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Aerobic Microorganisms Associated with Alfalfa Leafcutter Bees (*Megachile rotundata*)

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Abstract. Characterization of microorganisms associated with alfalfa leafcutter bee (*Megachile rotundata*) nectar, pollen, provisions, larval guts, and frass (excreta) in Alberta demonstrated a varied aerobic microflora. Yeasts were isolated frequently from nectar, pollen, and provisions but rarely from guts or frass. The most prevalent yeast taxa were: *Candida bombicola, Cryptococcus albidus, Metschnikowia reukaufii,* and *Rhodotorula glutinis.* Although few filamentous fungi were found in nectar, they were frequently isolated from pollen and provisions; the predominant taxa were *Alternaria alternata, Cladosporium cladosporioides, C. herbarum, Epicoccum nigrum,* and *Penicillium chrysogenum.* Bacteria, including species of *Bacillus, Corynebacterium, Micrococcus,* and the actinomycete *Streptomyces,* also were prevalent in provisions and/or on pollen. In general, the diversity of microorganisms isolated from alimentary canals and frass was lower than from nectar, pollen, and provisions. *Bacillus firmus, B. licheniformis, B. megaterium, B. pumilus,* and *Streptomyces* spp. were the most frequently isolated bacteria, whereas *Trichosporonoides megachiliensis* was the most common filamentous fungus isolated from larval guts and/or frass. These taxa may be part of the resident microflora of the alimentary canal. Populations of bacteria and filamentous fungi, but not yeasts, were larger from *Ascosphaera aggregata*-infected larvae than from healthy larvae. However, with the exception of *Aspergillus niger* and *T. megachiliensis* in frass from healthy larvae, no taxon of filamentous fungi was conspicuously present or absent in infected larvae, healthy larvae, or their frass.

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Introduction

Alfalfa leafcutter bees (*Megachile rotundata*) are the principal pollinators of alfalfa (*Medicago sativa*) in northwestern North America. Chalkbrood, caused by the fungus *Ascosphaera aggregata,* has become an economically important disease of leafcutter bees. It is initiated from ascospores which are introduced into larval provisions (consisting of nectar and pollen) by adult female bees [50], and subsequently ingested by larvae. Ascospores then germinate in the larval midgut, and hyphae penetrate into the hemocoel, eventually killing the larvae [41, 48, 49]. Larval provisions also contain a variety of other microorganisms [3, 26], but their contribution to the ecology of the larval alimentary canal is unknown. Although the insect gut microflorae may be antagonistic to some pathogens [8, 27, 29, 36], the influence of microorganisms associated with alfalfa leafcutter bees on *A. aggregata* is not yet clear. In order to evaluate the possible influence of specific microorganisms, it was first necessary to comprehensively examine the indigenous microflora. This study examined the aerobic microorganisms associated with nectar and pollen from adult female bees, larval provisions, pre- and post-defecation larval guts, and frass (excreta) from healthy larvae, and compared microbial populations from healthy and *A. aggregata*-infected larvae.

Methods

Media Selection

A preliminary experiment was conducted to assess the efficacy of different agar media for recovery of microorganisms. Provisions from five leafcutter bee nest cells and frass from five larvae (fourth to fifth instar) were aseptically removed and weighed. Provisions and frass were then macerated in 2.0 ml 0.01 M sodium phosphate buffer (pH = 7.0) containing 0.01% Tween 80 (Sigma, St. Louis, Missouri) using a Potter-Elvehjem grinder. Homogenates were diluted in a tenfold dilution series, and aliquots of 100 μl from each dilution were spread in duplicate onto several media; media for bacteria were plate count agar (PCA; Difco, Detroit, Michigan) and nutrient agar (NA; Difco), each amended with 50 mg liter^{-1} of Nystatin (Sigma). Media for fungi were malt extract agar (MEA; consisting of 20 g of malt extract, 20 g glucose, 1 g peptone, 15 g agar in 1 liter of water; pH = 5.6), phytone yeast extract agar (PYE; BBL, Baltimore, Maryland), potato dextrose agar (PDA; Difco), Sabouraud's dextrose agar (SAB; Difco), and sorbose yeast extract agar (SYE; consisting of 4 g sorbose, 0.5 g yeast extract, 15 g agar in 1 liter of deionized water; pH = 7), each amended with 100 mg liter^{-1} of tetracycline (Sigma). Cultures were incubated at 25 ± 1°C for 72–120 h as a completely randomized design with 3–4 replicates per treatment. Colonies of bacteria and fungi were enumerated at the dilution yielding 20–200 colonies per dish, and data were calculated as $\log_{10}$ colony-forming-units (cfu) g^{-1} fresh weight of provisions or frass.

Sample Collection and Preparation

Adult female leafcutter bees and nest cells containing eggs and larvae were randomly collected in August 1990 from a hive situated in an irrigated field of alfalfa near Lethbridge, Alberta, transported to the laboratory, and maintained at 5°C for a maximum of 5 days. Samples consisted of pollen, nectar, larval provisions, pre-defecation guts (second to third instar larval alimentary canals prior to completion of the gut), post-defecation guts (fourth to fifth instar larval alimentary canals after completion of the gut), and frass. Pollen and nectar were collected from adult female bees anaesthetized with CO_2. Pollen was aseptically collected from scopae on abdomens and nectar from bee crops. Prior to nectar

collection, bees were surface-sterilized in 1% sodium hypochlorite amended with 0.05% Tween 80 for 2 min and then rinsed twice in sterile distilled water. Bees were induced to regurgitate nectar by applying pressure to the abdomen. Nectar was then collected in 5- to 10-μl capillary tubes.

Larval provisions, larvae, and frass were removed from nest cells after detachment of the leaf caps. Provisions were removed from cells containing eggs or first instar larvae with a microspatula. Frass was collected from post-defecation larvae with forceps. We were unable to remove intact alimentary canals from longitudinally incised post-defecation larvae; therefore, both pre- and post-defecation larvae were surface-sterilized, as described for adults, and macerated. Initially the efficacy of the sterilization procedure was confirmed by agitating treated larvae in sterile nutrient broth for 30 sec, removing the larvae, and incubating the broth at 25°C for 5–7 days. The sterility of larval hemolymph also was confirmed by puncturing the cuticle of surface-sterilized larvae, and incubating 10-μl droplets of the collected hemolymph on NA. All treatments consisted of 10 weighed samples (fresh weight) in each of 5 replicates.

