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Influence of Microorganisms on Alfalfa Leafcutter Bee (*Megachile rotundata*) Larval Development and Susceptibility to *Ascosphaera aggregata*

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The influence of microorganisms associated with alfalfa leafcutter bee (*Megachile rotundata*) larvae on chalkbrood caused by *Ascosphaera aggregata* was determined by comparing disease in agnotobiotic and axenic larvae, with and without an introduced microflora (gnotobiotic). Bacterial and fungal populations in the frass of larvae reared on natural provisions (agnotobiotic) were log 4.24 and log 3.90 cfu/g of frass, respectively. No fungi were recovered from the frass of larvae reared on γ-irradiated provisions, but bacterial populations averaged log 0.89 cfu/g (near-axenic). Following ingestion of ascospores of *A. aggregata*, the incidence of chalkbrood was significantly higher in agnotobiotic (67.6%) than near-axenic (50.2%) larvae, but the time to death of infected larvae was the same for both. Microorganisms were inoculated onto the surface of γ-irradiated provisions adjacent to leafcutter bee eggs and were ingested by larvae; 11 of 13 bacteria, 9 of 10 filamentous fungi, but 0 of the 7 yeasts were recovered from frass (gnotobiotic). Populations of inoculated bacteria and filamentous fungi recovered in frass ranged from log 5.40 to 7.36 cfu/g and log 5.21 to 6.52 cfu/g of frass, respectively. Of the 20 microorganisms recovered in frass, only *Bacillus brevis* and a *Streptomyces* sp. significantly increased the incidence of chalkbrood (74.0 and 74.8%, respectively) when larvae were coinoculated with ascospores of *A. aggregata* (1:1 ratio); the incidence of chalkbrood in larvae inoculated with the pathogen alone was 52.1%. None of the microorganisms themselves appeared to be deleterious to larvae. There were no significant differences in microbial populations in frass from healthy larvae or chalkbrood cadavers; however, noninoculated bacteria were associated with all nine fungal treatments. The results of this study suggest that microorganisms associated with the alimentary tracts of alfalfa leafcutter bee larvae have a minimal impact if any on chalkbrood.

KEY WORDS: ***Ascosphaera aggregata; Megachile rotundata;* alfalfa leafcutter bee; chalkbrood; indigenous microflora; γ-irradiated; antagonism.**

INTRODUCTION

Chalkbrood, caused by the fungus *Ascosphaera aggregata*, is a serious disease of alfalfa leafcutter bees (*Megachile rotundata*) in western North America. Chalkbrood is initiated by ascospores which are introduced into larval provisions by adult bees (Vandenberg *et al.*, 1980). The ascospores ingested by larvae germinate in the midgut (putatively stimulated by elevated CO_2 concentrations), and hyphae penetrate the hemocoel eventually killing the larvae (Kish, 1980; Vandenberg and Stephen, 1982, 1983; McManus and Youssef, 1984). Once the hemocoel has been colonized by the fungus, subcutaneous ascogenesis may occur resulting in the formation of sporulating or partially sporulating chalkbrood cadavers (Skou and Holm, 1989; Goettel *et al.*, 1991). Following disruption of the cuticle, ascospores are released from the cadaver and disseminated on nesting materials and/or emerging adults (Vandenberg *et al.*, 1980).

The indigenous microflorae of mammalian gastrointestinal tracts may provide an inherent level of protection from potential pathogens. This is supported by observations of increased resistance to infection in conventionally reared individuals relative to germ-free ones (Tannock, 1984). Further, the use of antibiotics has been associated with decreased populations of nontarget microorganisms and the development of iatrogenic diseases (Finland, 1951; George *et al.*, 1979). Exposure to antibiotics and agrochemicals has been shown to alter the composition of gut microorganisms of many insects, including adult honeybees (Gilliam and Morton, 1978; Gilliam *et al.*, 1974a,b, 1977), but the impact of altered microflorae on entomopathogens and/or disease development has received little attention. Some evidence suggests that the indigenous microflorae of insect alimentary canals also affect patho-

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gens. The microflora of greater wax moth larvae is naturally monobiotic for *Streptococcus faecium* (Jarosz, 1983). The introduction of several entomopathogenic bacteria (*Pseudomonas aeruginosa, Proteus mirabilis,* and *Bacillus thuringiensis*) into larval guts did not initiate disease (Jarosz, 1979). Although the evidence was circumstantial, inhibition of the inoculated bacteria was attributed to the presence of *S. faecium* (Jarosz, 1979). By comparing the excretion of *Salmonella typhimurium* in frass from houseflies, with and without an introduced bacterial flora, Greenberg *et al.* (1970) determined that the excretion of the pathogen was substantially reduced in flies inoculated with bacteria. Dillon and Charnley (1986) reported that *Metarhizium anisopliae* was inhibited in starved, conventionally reared desert locusts, but not in axenic ones. This was attributed to the bacteria-facilitated release of inhibitory phenolic compounds from plant tissues (Dillon and Charnley, 1988).

