, and colony context influence honey bee gut microbiota composition, however interactions among these factors are likely complex and will require more detailed investigations to elucidate.

Our findings expand on previously reported carbon source utilization experiments and indicate that the honey bee gut microbiota is capable of hydrolyzing sugars that are prevalent in pollen biomass, thus liberating them from larger carbohydrates. Strains of the bee symbiont *G. apicola* (*Gammaproteobacterium)* can utilize sugars such as mannose, and

the bee-specific *B. asteroides* species cluster exhibits a broader range of carbon source utilization than any other tested species within the genus *Bifidobacterium*, a large and diverse group of intestinal commensals (Bottacini et al., 2012). We characterized the GH activities of bacterial isolates representing the *Lactobacillus* Firm 5, *Bifidobacterium*, and *Gammaproteobacterium* phylotypes. The bacterial isolates featured primarily insoluble or membrane-bound GH activities with similar substrate specificities to the hydrolytic repertoire of the bee. Crailsheim et al. (1992) estimated the amount of pollen biomass contained in the digestive tracts of all workers in a normal-sized bee colony to be between 81 and 107 g. Taken together with bacterial abundance data and in vitro enzyme activities, the saccharification potential of the collective gut microbiota of an entire bee colony could be significant. Further investigations are necessary to characterize the effect of GH enzymes of various origins and their collective potential to influence microbiota establishment and host physiology.

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SUPPORTING INFORMATION

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