Isolation and Enumeration of Microorganisms

Samples of surface-sterilized pre- and post-defecation larvae, pollen, provisions, and frass were each macerated in 1 ml chilled buffer amended with Tween 80. The macerates and nectar for each replicate were immediately diluted 2 times in a tenfold dilution series, and 100-μl aliquots from each dilution were spread in duplicate onto NA for the isolation of bacteria, MEA for yeasts, and SYE for filamentous fungi. In addition, MEA amended with 30% dextrose (M30) was used for the nectar and provision treatments. Cultures were incubated as previously described as a completely randomized design. Colonies of bacteria and yeasts were enumerated at 72 and 96 h, and filamentous fungi at 120 h at the appropriate dilution, and data were calculated as $\log_{10}$ cfu g^{-1} fresh weight of substrate. Five randomly-selected colonies of bacteria and yeasts, and 20 colonies of filamentous fungi per dish at the dilution used for enumeration were subcultured onto NA, MEA, and cornmeal agar (CMA; consisting of 50 g cornmeal, 15 g agar in 1 liter of deionized water) slants, respectively. Due to the diversity of colonial morphologies encountered, 8 bacterial colonies per dish were subcultured from the pollen treatment. All slant cultures were maintained at 25°C for 2–10 days in the dark and then placed at 4°C until isolates were identified.

Characterization of Isolates

Most isolates of filamentous fungi were grown in slide culture preparations on CER and identified according to morphological characteristics using standard mycological references and by comparison with known isolates maintained at the University of Alberta Microfungus Collection and Herbarium (UAMH). Morphology of yeasts was examined in glucose-yeast-peptone broth [51] and on MEA in Dalmau culture; in addition, filamentous yeasts were grown in slide culture preparations. Yeast isolates also were tested for their ability to assimilate 23 carbohydrates, utilize nitrate and urea, and grow in the presence of cycloheximide (6.0 mg ml^{-1}) using an automated AutoMicrobic system (AMS, Vitek Systems, Inc., Hazelwood, Missouri). However, the AMS-yeast biochemical cards (YBC) were incubated at lower than recommended temperature (25°C) and read at 24-h intervals for a total of 96 or 120 h. Since the AMS system database is oriented to medically important species, the system generally did not provide an identification. Therefore, identifications based on morphology and the assimilation profiles provided by the AMS-YBC were made according to standard references [1, 51]. To verify the identifications, the ability of representative isolates to ferment glucose, galactose, lactose, maltose, sucrose, and trehalose using the Wickerham procedure [44] at 25°C for 28 days also was tested.

Bacteria were grown on sheep's blood agar and/or NA at 30° and/or 25°C and their gram stain reactions determined at 24 and 48 h. Gram-negative isolates were tested for catalase and oxidase capability, and characterized using a Biolog GN system at 25°C (Biolog, Inc., Hayward, California). Gram-positive cocci were tested for pigmentation, cell configuration, catalase, hemolysis, anaerobosis, acetoin (acetylmethyl-carbinol) production, oxidase capability, utilization of nitrate, growth on 7%

sodium chloride and Simmon's citrate media, growth at 37°C, sensitivity to Bacitracin (Difco) and Novobiocin (Difco), and were identified according to Kocur [38]. Gram-positive bacilli producing endospores were identified using the AMS-*Bacillus* card at 37°C. Isolates that could not be definitively identified were further characterized using other references [7, 28, 40]. Gram-positive coryneforms were characterized with the API Rapid Coryne system (API Laboratory Products Ltd., St. Laurent, Quebec). Isolates of actinomycetes were characterized according to spore chain morphology, spore mass color, melanin production on tyrosine agar, diffusing pigment, hydrolysis of casein, tyrosine and xanthine, and assimilation of glucose, xylose, arabinose, rhamnose, fructose, galactose, raffinose, mannitol, inositol, salicin, and sucrose [42].

At least one representative of each taxon of filamentous fungi and yeasts was deposited at UAMH; isolates were lyophilized and also stored over liquid nitrogen. Selected taxa of bacteria were accessioned and lyophilized.

Scanning Electron Microscopy

Intact guts were removed from pre-defecation larvae collected in August 1991. They were immediately washed with a sterile 0.85% sodium chloride solution, then fixed in 2% glutaraldehyde in 0.05 M phosphate buffer, post-fixed in 2% osmium tetroxide, washed with buffer, dehydrated in ethanol, critical point dried in liquid CO_2, sputter coated in gold, and examined with a Hitachi S-570 scanning electron microscope at an accelerator voltage of 10 kV.

Microorganisms Associated with Diseased Larvae

Sporulating chalkbrood cadavers were supplied by the Canadian Leafcutter Bee Cocoon Testing Centre, Brooks, Alberta. Ascospores of *A. aggregata* were recovered and viability tested as described previously [32]. A germination percentage of 76% was observed after 24 h. Immediately prior to use, asci (spore balls) were crushed between two sterile glass slides to release ascospores which were then suspended in a sterile buffer composed of 0.02 M BES (N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid), 0.15 M sodium chloride, and 0.017 mM DOSS (dioctyl sulfosuccinate) (pH = 7.1). Ascospore concentrations were then estimated with a hemocytometer and adjusted to 5×10^8 ascospores ml^{-1}.

Leafcutter bee cells were collected in July and August 1991 from the same field site as previously described, leaf caps were immediately removed, and the cells containing eggs placed in 96-well tissue culture plates. Eggs on provisions were maintained at 5°C for a maximum of 5 days. A 2-μl aliquot of the ascospore suspension was then placed adjacent to each individual egg, and the plates were subsequently maintained at 30 ± 1°C. At 7–10 days post-inoculation, healthy larvae and chalkbrood cadavers, and the associated frass from 5 individuals per replicate were aseptically collected and weighed. Samples consisted of healthy larvae, chalkbrood cadavers, frass from healthy larvae, and frass from larvae developing chalkbrood. Larvae were surface-sterilized as described previously. Samples were macerated and spread onto SYE and NA as before, except that duplicate dishes per dilution were not used. Where possible, 10 randomly-selected colonies of filamentous fungi per plate also were transferred onto CMA, incubated for 7–10 days, and then maintained at 5°C until identified. The experiment was arranged as a completely randomized design with 3–4 replicates per treatment, and was repeated 2 times for the larval and once for the frass treatments.