The influence of microorganisms on chalkbrood of alfalfa leafcutter bees is not clear. Although chalkbrood is markedly increased and larval development delayed in larvae reared on propylene-oxide-sterilized provisions, this may be due to inadequate detoxification rather than to the absence of viable microorganisms in provisions (Inglis *et al.,* 1992a). The incidence of chalkbrood is also higher in larvae reared on a sterile artificial diet (Goettel *et al.,* 1993) and, because the alimentary canals of leafcutter bee larvae reared on natural provisions contain greater numbers of microorganisms than those maintained on the artificial diet (Inglis *et al.,* 1992b), one hypothesis is that microorganisms in the gut influence chalkbrood. The artificial diet differs substantially from the natural provisions in its chemical composition (Inglis *et al.,* 1992a), however, and the differential susceptibilities of larvae may be due to nutrition or to chemical stressors present in the diet.

Since most microorganisms found in larval guts originate in the provisions (Inglis *et al.,* unpublished), a nondestructive method for the sterilization of provisions was required to obtain axenic larvae. Gamma (γ)-irradiation of provisions at a dose of 15 kGy effectively sterilized them and did not affect larval development, and although larval guts were not axenic, microbial populations were reduced by more than 97% compared with those of larvae maintained on natural provisions (Inglis *et al.,* 1992b). Using γ-irradiated provisions the objective of this study was to assess the influence of microorganisms associated with alfalfa leafcutter bees on larval development and susceptibility to *A. aggregata*. To meet this objective, we (1) compared disease in agnotobiotic larvae with larvae reared on γ-irradiated provisions to elucidate the role of the indigenous microflora, (2) verified that monobiotic or near-monobiotic conditions could be achieved by the per os inoculation of larvae with selected microorganisms, and (3) assessed whether the selected microorganisms influenced development and/or chalkbrood in the monobiotic larvae.

MATERIALS AND METHODS

Collection and Preparation of Microogranisms

Ascosphaera aggregata. Asci (spore balls) collected from sporulating chalkbrood cadavers from Idaho were provided by J. D. Vandenberg (USDA, Utah State University, Logan, UT) and maintained at 5°C. Immediately prior to use, ascospores were released by crushing the spore balls between two sterile glass slides in 0.02 M BES (*N,N*-bis (2-hydroxyethyl)-2-aminoethane sulfonic acid)–0.017 mM DOSS (dioctyl sulfosuccinate), and 0.15 M saline buffer (pH 7.1). Ascospores were then suspended in BES, their concentrations estimated with a hemocytometer and adjusted to 2×10^7 or 1×10^7 ascospores/ml. Ascospore viability, determined in a high CO_2 environment as previously described by Goettel *et al.* (1991), was 25%. Microorganisms associated with ascospores were enumerated by spreading 100-μl aliquots of the ascospore suspension (10^7 ascospores/ml) onto nutrient agar (NA; Difco) containing 50 mg/liter of nystatin, and onto potato dextrose agar (PDA; Difco) containing 100 mg/liter of tetracycline. Cultures were maintained at 25°C in the dark and colony-forming units (cfu) enumerated at 3–4 days. Data were calculated as the mean number of microbial cfu per 10^4 ascospores.

Test microorganisms. Selected microorganisms associated with alfalfa leafcutter bees (Inglis *et al.,* unpublished), deposited at the University of Alberta Microfungus Collection and Herbarium (UAMH), were used. Bacterial isolates included *Bacillus coagulans* (UAMH 6937), *B. lentus* (6944), and *Streptomyces* sp. (6912) from larval gut contents; *B. brevis* (6938), *B. firmus* (6925, 6940, and 6942), *B. licheniformis* (6929 and 6932), *B. megaterium* (6927), *B. pumilus* (6941), and *Streptomyces* sp. (6915) from frass; and *Corynebacterium* group B sp. (6884) from alfalfa (*Medicago sativa*) pollen. Yeast isolates included *Candida bombicola* (7074), *Cryptococcus albidus* (7076), *Leucosporidium scottii* (7078), *Rhodotorula glutinis* (7080), *R. mucilaginosa* (7081), and *Torulopsis glabrata* (7075) from frass and *Zygosaccharomyces bailii* (7083) from provisions. Filamentous fungal isolates included *Aspergillus versicolor* (6984), *Mucor plumbeus* (7013), *Rhizopus oryzae* (7019), *Scopulariopsis brevicaulis* (6877), *Stachybotrys chartarum* (7044), and *Trichosporonoides megachiliensis* (6823) (Inglis *et al.,* 1992c) from larval gut contents and *Aspergillus niger* (7040), *Penicillium granulatum* (6879), *Penicillium viridicatum* (7039), and *T. megachiliensis* (6821) from frass.

Cultures were established from freeze-dried stock material. Bacteria were grown on NA, yeasts on malt extract agar (MEA; consisting of 20 g Difco malt extract, 20 g glucose, 1 g peptone, 15 g agar in 1 liter of deionized water), and filamentous fungi on PDA at 25°C in the dark. Propagule suspensions were prepared by flooding 2- to 10-day-old cultures twice with 4 ml of BES, agitating with a sterile glass rod, and collecting the suspension. The suspensions were then centrifuged at 3726*g* for 10 min, the supernatant was removed, the propagules were resuspended in BES, and the process was repeated. All propagule concentrations were estimated with a hemocytometer and adjusted to 2×10^7 or 1×10^7 propagules/ml. Viabilities were determined by diluting the suspensions in a 10-fold dilution series; spreading 100-μl aliquots onto NA, MEA, or PDA; incubating at 25°C for 72 hr; and enumerating the number of cfu at the dilution yielding 20–200 cfu/dish. Propagule viability for all microorganisms were greater than 75%.

Collection and Preparation of Leafcutter Bee Eggs and Provisions

Leafcutter bee nest cells were collected from hives in an irrigated field of alfalfa near Lethbridge, Alberta, in July and August, 1991. Cells were removed from the hives and leaf caps removed, and cells containing eggs were placed in 96-well tissue culture plates and transported to the laboratory. Eggs were transferred onto a 0.5% water agar medium (WA) and were used immediately or stored at 5°C for a maximum of 24 hr. Following egg removal, provisions were either maintained at 5°C or transported to Pinawa, Manitoba (AECL Research), where they were sterilized with γ-irradiation at a dose of 15 kGy using a I-10/1 linear accelerator (Inglis *et al.*, 1992b). Prior to transfer onto provisions, eggs on the surface of the WA medium were submerged in 0.3% (w/v) sodium dichloro-*s*-triazine (dry chlorine) (Fichter *et al.*, 1981) in BES with 0.01% Tween 80 (BES–Tween) for 1 min and then rinsed with BES–Tween alone. The surface-treated eggs were then aseptically placed onto the surface of provisions in the γ-irradiated or untreated bee cells housed in the tissue culture plates.

Influence of the Indigenous Gut Microflora on Chalkbrood

Surface-treated eggs were placed on either untreated or γ-irradiated natural provisions with 12–16 individuals in each of four replicates arranged as a completely randomized design (CRD). A 2-μl aliquot containing 10^4 ascospores of *A. aggregata* was then placed adjacent to each egg and plates were incubated at 30 ± 1°C under a 16-hr photoperiod. Eggs collapsing within the first 2 days were considered to be either mechanically or chemically damaged during transfer and were replaced. Eggs subsequently dying were not included in the analyses. Larval development was recorded daily for 15 days at which time frass was aseptically collected from 3 to 4 randomly selected individuals per replicate and microorganisms were enumerated. Cocoons were opened, if necessary, and the incidence of sporulating chalkbrood (presence of subcutaneous ascomata) corresponding to categories 1 and 2 (Skou and Holm, 1989) in larvae reared on both the untreated and γ-irradiated provisions was recorded. The incidence of other mortality, consisting of nonsporulating (category 3) and greasy brown (category 4) cadavers, also was determined. The experiment was conducted three times.

Effect of Applied Microorganisms on Larval Development and Chalkbrood

The influence of individual microorganisms on larval development was tested. However, due to the shortage of eggs available for use in August, only 20 (11 bacteria, 5 yeasts, and 4 filamentous fungi) of the 30 test isolates were included in this experiment. A 2-μl aliquot of each propagule suspension (10^4 propagules) was placed adjacent to each of 12 eggs per replicate on γ-irradiated provisions. The experiment was arranged as a CRD with four replicates per treatment. The control treatment was inoculated with 2 μl of BES alone. Eggs were maintained and collapsed eggs replaced as previously described. Larvae were observed daily for 15 days and the time from hatching (instar 1) to defecation and initiation and termination of cocoon construction were recorded. At 15 days, frass was collected aseptically from three to five individuals per replicate per treatment and microorganisms were enumerated. Frass samples were air-dried, sputter-coated with gold, and observed with a Hitachi S-570 scanning electron microscope at an accelerator voltage of 10 kV. Following collection of frass, cocoons were opened and mortality was recorded.

To evaluate the influence of 30 microorganisms on chalkbrood, co-inoculum propagule suspensions were prepared by combining a 100-μl aliquot of the *A. aggregata* ascospore suspension (2×10^7 ascospores/ml) with a 100-μl aliquot of a propagule suspension (2×10^7 propagules/ml) of the test microorganism. The control treatment was *A. aggregata* alone. A 2-μl aliquot of the co-inoculum was placed adjacent to each of 12 eggs per replicate on γ-irradiated provisions. There were four replicates per treatment arranged as a CRD. Plates were maintained and collapsed eggs replaced as reported above. Larvae were observed daily for 15 days, whereupon frass was collected aseptically from three to five sporulating cadavers and two to three healthy prepupae per replicate per treatment and mi-

croorganisms were enumerated. Cocoons were opened if required and mortality was recorded.