Analyses

Computations were performed with Statistical Analysis System Software (SAS Institute Inc., Cary, North Carolina) using the ANOVA, GLM, TTEST and CLUSTER procedures. Normality of data was examined with residual plots; where appropriate, $\log_{10}$-transformations were used to normalize the

Table 1. Colony counts of filamentous fungi, yeasts, and bacteria recovered from nectar (NR), pollen (PO), provisions (PR), pre-defecation larval guts (PRE), post-defecation larval guts (POST), and frass (FR)[a]

	Log cfu g^{-1} (fresh weight)					
Treatment	NR	PO	PR	PRE	POST	FR
Yeasts	2.17 (0.89)ab[b]	3.78 (0.26)a	3.10 (0.11)a	1.61 (0.67)	0.73 (0.45)a	1.54 (0.97)a
Filamentous	1.06 (0.65)a	4.90 (0.12)b	3.84 (0.14)b	2.48 (0.77)	2.81 (0.54)b	4.87 (0.44)b
Bacteria	3.73 (0.11)b	4.94 (0.14)b	3.61 (0.07)b	2.16 (0.27)	2.14 (0.26)b	4.27 (0.20)b

[a]Experiment arranged as a completely randomized design with 5 replicates per treatment. Each replicate consisted of 10 bulked samples

[b]Standard errors of the means are presented in parentheses. Means not followed by the same letter within each column are not significantly different ($\alpha = 0.05$) as determined by Duncan's multiple range test for log-transformed data

data. Repeated experiments were tested for homogeneity of variance [47] prior to pooling the data. Standard errors of the means were calculated from individual treatments and are presented in parenthesis. Pairwise t-tests were used to separate two treatments; Duncan's multiple range test in conjunction with a significant F-test was used to separate three means. Log cfu of individual taxa were calculated as log [(% isolation)(total cfu g^{-1})]. Taxa diversity indexes (DI) were calculated as the number of taxa/number of isolates. For *Bacillus* and *Streptomyces,* results from the physiological and growth tests were subjected to SAS cluster analysis and TAXMAP [6].

Results

Media Selection

Bacteria isolated from provisions on NA and PCA were 3.72 (0.13) and 3.73 (0.16) log cfu g^{-1}; from frass, populations were 3.92 (0.06) and 4.03 (0.13) log cfu g^{-1}, respectively. Since there were no differences in total numbers of colony forming units recovered from either provisions ($P = 0.80$) or frass ($P = 0.30$) on the two media, NA was used to isolate bacteria in all subsequent experiments. Fungi (including yeasts) recovered on MEA, PDA, PYE, SAB, and SYE from provisions ranged from 3.68–3.93 log cfu g^{-1}; from frass, counts ranged from 5.30–5.41 log cfu g^{-1}. There were no differences in recovery of fungi between the media tested ($P = 0.97$ and $P = 0.99$), and MEA was subsequently selected for the isolation of yeasts and SYE for filamentous fungi; MEA is a conventional yeast medium, and the growth of filamentous fungi on SYE was slower than that on other media tested.

Associated Microflora

More bacteria ($\alpha = 0.05$) than filamentous fungi were isolated from regurgitated nectar; however, there were no differences between bacterial and fungal colony forming units from all other treatments (Table 1). With the exception of the pre-defecation larval gut treatment for bacteria and filamentous fungi, and the nectar treatment for filamentous fungi, yeasts were significantly less numerous. Eleven species of yeasts were identified (Table 2). One taxon from pollen could not

Table 2. Prevalence of yeasts recovered from nectar (NR), pollen (PO), provisions (PR), pre-defecation larvae (PRE; instar 2–3), post-defecation larvae (POST; instar 4–5) and frass (FR)

Species	Log cfu g^{-1} (fresh weight)					
	NR	PO	PR	PRE	POST	FR
Candida bombicola	2.13 (0.58)[a]		1.63 (0.75)	1.15 (0.39)	0.47 (0.29)	1.21 (0.79)
Candida glabrata						0.44 (0.44)
Cryptococcus albidus	0.54 (0.36)	3.41 (0.29)	2.46 (0.03)	0.78 (0.42)	0.65 (0.41)	1.44 (0.92)
Cryptococcus hungaricus		1.83 (0.77)				
Leucosporidium scottii						1.15 (0.73)
Metschnikowia reukaufii	0.32 (0.32)	1.09 (0.67)	1.54 (0.63)			
Rhodotorula glutinis	0.32 (0.32)	2.38 (0.63)	0.99 (0.60)	0.17 (0.17)	0.26 (0.26)	0.44 (0.44)
Rhodotorula mucilaginosa		0.72 (0.72)				0.44 (0.44)
Sporobolomyces antarcticus[b]		0.78 (0.78)				
Trichosporon pullulans			0.48 (0.48)			
Zygosaccharomyces bisporus	0.28 (0.28)		1.65 (0.68)			
Unidentified		0.72 (0.72)				
Number of isolates	39	31	25	35	9	19
Diversity index[c]	0.13	0.23	0.24	0.09	0.33	0.32

[a] Standard errors of the means are presented in parentheses
[b] Identification indefinite
[c] Taxa diversity indexes were calculated as the number of taxa/number of isolates