Enumeration of Microorganisms in Frass

Frass samples were weighed to 1 mg, suspended in 0.5 ml of 10 mM phosphate buffer with 0.01% Tween 80 (pH 7), and macerated, and the homogenate was diluted two times in a 10-fold dilution series. Aliquots of 100 μl from each dilution were then spread onto NA amended with nystatin for the recovery of bacteria and onto MEA and PDA amended with tetracycline for the recovery of yeasts and filamentous fungi, respectively. Cultures were incubated aerobically at 25°C for 3–4 days and cfu were enumerated at the appropriate dilution and calculated as $\log_{10}$ cfu per gram fresh weight of frass. In addition to total cfu counts, isolates from each replicate were collected randomly for identification. The mean weight of frass produced by individual larvae was 88 (0.074) mg (n = 10 larvae).

Data Analysis

Computations were performed with Statistical Analysis System Software (SAS Institute Inc., Cary, NC) using the ANOVA, GLM, and TTEST procedures. Normality of data was examined with residual plots; where appropriate, transformations were used to normalize data. Prior to pooling data, homogeneity of variance was checked according to Snedecor and Cochran (1980). Standard errors of means were calculated for individual treatments and are presented in parentheses. Where applicable, means were compared to the control treatment using a Dunnett's test (α = 0.05). Populations of bacteria and fungi in frass from healthy larvae and from those with chalkbrood were compared as a 2 (substrate) × 20 (microorganism) factorial arranged as a CRD. Total populations of cfu in frass were calculated as (antilog cfu/g in frass) × (mean wt frass/larva).

RESULTS

Influence of the Indigenous Gut Microflora on Chalkbrood

Bacterial and fungal populations in the frass of larvae reared on natural provisions were log 4.24 and log 3.90 cfu/g, respectively. Bacterial populations were reduced by 99.9% and no fungi were recovered from the frass of larvae reared on γ-irradiated provisions. Ascospore inoculum was not axenic; mean numbers of bacteria and fungi associated with ascospores were 0.13 (0.04) and 0.24 (0.06) cfu/10^4 ascospores (n = 12 replicates), respectively. Reduction of the gut microflora was associated with a decreased (P = 0.04) incidence of chalkbrood (Table 1). There were no differences in egg mortality (P = 0.62), other larval mortality (P = 0.10), or average time to death (P = 0.45) between the two diet treatments.

Effect of Applied Microorganisms on Larval Development and Chalkbrood

A total of 20 of the 23 isolates of bacteria and filamentous fungi applied onto γ-irradiated provisions adjacent to eggs were ingested by larvae and subsequently recovered in frass as bacterial cells or fungal propagules (Figs. 1 and 2). the only isolates not recovered were *Corynebacterium* sp., *Streptomyces* sp. 6912, and *S. chartarum*. None of the yeasts applied were recovered in frass. Inoculated bacteria ranged from log 5.40 to log 7.36 cfu/g of frass, and filamentous fungi ranged from log 5.21 to log 6.62 cfu/g (Tables 2 and 3). Examination of frass indicated the predominance of pollen and the absence of the nectar component; bacteria were observed on pollen surfaces (Fig. 2). Bacterial contaminants were recovered from the frass of larvae inoculated with fungi; in six instances the bacterial populations were larger (α = 0.05) than those of the near-axenic larvae. No fungal contaminants were isolated from the frass of larvae inoculated with bac-

TABLE 1

The Influence of γ-Irradiated Provisions on Alfalfa Leafcutter Bee Mortality Caused by *Ascosphaera aggregata*[a]

Diet	% Chalkbrood	% Other mortality	Bacteria (log cfu/g fresh wt frass)	Fungi (log cfu/g fresh wt frass)	Time to death[b] (days)
γ-irradiated	50.2 (4.9)a[c]	15.6 (3.6)a[c]	0.89 (0.47)a[c]	0.00a[c]	7.1 (0.05)a[c]
Nonsterilized	67.6 (6.3)b	15.4 (5.9)a	4.24 (0.11)b	3.90 (0.77)b	7.3 (0.04)a

[a] Larvae were inoculated with 10^4 ascospores of *A. aggregata*. Mortality and microbial cfu data are the pooled results of three trials (n = 93–98 larvae/treatment).

[b] Time to death was determined in two trials (n = 32–39 larvae/treatment).

[c] Standard errors are given in parentheses following means. Means not followed by the same letter were significantly different (α = 0.05) as determined by a two-tailed t test for arcsine-transformed (sporulating cadavers, total mortality), logarithmic-transformed (bacterial and fungal cfu), and untransformed (other mortality and time to death) data. With the exception of the microbial cfu data, untransformed means and standard errors are presented.