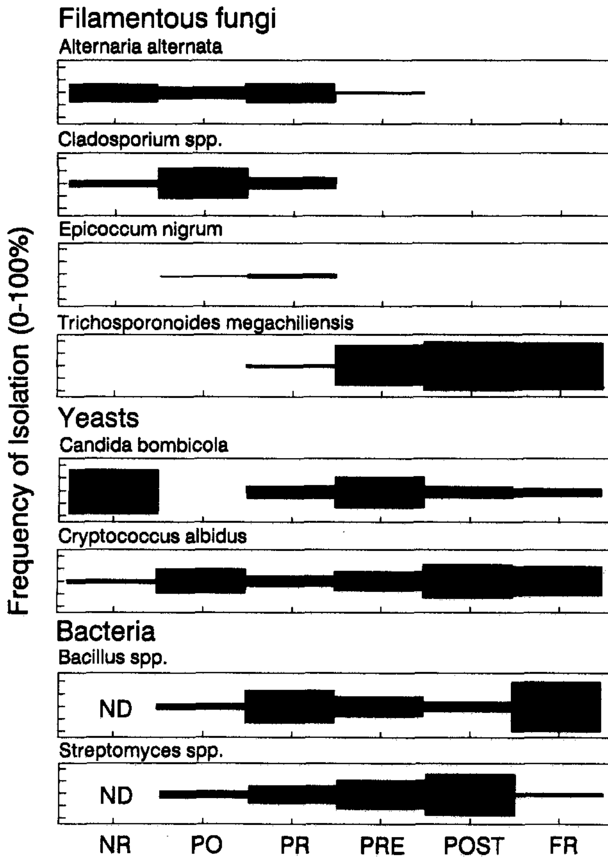

Fig. 1. Incidence of recovery (0–100%) of selected taxa of filamentous fungi, yeasts, and bacteria from alfalfa leafcutter bee (*Megachile rotundata*) nectar (NR), pollen (PO), provisions (PR), predefecation (PRE) and post-defecation (POST) larval guts, and frass (FR). No data (ND) is available for bacteria from nectar.

be identified. Since there was no difference in total colony forming units recovered or in diversity or variety of taxa between MEA and M30 for the nectar and provision treatments, data for the two media were combined. The smallest numbers of yeast isolates were recovered from post-defecation larvae and frass, and DI were highest for these treatments (Table 2). The yeasts most frequently recovered were *Candida bombicola* and *Cryptococcus albidus* (Fig. 1); *Rhodotorula glutinis* was also isolated consistently from all treatments, but at low levels.

A total of 47 taxa of filamentous fungi was isolated from the various samples (Table 3). Only 9 isolates, of which 5 were different taxa, were recovered from nectar (DI = 0.56). In contrast, 22 (DI = 0.11) and 31 (DI = 0.12) taxa were isolated from pollen and provisions, respectively. *Alternaria alternata, Cladosporium cladosporioides, Cladosporium herbarum, Epicoccum nigrum,* and *Penicillium chrysogenum* were frequently recovered. The diversity of taxa was lower from predefecation larvae (DI = 0.09), post-defecation larvae (DI = 0.04), and

Table 3. Prevalence of filamentous fungi recovered from nectar (NR), pollen (PO), provisions (PR), pre-defecation larvae (PRE; instar 2–3), post-defecation larvae (POST; instar 4–5), and frass (FR)

Species	Log cfu g^{-1} (fresh weight)					
	NR	PO	PR	PRE	POST	FR
Acremonium sp.		0.70 (0.70)[a]		0.17 (0.17)	0.39 (0.39)	
Alternaria alternata	0.55 (0.30)	4.20 (0.17)	3.44 (0.09)	0.80 (0.35)		
Arthrinium arundinis			1.26 (0.43)	0.15 (0.15)		
Aspergillus fumigatus			0.24 (0.24)			
Aspergillus ornatus			0.21 (0.21)			
Aspergillus subolivaceous		0.69 (0.69)	0.23 (0.23)			
Aspergillus versicolor				0.20 (0.20)		
Basipetospora chlamydosporis				0.16 (0.16)		
Beauveria bassiana		0.60 (0.60)				
Botrytis allii			0.29 (0.29)			
Camarosporium propinquum		0.69 (0.69)	0.21 (0.21)			
Cladosporium cladosporioides		4.36 (0.16)	2.91 (0.15)			
Cladosporium colocasiae[b]			0.32 (0.32)			
Cladosporium cucumerinum[b]		1.38 (0.62)	0.27 (0.27)			
Cladosporium herbarum	0.55 (0.29)	4.19 (0.14)	1.84 (0.41)			
Cladosporium macrocarpon		2.95 (0.33)	0.84 (0.43)			
Cladosporium sp.		0.70 (0.70)	0.21 (0.21)			
Cochliobolus sativa			0.24 (0.24)			
Dreschlera ravenelii		0.60 (0.60)				
Epicoccum nigrum		1.75 (0.90)	2.30 (0.40)			
Eurotium amstelodami	0.18 (0.18)		0.26 (0.26)			
Fusarium culmorum		0.70 (0.70)				
Mucor plumbeus			0.24 (0.24)		0.59 (0.38)	0.71 (0.71)
Penicillium chrysogenum		3.78 (0.15)	1.09 (0.46)	0.55 (0.28)		
Penicillium corylophilum						0.47 (0.47)

Penicillium crustosum	0.18 (0.18)		0.63 (0.42)	0.17 (0.17)		
Penicillium decumbens				0.45 (0.30)		
Penicillium granulatum						0.73 (0.73)
Penicillium implicatum				0.15 (0.15)		
Penicillium janczewskii		1.28 (0.79)				
Penicillium viridicatum						1.13 (0.69)
Phoma sp.	0.17 (0.17)		0.46 (0.31)			
Pleospora herbarum		0.71 (0.71)	0.52 (0.52)			
Pleospora infectoria			0.23 (0.23)			
Polyscytalum fecundissium		0.60 (0.60)	0.26 (0.26)			
Rhinocladiella atrovirens		0.60 (0.60)				
Rhizopus oryzae		0.70 (0.70)	0.85 (0.43)			
Scopulariopsis brevicaulis				0.16 (0.16)	0.49 (0.49)	
Tilletiopsis sp.		0.70 (0.70)				
Trichoderma viride			0.30 (0.30)			
Trichosporonoides megachiliensis			1.87 (0.42)	2.27 (0.51)	2.58 (0.71)	4.79 (0.49)
Ulocladium atrum		0.70 (0.70)	1.30 (0.44)			
Ulocladium botrytis			0.76 (0.39)			
Coelomycete A			0.23 (0.23)			0.53 (0.53)
Mycelia sterilia A		1.20 (0.73)	1.32 (0.44)	0.35 (0.23)	0.44 (0.44)	1.13 (0.69)
Mycelia sterilia B[c]					0.44 (0.44)	
Mycelia sterilia C[d]			0.46 (0.31)			
Number of isolates	9	200	256	135	135	165
Diversity index[e]	0.56	0.11	0.12	0.09	0.04	0.04