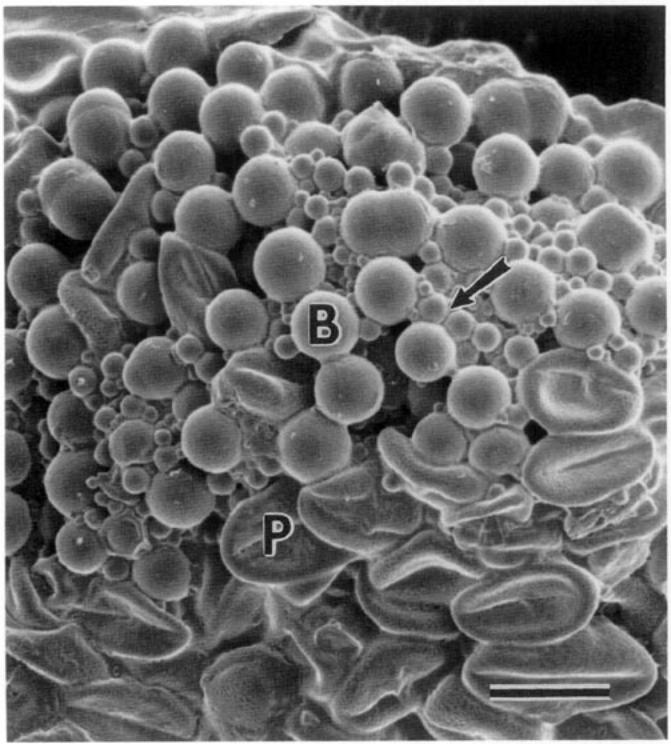

FIG. 1. Frass from larvae inoculated with conidia of *T. megachiliensis* (UAMH 6823). Note mature blastoconidia (B) and pollen (P). The arrow points to young blastoconidia in acropetal chains. Bar = 20 μm.

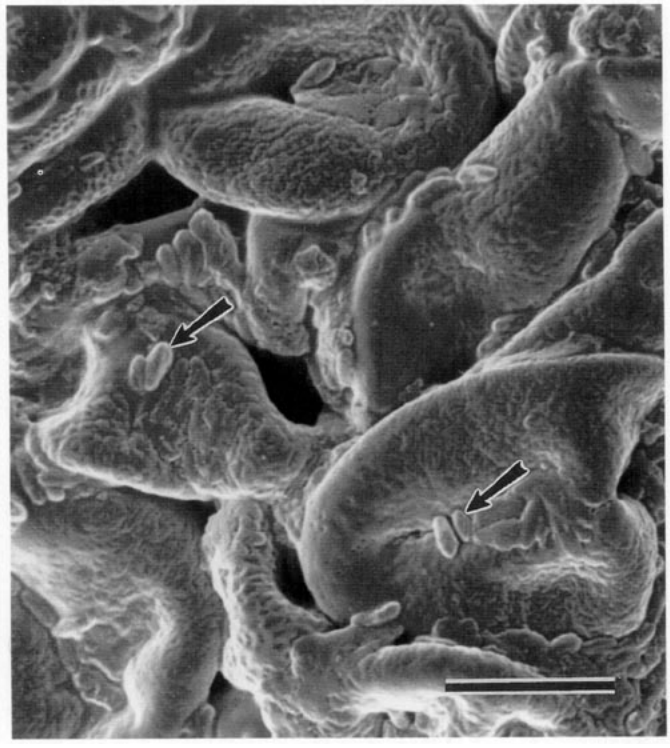

FIG. 2. Frass from larvae inoculated with *B. firmus* (UAMH 6942). Arrows point to bacilli on the surfaces of pollen. Bar = 10 μm.

teria and we did not observe fungal propagules in such frass (Fig. 2).

Mortality of eggs on provisions infested with microorganisms was not significantly ($P = 0.52$) different from that of the control treatment and ranged from 25.0 (14.8) to 45.9 (4.2)%. None of the 20 isolates tested without *A. aggregata* affected total mortality ($P = 0.07$), time to defecation ($P = 0.26$), or completion of cocoon construction ($P = 0.057$) (Table 2). Time to initiation of spinning was similarly not influenced ($\alpha = 0.05$) except when *B. lentus* was inoculated (8.8 (0.5) days). Although larvae were not inoculated with *A. aggregata,* the incidence of chalkbrood ranged from 0.0 to 7.2%.

Of the 30 microorganisms co-inoculated with *A. aggregata,* only *Streptomyces* sp. 6915 and *B. brevis* affected ($\alpha = 0.05$) the incidence of chalkbrood when co-inoculated with *A. aggregata* (Table 3); chalkbrood incidence was 30% greater than that in the control treatment. None of the isolates affected ($P = 0.15$) the incidence of other mortality (nonsporulating and greasy brown cadavers). The differences in number of microorganisms recovered in the frass from healthy larvae and sporulating cadavers were not significant ($P = 0.57$ for bacteria and $P = 0.07$ for filamentous fungi).

DISCUSSION

We evaluated the influence of microorganisms associated with the alfalfa leafcutter bee gut on chalkbrood by comparing disease incidence in agnotobiotic and axenic larvae. We previously showed that rearing larvae on γ-irradiated provisions did not affect development and that larvae were axenic for fungi but not for bacteria, although bacterial populations were reduced by 97% (Inglis *et al.,* 1992b). In the present study, we also recovered a small number of bacteria in the frass of larvae reared on γ-irradiated provisions. We were able to achieve near-axenic conditions (bacterial populations reduced by more than 99.9%) and the incidence of chalkbrood (67.6%) was significantly higher in agnotobiotic larvae than in near-axenic larvae (50.2%). Rather than having an antagonistic role, the indigenous microflora appears to stimulate *A. aggregata.* Explanations for this are speculative. However, one hypothesis is that the microbial activity within the gut increased the CO_2 concentration. Since germination of *Ascosphaera* spp. ascospores is initiated, at least in part, by elevated CO_2 levels (Kish, 1980; Heath and Gaze, 1987), the increased concentration of CO_2 may accentuate chalkbrood.

With the exception of *Corynebacterium* sp. 6884 and *Z. bailii* which were isolated from pollen and provisions, all other isolates were originally recovered from the larval gut contents or frass of alfalfa leafcutter bees

TABLE 2

The Influence of Introduced Microorganisms on Alfalfa Leafcutter Bee Larvae[a]

Microorganism	UAMH no.	% Chalkbrood	% Total mortality[b]	Bacteria (log cfu/g frass)	Fungi (log cfu/g frass)	Defecation (days)	Cocoon (days)	
							Started	Completed
Bacteria								
B. brevis	6938	0.0	10.0 (10.1)	5.72 (0.06)*	0.0	4.2 (0.1)	7.2 (0.4)	9.6 (0.4)
B. coagulans	6937	0.0	16.2 (7.3)	6.01 (0.08)*	0.0	4.5 (0.1)	7.5 (0.2)	9.6 (0.3)
B. firmus	6925	7.2 (7.2)	7.2 (7.2)	5.87 (0.14)*	0.0	4.6 (0.2)	7.5 (0.3)	9.4 (0.4)
B. firmus	6942	0.0	3.1 (3.1)	6.09 (0.10)*	0.0	4.3 (0.1)	8.1 (0.3)	10.2 (0.2)
B. lentus	6944	0.0	0.0	6.55 (0.07)*	0.0	4.9 (0.3)	8.8 (0.5)	10.1 (0.3)
B. licheniformis	6929	3.1 (3.1)	6.7 (3.9)	6.76 (0.23)*	0.0	4.4 (0.1)	7.3 (0.2)	9.9 (0.5)
B. licheniformis	6932	0.0	2.8 (2.8)	7.36 (0.08)*	0.0	4.5 (0.1)	7.6 (0.5)	9.2 (0.2)
B. megaterium	6927	0.0	20.5 (7.4)	5.89 (0.23)*	0.0	4.5 (0.2)	7.7 (0.3)	10.3 (0.4)
B. pumilus	6941	0.0	3.1 (3.1)	5.46 (0.13)*	0.0	4.4 (0.2)	7.8 (0.3)	9.6 (0.4)
Corynebacterium sp.	6884	0.0	2.1 (2.1)	3.35 (0.23)[c]	0.0	4.4 (0.2)	8.0 (0.1)	9.9 (0.2)
Streptomyces sp.	6915	3.6 (3.6)	25.5 (5.8)	6.59 (0.18)*	0.0	4.5 (0.1)	7.5 (0.2)	10.4 (0.3)
Fungi								
A. versicolor	6984	0.0	6.3 (6.3)	3.55 (0.22)[c]	5.50 (0.20)*	4.2 (0.1)	7.7 (0.1)	9.4 (0.3)
C. bombicola	7074	0.0	0.0	0.0	0.73 (0.73)[c]	4.2 (0.2)	7.0 (0.3)	9.3 (0.4)
C. albidus	7076	5.0 (5.0)	13.6 (4.7)	4.25 (0.19)[c,*]	0.75 (0.75)[c]	4.5 (0.1)	7.4 (0.2)	10.0 (0.2)
L. scottii	7078	0.0	0.0	1.88 (1.09)[c]	0.0	4.3 (0.1)	8.5 (0.3)	10.8 (0.4)
M. plumbeus	7013	0.0	0.0	3.63 (0.08)[c]	5.54 (0.05)*	4.5 (0.2)	7.9 (0.3)	9.6 (0.2)
R. glutinis	7080	0.0	2.8 (2.8)	2.28 (0.78)[c]	0.0	4.2 (0.2)	7.8 (0.4)	9.4 (0.3)
R. mucilaginosa	7081	2.5 (2.5)	10.0 (10.0)	2.12 (1.23)[c]	0.0	4.4 (0.1)	8.2 (0.3)	10.1 (0.3)
S. brevicaulis	6877	0.0	11.5 (7.9)	4.50 (0.02)[c,*]	5.71 (0.05)*	4.5 (0.2)	7.5 (0.2)	9.7 (0.1)
T. megachiliensis	6821	3.1 (3.1)	10.6 (4.1)	2.05 (1.18)[c]	6.21 (0.10)*	4.4 (0.1)	7.5 (0.4)	10.0 (0.2)
Control		2.5 (2.5)	9.7 (4.4)	1.86 (0.72)	0.84 (0.55)	4.6 (0.1)	7.6 (0.2)	10.1 (0.4)

[a] A suspension (2 μl) containing 10^4 propagules/cell for each microorganism was placed adjacent to each of 12 eggs per replicate on γ-irradiated (15 kGy) provisions. The control treatment was inoculated with 2 μl of sterile BES-buffer.