[a] Standard errors of the means are presented in parentheses
[b] Identification indefinite
[c] Arthroconidial basidiomycete
[d] Nonsporulating basidiomycete
[e] Taxa diversity indexes were calculated as the number of taxa/number of isolates

Table 4. Prevalence of bacteria recovered from pollen (PO), provisions (PR), pre-defecation larvae (PRE; instar 2–3), post-defecation larvae (POST; instar 4–5), and frass (FR)

Species	Log cfu g^{-1} (fresh weight)				
	PO	PR	PRE	POST	FR
Bacillus brevis		0.55 (0.55)[a]			0.59 (0.59)
Bacillus circulans		0.53 (0.53)			
Bacillus coagulans				0.51 (0.32)	0.62 (0.62)
Bacillus firmus	0.87 (0.87)	1.65 (0.67)	0.30 (0.30)	0.27 (0.27)	2.68 (0.69)
Bacillus lentus				0.27 (0.27)	
Bacillus licheniformis	1.96 (1.13)	2.91 (0.08)	0.77 (0.47)	0.29 (0.29)	3.19 (0.82)
Bacillus megaterium	0.98 (0.98)	0.60 (0.60)	0.74 (0.45)		1.36 (0.84)
Bacillus pumilus	0.87 (0.87)				2.81 (0.74)
Bacillus subtilis		0.55 (0.55)			
Bacillus thuringiensis		0.66 (0.66)			
Bacillus spp.	1.93 (1.11)	2.24 (0.56)	0.32 (0.32)		1.59 (0.99)
Corynebacterium aquaticum	1.21 (1.21)	0.54 (0.54)			
Corynebacterium group 1	3.21 (1.08)				
Corynebacterium group 2	4.11 (0.32)				
Corynebacterium group 3	2.94 (1.00)				
Erwinia amylovora	0.98 (0.98)				
Micrococcus kristanae	1.86 (1.07)				
Micrococcus roseus	3.00 (1.02)				
Micrococcus sedentarius	0.98 (0.98)				
Micrococcus spp.	3.89 (0.09)		0.30 (0.30)		
Pantoea agglomerans	0.98 (0.98)				
Pantoea dispersa	0.91 (0.91)				
Pseudomonas sp.	1.10 (1.01)				
Streptomyces group 1 (Grey)	1.93 (1.12)	2.28 (0.58)		1.51 (0.62)	1.46 (0.90)
Streptomyces group 2 (White)	3.97 (0.18)	2.37 (0.59)	1.32 (0.58)	0.28 (0.28)	0.61 (0.61)
Streptomyces group 3			0.33 (0.33)		
Unidentified	3.89 (0.09)				
Number of isolates	76	38	24	35	49
Diversity index[b]	0.26	0.30	0.29	0.17	0.18

[a] Standard errors of the means are presented in parentheses
[b] Taxa diversity indexes were calculated as the number of taxa/number of isolates

frass (DI = 0.04) than from pollen and provisions. From pre- and post-defecation larvae and frass,*Trichosporonoides megachiliensis* [34] was the most prevalent fungus (79–91% of the isolates; Fig. 1).

Twenty bacterial taxa were isolated from pollen and 11 from provisions (Table 4). *Corynebacterium* (≥4 taxa), *Pantoea agglomerans* and *P. dispersa, Erwinia amylovora*, and a *Pseudomonas* species were recovered only from these substrates. Although *Micrococcus* (≥3 taxa) was primarily isolated from pollen, one isolate was associated with the alimentary canal of a pre-defecation larva. Species of *Micrococcus* were identified as *M. kristanae, M. roseus,* and *M. sedentarius*. The unidentified species produced colonies that were either pale orange or cream, grew at 37°C, were catalase positive, obligate aerobes, did not produce acetoin, grew in

the presence of 7% sodium chloride, produced variable growth on citrate agar, were oxidase positive, and were resistant to Novobiocin and sensitive to Bacitracin. Only one species of *Corynebacterium*, *C. aquaticum*, could be identified using the API Rapid Coryne system. Other isolates were assigned to three taxonomic groups. Group 1 isolates were grey to white, catabolized nitrate, were positive for pyrazinamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, and esculin, variable for urease and gelatin hydrolysis, fermented glucose, ribose, xylose, mannitol, and sucrose. Group 2 isolates were white to pale orange, were positive for pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, α-glucosidase, esculin and urease, fermented glucose, ribose, xylose, mannitol, maltose, lactose, and sucrose. Group 3 isolates were yellow to green, positive for nitrate, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, α-glucosidase, and urease, but were nonfermentative. Most of the bacteria isolated from nectar did not survive long-term storage (>12 months) at 4°C; however, 12 of 49 isolates that could be recovered were identified as *Bacillus firmus*, *B. licheniformis*, *B. megaterium*, or *B. pumilus*.

The diversity of recovered bacterial taxa from post-defecation larval guts (DI = 0.17) and frass (DI = 0.18) was lower than from other substrates (Table 4). Species of *Bacillus* and *Streptomyces* were the most prevalent bacteria from larval alimentary canals and frass (Fig. 1); moreover, 2 species of *Bacillus* (*B. licheniformis* and *B. firmus*) and 1 species of *Streptomyces* (group 2) were recovered from all treatments. *Streptomyces* isolates were divided into either white or grey groups according to spore color "en masse" [42]. White taxa (group 2) formed straight or flexuous ("rectus flexibilis") chains of spores [46], catabolized casein, xanthine, and tyrosine, and assimilated glucose, L-arabinose, fructose, galactose, mannitol, and salicin. Grey taxa (group 1) formed flexuous chains of spores, catabolized casein and xanthine but not tyrosine, and assimilated glucose, rhamnose, and galactose. Assimilation of arabinose and salicin was variable. One grey isolate (group 3) was unique. It did not utilize xanthine and formed melanoid pigments on tyrosine agar, producing a brown, diffusing pigment and becoming brown-black in reverse. Observations of excised guts showed numerous rod-shaped bacilli on the surface of the gut epithelium (Fig. 2), and on the surface of pollen in the gut lumen. In contrast, spores or hyphae which could be verified as those of *Streptomyces* were not observed. Propagules of filamentous fungi were infrequently observed in the washed alimentary canals; a single conidium of *Scopulariopsis* is seen in Fig. 2 (*arrow*).

Microorganisms Associated with Diseased Larvae

The incidence of chalkbrood in larvae inoculated with *A. aggregata* ranged from 22.4–35.4%. Bacteria were more numerous in both chalkbrood cadavers and frass from infected larvae than from healthy larvae ($P = 0.0001$) and their frass ($P = 0.0249$) (Table 5). Numbers of filamentous fungi also were significantly greater ($P = 0.0018$) in chalkbrood cadavers than in healthy larvae. In contrast, there was no difference ($P = 0.34$) in numbers of filamentous fungal colony forming units in frass from healthy and diseased larvae. Yeasts were isolated infrequently from healthy larval guts and associated frass, and no yeasts were recovered from diseased larvae and their frass.

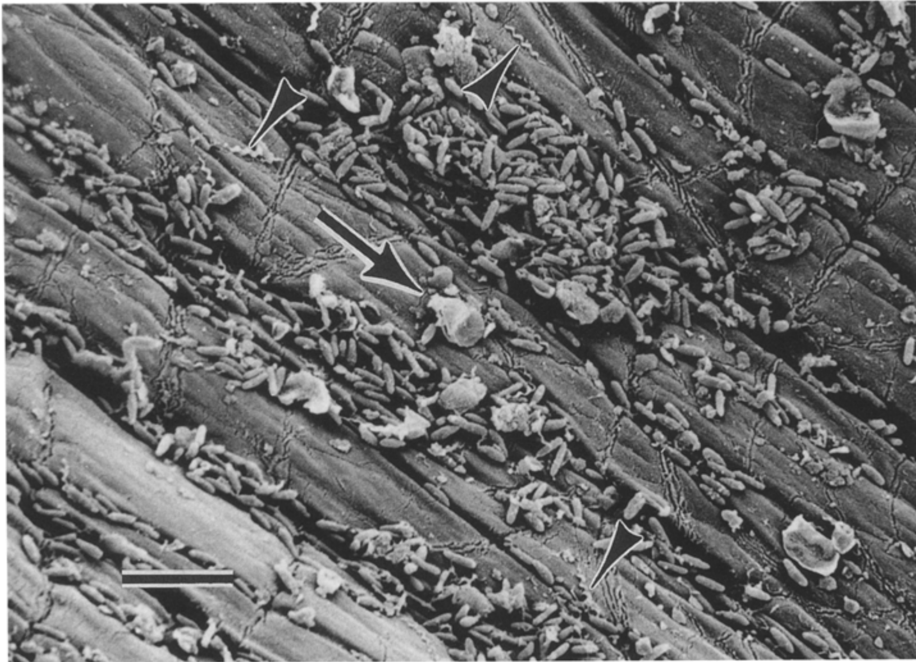

Fig. 2. Scanning electron micrograph of a washed alimentary canal of a pre-defecation alfalfa leafcutter bee (*Megachile rotundata*) larva showing numerous bacilli on epithelial tissue. The *arrow* points to a conidium of *Scopulariopsis*; *arrowheads* indicate spiral-shaped bacteria. *Bar* = 10 μm.

Table 5. Colony forming units of bacteria, filamentous fungi, and yeasts recovered from healthy larvae, *Ascosphaera aggregata*-infected (chalkbrood) larvae, and their frass

	log cfu g^{-1} (fresh weight)		
	Bacteria	Filamentous fungi	Yeasts
Larvae[a]			
Chalkbrood	1.80 (0.24)a[c]	4.49 (0.08)a[c]	0.00
Healthy	0.12 (0.12)b	1.99 (0.43)b	0.47 (0.24)
Frass[b]			
Chalkbrood	4.33 (0.24)a[c]	3.55 (0.17)a[c]	0.00
Healthy	3.44 (0.24)b	3.84 (0.25)a	1.16 (0.46)

[a] Data are the pooled results of three trials ($n = 10$)
[b] Data are the pooled results of two trials ($n = 7$)
[c] Means not followed by the same letter within each of the larval or frass treatment groups are significantly different as determined by a two-tailed *t*-test for log-transformed data. Standard errors of the means are presented in parentheses

Twenty-seven species of filamentous fungi were isolated and subsequently identified from healthy larvae, chalkbrood cadavers, and their frass (Table 6). Although *Aspergillus niger, Eurotium repens, T. megachiliensis,* an unidentified coelomycete, and a nonsporulating fungus (A) were recovered at greater than 1.0 cfu g^{-1}

Table 6. Prevalence[a] of filamentous fungi associated with healthy larvae, *Ascosphaera aggregata*-infected (chalkbrood) larvae, and their frass

	Larvae		Frass	
	Healthy	Chalkbrood	Healthy	Chalkbrood
Absidia corymbifera			+	+
Absidia glauca	+			
Absidia spinosa	+			
Arthrinium arundinis		+	+	
Ascosphaera apis	+			
Ascosphaera atra			+	+
Aspergillus fumigatus				+
Aspergillus niger			++	
Aspergillus sydowii			+	
Aspergillus terrus[b]			+	
Alternaria alternata	+			
Beauveria brongniartii		+		
Chaetomium globosum		+		
Chaetomium indicum		+		+
Eurotium amstelodami		+	+	+
Eurotium chevalieri			+	+
Eurotium repens			++	++
Penicillium brevicompactum			+	
Penicillium chrysogenum				+
Preussia fleischhakii			+	
Preussia terricola			+	
Rhizopus oryzae	+		+	
Scopulariopsis acremonium		+		
Scopulariopsis brevicaulis	+			
Sporormiella intermedia	+	+	+	
Trichosporonoides megachiliensis	++	+++	+++	+
Ulocladium botrytis	+			
Coelomycete spp.	+		++	+
Mycelia sterilia A	+	+	+	++
Mycelia sterilia B[c]		+	+	
Number of isolates	74	51	110	34
Diversity index[d]	0.15	0.20	0.16	0.32