[b] Incidence of total mortality (sporulating, nonsporulating, and greasy-brown cadavers).

[c] Noninoculated microorganisms.

* Mean is significantly different from the control treatment as determined by Dunnett's test ($\alpha = 0.05$) for untransformed (sporulating cadavers, total mortality and time to defecation, initiation and completion of cocoon construction) and logarithmic-transformed (bacterial and fungal cfu) data. The control treatment consisted of 47 larvae; n = 24–34 larvae/treatment for microorganisms. Standard errors are given in parentheses following means.

and, therefore, would be expected to survive passage through the gut. Of the ingested microorganisms, only bacteria and filamentous fungi (20 of 23 isolates) but not yeasts (0 of 7 isolates) were subsequently recovered from frass. Populations of applied bacteria and filamentous fungi were one to three orders of magnitude greater than microbial populations from the frass of larvae reared on untreated provisions. Although yeasts are commonly associated with wild and domestic bees (Batra *et al.*, 1973), they were isolated infrequently from the gut contents and frass of leafcutter bee larvae (Inglis *et al.*, unpublished). Therefore, it would appear that yeasts do not contribute substantially to the ecology of the leafcutter bee larval alimentary canal.

Research on the colonization of insect alimentary canals by microorganisms has focused almost entirely on entomopathogens. Histopathological studies have indicated that ascospores of *A. aggregata* germinate in the larval midgut and penetrate the hemocoel within 2 days after ingestion (Vandenberg and Stephen, 1983), but little is known about environmental conditions within the alimentary canal. Vandenberg and Stephen (1984) found a neutral to slightly alkaline pH throughout, and a redox potential that was slightly to highly reduced. The pH is conducive for microbial activity, but the reduced conditions limit microbial growth. Larval provisions possess a high sugar content (66%) which is comprised primarily of glucose and fructose (Inglis *et al.*, 1992a). Although, *T. megachiliensis*, the most prevalent fungus in leafcutter bee larval guts, is sufficiently osmotolerant to grow on media containing 66% glucose (Inglis *et al.*, 1992c), the high concentration of sugars would inhibit the growth of most microorganisms. Digestion and subsequent absorption of the carbohydrates could result in a more suitable environment for microorganisms especially in alfalfa leafcutter bees where provisions are held in a blind gut for up to 6 days. Our findings suggest that, in addition to surviving passage, at least some microorganisms were actively growing within the leafcutter bee gut. More propagules were recovered from frass than were originally inoculated onto the surface of provisions for 16 of 20 isolates. In addition, the presence of small blastoconidia of *T. megachiliensis* in acropetal chains (Fig. 1) suggests that growth occurred in the gut.

Our attempts to achieve monobiotic conditions within larval guts were not entirely successful. Bacterial contaminants were recovered from the frass of larvae inoculated with most fungi. In several instances populations were significantly larger than those in the control treatment and comparable to those in the frass of larvae reared on natural provisions. Although contaminant numbers were low (between 0.001 and 6.2%

TABLE 3
The Influence of Introduced Microorganisms on Chalkbrood[a]