[a] + $< \log 1.0$ cfu g^{-1}; ++ $> \log 1.0 < \log 2.0$ cfu g^{-1}; +++ $> \log 2.0 < \log 3.0$ cfu g^{-1}
[b] Identification indefinite
[c] Arthroconidial basidiomycete
[d] Taxa diversity indexes were calculated as the number of taxa/number of isolates

from the various samples, most fungi were recovered at low frequencies ($<$1.0 log cfu g^{-1}). Several taxa were isolated from one but not both treatments (15 for larval guts and 13 for frass). The DI for healthy larvae was 0.15 and for chalkbrood cadavers was 0.20. Larvae developing chalkbrood produced less frass than healthy larvae and the diversity of taxa from chalkbrood cadavers was lower (DI = 0.16) compared to healthy larvae (DI = 0.32). Only *A. niger* and *T. megachiliensis* were conspicuously more prevalent in one of the treatments; both were two orders of magnitude higher in frass from healthy larvae than from diseased larvae.

Discussion

Abundant microorganisms were recovered from larval provisions and its components (nectar and pollen), several of which have been implicated in the spoilage of leafcutter bee provisions [26]. In general, the diversity of aerobic microorganisms was lower from alimentary canals and frass than from nectar, pollen, and provisions. Yeasts were recovered frequently from nectar in bee crops and in pollen from abdominal scopae, but rarely from guts or frass. *Candida bombicola,* a species originally isolated from honeybees [1], was the most prevalent taxon (Fig. 1). Several yeasts that we recovered from pollen or provisions, including *Cryptococcus albidus, C. hungaricus, M. reukaufii,* and *R. glutinis,* have also been previously reported from bees or their provisions [2, 3, 12]. Leafcutter bee provisions comprise alfalfa pollen and nectar [32]. Except for *Leucosporidium scottii,* found only in frass, and *Trichosporon pullulans,* found only in provisions, all yeasts that were recovered from larval guts and frass were also recovered in pollen or in nectar. This suggests that most yeasts associated with alfalfa leafcutter bee larvae originate from pollen or nectar. Yeasts are commonly associated with flowers [39], including pollen [12] and nectar [2, 3, 20, 45], and also have been isolated from crops and honey stomachs of various bees [2, 3, 19, 45]. Yeasts do not appear to survive passage through the leafcutter bee larval alimentary canal. We previously showed [35] that none of 7 yeasts (*C. bombicola, C. glabrata, C. albidus, L. scottii, R. glutinis, R. mucilaginosa,* and *Zygosaccharomyces bailii*) ingested by near-axenic larvae could be subsequently recovered in frass. Similar findings have been reported for honeybees in which yeasts are rarely isolated from adult intestinal contents [15] or larval frass [16]. They are found in the alimentary canal of worker honeybees only after they have been fed antibiotics or herbicides [22, 19], and may only be present in stressed bees [10].

Although few filamentous fungi were found in nectar from bee crops, they were isolated frequently from pollen and provisions. The predominant taxa from pollen were *Alternaria alternata, Cladosporium* spp., *Epicoccum nigrum* (Fig. 1), and *Penicillium chrysogenum.* These taxa, comprising 92% of the isolates, are commonly recovered from phylloplanes [31], including alfalfa [43], and from pollen carried by honeybees [23]. Larval provisions (DI = 0.12) contained a diversity of fungi similar to pollen (DI = 0.11). With the exception of *Trichosporonoides megachiliensis* and *Arthrinium arundinis,* 71% of isolates found at high frequencies in provisions were similarly isolated from pollen. It appears that pollen, not nectar, is the primary source of filamentous fungi in provisions. However, nest cells (composed of flower and leaf pieces) may also represent a source of microorganisms, since Goerzen [26] has isolated similar phylloplane fungi from their surfaces.

Although the diversity of filamentous fungi in larval provisions was relatively large, both total numbers and diversity appeared to decrease progressively following ingestion by larvae, with a DI of 0.12 in provisions, 0.09 in pre-defecation larval guts, and 0.04 in post-defecation guts. Insect guts are considered to be a relatively inhospitable environment for fungi [9], and evidence suggests that phylloplane fungi do not contribute substantially to the microflora of the alfalfa leafcutter bee larval alimentary canal. Indeed, ubiquitous phylloplane fungi (i.e., *Alternaria, Cladosporium,* and *Epicoccum*), prevalent on pollen and in provisions, comprised only 6.7% of the isolates from pre-defecation larval guts, and none was

isolated from either post-defecation larval guts or frass (Fig. 1). However, Gilliam and Prest [15] isolated species of *Aspergillus, Cladosporium,* and *Penicillium* from the intestinal contents or worker honeybees, and we previously demonstrated that we could recover species of *Aspergillus, Mucor, Penicillium, Rhizopus,* and *Scopulariopsis* from leafcutter bee frass when their spores were ingested by near-axenic larvae [35]. Moreover, representatives of these genera, along with *Absidia, Alternaria, Beauveria, Chaetomium, Eurotium, Preussia, Sporormiella,* and *Ulocladium,* were isolated from larvae and/or frass in the present study, most at low frequencies. Although the activity of these fungi within the alimentary canal is unknown, it seems likely that a relatively small number of propagules survive passage through the gut as latent propagules (e.g., *Scopulariopsis;* see Fig. 2).

A black yeast-like fungus, named by us as *Trichosporonoides megachiliensis* [34], was the most prevalent filamentous fungus recovered from pre- and post-defecation guts and frass, suggesting that it is the only fungus which may be considered part of the normal mycoflora of the bee gut. Moreover, it comprised only 5.5% of the isolates from larval provisions and was not isolated from nectar or pollen (Fig. 1). Evidence suggested that is was introduced into provisions at low frequencies by infested adults [26, 34]. A previous study showed that the incidence of chalkbrood in agnotobiotic larvae was 26% higher than in near-axenic larvae, suggesting that the gut microflora stimulates *A. aggregata* [35]. Leafcutter bee larval provisions possess a high sugar content (66%) which is composed primarily of glucose, fructose, sucrose, and palatinose [32], and *T. megachiliensis* is capable of fermenting and/or assimilating these sugars [34]. Since germination of *Ascosphaera* ascospores is stimulated by increased levels of CO_2 [30, 37], one hypothesis was that the prevalence of *T. megachiliensis* in larval guts could stimulate ascospore germination and thus contribute to the severity of chalkbrood. However, when *T. megachiliensis* was co-inoculated with ascospores of *A. aggregata,* there was no increase in chalkbrood between these larvae and those inoculated with the pathogen alone [35]. Although *T. megachiliensis* did not affect chalkbrood, its influence on the ecology of leafcutter bee larval guts is uncertain.

Bacteria were recovered from all substrates. The most common genera isolated from pollen and provisions were *Bacillus, Corynebacterium, Micrococcus,* and *Streptomyces.* Once ingested by larvae, survival in the alimentary canal appeared to be limited to species of *Bacillus* and *Streptomyces* (Fig. 1). Scanning electron microscopy (Fig. 2) of the gut showed mucosa-associated microorganisms, principally rod-shaped bacteria, some of which were coryneform or endospore-forming bacilli. The aerobic conditions and media used would preclude the isolation of anaerobic or fastidious organisms, and spiral-shaped bacteria were also present (Fig. 2, *arrowheads*). Goerzen [26] found *Bacillus* species to be common in/on adult leafcutter bees, provisions, and nest material. He also frequently isolated species of *Enterobacter and Pseudomonas* from these substrates, but these taxa were not or only infrequently isolated by us. *Bacillus* species have also been reported from honeybee adult guts [14] and larval frass [16], intestinal contents of worker and queen bees [11, 18], honeybee bread [13], and in provisions of other bees [21, 24, 25]. The metabolic activity of *Bacillus* species in provisions has been postulated to inhibit spoilage microorganisms [24, 25]. *Streptomyces* species also have been reported to be found in the intestines of feral bees [17] and in honeybee frass [16]. Although the complexity of *Streptomyces* identification did not allow us

to identify our taxa to species, we delimited three taxonomic groups. These were recovered at lower frequencies from guts and frass than from pollen or provisions. We showed previously that isolates of both *Streptomyces* and *Bacillus* survived passage through the alimentary canal [35]. Although most did not influence chalkbrood, an isolate of *B. brevis* and *Streptomyces* (group 2) increased chalkbrood slightly [35].

Due to our inability to remove intact alimentary canals from post-defecation larvae (gut contents voided), it was necessary to macerate larvae to isolate microorganisms from the alimentary canal. However, by calculating colony forming units per gram fresh weight of larvae, populations in alimentary canals would be underestimated relative to other substrates. This technique also rests on the assumption that hemolymph is sterile and that the subsequent immune response of the hemolymph in vitro is negligible. We found that larval hemolymph was sterile, and we chilled the buffer prior to maceration in an attempt to reduce a possible immune reaction. However, encapsulation of bacteria and fungi in hemolymph can occur rapidly [5], and the degree to which propagules were affected by leafcutter bee larval hemolymph was not addressed.

Populations of bacteria and filamentous fungi, but not yeasts, were higher from *Ascosphaera aggregata*-infected larvae than from healthy larvae (Table 5). Populations of yeasts and fungi in frass and guts of post-defecation larvae not developing chalkbrood (Table 5) were comparable to, although smaller than, those found previously (Table 1). Although bacterial populations in frass from healthy larvae were similar to those from frass of non-inoculated larvae, populations in post-defecation larval guts were lower in healthy larvae than in non-inoculated larvae (Tables 1 and 5). Reasons for this discrepancy are unknown.

We previously showed no difference in development between agnotobiotic and near-axenic larvae (microbial populations reduced by more than 97%) [33], suggesting that microorganisms are not required for larval nutrition. However, the presence of microorganisms in alimentary canals was associated with a slight increase in the susceptibility of larvae to *A. aggregata* [35]. Since populations of bacteria and filamentous fungi were higher in alimentary tracts of chalkbrood cadavers than in healthy larvae, it is possible that larger microbial populations predispose larvae to infection by *A. aggregata*. An alternative explanation is that *A. aggregata* provides access to additional substrates required for microbial growth by breaching the alimentary canal and killing the larva. Support for this explanation comes from our finding that there were no differences in the sizes of microbial populations of filamentous fungi in frass from healthy and diseased larvae, whereas populations were 99.7% smaller in the guts of healthy larvae. However, bacterial populations also were significantly larger in frass from infected larvae than from healthy larvae.

With the exception of *T. megachiliensis* and *Aspergillus niger* in frass from healthy larvae, no taxon was conspicuously present or absent from infected larvae, healthy larvae, or their frass (Table 6). *Aspergillus niger,* prevalent in frass of healthy larvae inoculated with *A. aggregata,* was not previously isolated in non-inoculated larvae or their frass (Table 3). Goerzen [26] also frequently recovered this fungus from larval cadavers in Saskatchewan. *Ascosphaera atra* and *A. apis* were recovered from healthy larvae and frass, respectively; both taxa have previously been isolated from alfalfa leafcutter bees [4]. Copious numbers of subcutane-

ous ascomata of *A. aggregata* were present in chalkbrood cadavers; however, this species was not isolated since its ascospores do not germinate readily in ambient CO_2. Additional potential entomopathogens recovered included *A. niger, Beauveria bassiana, B. brongniartii, P. chrysogenum,* and *Bacillus thuringiensis.* Several coprophilous fungi, including *Preussia fleishhakii, P. terricola,* and *Sporormiella intermedia,* were also isolated from larval guts/frass. To our knowledge this is the first report of their isolation from insect frass. We did not attempt to isolate anaerobic microorganisms, and the contribution of both aerobic and anaerobic microorganisms to the ecology of the alimentary canal of leafcutter bee larvae requires further study.

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