Microorganism	UAMH no.	% Chalkbrood	% Other mortality[b]	Healthy prepupae (log cfu/g frass)		Chalkbrood (log cfu/g frass)	
				Bacteria	Fungi	Bacteria	Fungi
Bacteria							
B. coagulans	6937	59.4 (3.7)	18.7 (3.7)	6.11 (0.08)	0.0	6.02 (0.16)*	0.0
B. brevis	6938	74.0 (6.5)*	3.6 (3.6)	6.13 (0.16)	0.0	5.98 (0.14)*	0.0
B. firmus	6925	55.6 (3.3)	13.4 (4.5)	6.42 (0.30)	0.0	6.35 (0.28)*	0.0
B. firmus	6942	56.0 (6.4)	16.1 (10.5)	6.44 (0.20)	0.0	6.55 (0.17)*	0.0
B. firmus	6940	50.9 (8.1)	6.7 (3.9)	7.08 (0.09)	0.0	7.26 (0.08)*	0.0
B. lentus	6944	72.0 (9.3)	10.8 (4.5)	6.99 (0.18)	0.0	7.00 (0.13)*	0.0
B. licheniformis	6929	63.8 (3.2)	11.3 (7.0)	6.70 (0.23)	0.0	6.87 (0.23)*	0.0
B. licheniformis	6932	65.4 (6.6)	4.2 (4.2)	7.26 (0.42)	0.0	7.76 (0.11)*	0.0
B. megaterium	6927	70.3 (9.3)	10.9 (3.7)	6.13 (0.09)	0.0	6.08 (0.19)*	0.0
B. pumilus	6941	64.8 (4.9)	6.4 (3.7)	5.40 (0.14)	0.0	6.09 (0.20)*	0.0
Streptomyces sp.	6915	74.8 (6.8)*	14.9 (5.9)	6.31 (0.08)	0.0	6.45 (0.14)*	0.0
Filamentous fungi							
A. niger	7040	53.6 (10.6)	10.5 (3.7)	3.39 (0.32)[c]	6.52 (0.10)	3.97 (0.10)[c,*]	6.78 (0.18)*
A. versicolor	6984	68.1 (6.9)	7.0 (4.2)	3.14 (1.07)[c]	5.21 (0.18)	4.18 (0.20)[c,*]	5.44 (0.19)*
M. plumbeus	7013	50.4 (9.2)	2.5 (2.5)	3.91 (0.24)[c]	5.86 (0.14)	4.38 (0.04)[c,*]	6.13 (0.18)*
P. granulatum	6879	61.0 (4.4)	14.8 (1.8)	4.12 (0.06)[c]	6.33 (0.14)	1.41 (1.41)[c]	6.71 (0.30)*
P. viridicatum	7039	46.3 (6.3)	25.0 (7.4)	2.57 (1.11)[c]	6.62 (0.02)	1.78 (1.03)[c]	6.50 (0.17)*
R. oryzae	7019	43.3 (2.6)	16.5 (2.9)	4.20 (0.12)[c]	5.56 (0.35)	4.13 (0.13)[c,*]	6.18 (0.05)*
S. brevicaulis	6877	56.9 (5.9)	24.6 (8.7)	4.12 (0.28)[c]	5.91 (0.19)	2.05 (1.18)[c]	6.09 (0.12)*
T. megachiliensis	6821	66.2 (11.1)	9.1 (5.5)	2.78 (0.93)[c]	6.30 (0.09)	2.37 (1.19)[c]	6.13 (0.08)*
T. megachiliensis	6823	48.7 (8.3)	13.4 (4.5)	1.88 (1.09)[c]	6.13 (0.10)	1.48 (1.48)[c]	5.94 (0.19)*
Control		52.1 (4.5)	17.2 (3.6)	—	—	1.24 (0.54)	0.27 (0.27)

[a] A coinoculum suspension (2 μl) containing 10^4 ascospores of *A. aggregata* and 10^4 microbial propagules for each isolate was placed adjacent to each of 12 eggs per replicate on γ-irradiated (15 kGy) provisions. The control treatment was inoculated with 2 μl of the ascospores suspension alone. Twenty of the 30 isolates tested were recovered in frass.

[b] Incidence of other mortality (nonsporulating and greasy-brown cadavers).

[c] Noninoculated microorganisms.

* Mean is significantly different from the control treatment as determined by Dunnett's test ($\alpha = 0.05$) for arcsine-transformed (sporulating cadavers), logarithmic-transformed (bacterial and fungal cfu), and untransformed data (other mortality) data. Standard errors are given in parentheses following means. Untransformed means and standard errors are reported for the mortality data. The control treatment consisted of 83 larvae compared with 23–35 larvae for the microorganism treatments.

of numbers of inoculated microorganisms), their presence nevertheless presents a confounding factor. Comparable contamination probably occurred in larvae inoculated with bacteria, but contaminants were not detected by the dilution spread-plate technique. In a previous study, bacterial contamination was attributed to inadequate decontamination of leafcutter bee eggs (Inglis *et al.*, 1992b). The extreme susceptibility of eggs to either mechanical or chemical injury made efficacious decontamination difficult and we observed high egg mortality (17–58%) following surface treatment of eggs with 0.3% dry chlorine. Microorganisms were associated with the ascospore inoculum (less than 1 cfu per dose) and were another source of contamination. Attempts to purify ascospore suspensions in a Percoll density gradient were unsuccessful, but a recently described method of inducing sporulation *in vitro* (Youssef and McManus, 1991) may provide an axenic supply of ascospores for future studies.

The use of antagonistic microorganisms to control pathogens has been extensively studied in plant systems, but few studies have been conducted on entomopathogens. Co-inoculation of armyworm larvae with *Streptococcus faecalis* var. *liquefaciens* and *P. aeruginosa* or *Serratia marcescens* reduced disease caused by the former, but increased the incidence of *S. marcescens*-incited mortality, indicating that interactions are not always antagonistic (Goodwin, 1968). Gilliam *et al.* (1988) observed that several microorganisms associated with honeybees, including species of *Aspergillus, Mucor, Rhizopus,* and *Penicillium,* produced metabolites *in vitro* that were inhibitory to vegetative growth of *Ascosphaera apis*. We estimated the efficacy of applied microorganisms in reducing chalkbrood by comparing disease in monobiotic and axenic larvae since *in vitro* antagonism rarely correlates with efficacy *in vivo* (Hentges and Freter, 1962; Andrews, 1985). None of the microorganisms tested were antagonistic toward *A. aggregata;* indeed two isolates increased chalkbrood, if only marginally. There were no differences in the sizes of microbial populations in the frass of healthy and diseased larvae, dismissing the possibility of differential ingestion of the propagules. We conclude that the microorganisms associated with

alfalfa leafcutter bee guts have a minimal, if any, effect on the incidence of chalkbrood.

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