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PROBIOTICS MAY PROTECT *DROSOPHILA* FROM INFECTION BY *ASPERGILLUS FLAVUS*

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ABSTRACT: Probiotics have been used to protect hosts from pathogens of gastrointestinal and reproductive systems, but their ability to protect against systemic pathogens is largely unexplored. In this study we ask whether orally administered bacteria and yeasts can protect *Drosophila melanogaster* against the opportunistic fungal pathogen *Aspergillus flavus*. Flies were fed an artificial diet mixed with live microorganisms for one day prior to infection with *A. flavus*, and mortality was recorded every day for 8 days. Seven microorganisms were tested; of these, *Bacillus cereus* (ATCC 13061), *Candida inconspicua*, *Issatchenkia hanoiensis*, and *Klebsiella* sp. significantly decreased mortality of flies subsequently infected with *A. flavus* compared to controls infected with *A. flavus* alone. Heat-killed microorganisms did not protect flies, suggesting that the probiotic effect observed was not caused by improved nutrition. *D. melanogaster* is a good model organism to study microbial interactions with hosts and test the effects of potential probiotics against pathogens.

INTRODUCTION: Probiotics: Probiotics are defined as live microorganisms that confer beneficial effects when administered to hosts [1]. Orally administered probiotics are increasingly important tools for protecting humans and domestic animals against pathogens [2–5]. Despite an extensive and growing literature on probiotics, relatively few studies have tested their effects on model invertebrates such as *Caenorhabditis elegans* [6, 7] and *Drosophila melanogaster* [8]. Probiotic-induced protection against infectious disease may occur by various mechanisms, principally competitive exclusion of pathogens, antibiotic production, and stimulation of the immune system [3, 9, 10].

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Most work on protection against pathogens has focused on the gastrointestinal system and to a lesser extent the reproductive system, in which all these mechanisms of protection have been demonstrated. Much less is known about the potential of probiotics to prevent or ameliorate systemic infections. However, the third mechanism of protection mentioned above, immune stimulation, is partly systemic - implying that oral probiotics may have potential to protect the organism from systemic pathogens. In this study we test whether feeding *Drosophila melanogaster* with potentially probiotic bacteria and yeasts protects flies against systemic infection by the opportunistic pathogen *Aspergillus flavus*.

***Aspergillus* and human aspergillosis:** *Aspergillus flavus* (among other *Aspergillus species*) *causes* aspergillosis in humans and animals, and also infects insects [11–15]. Immunocompromised patients, one of the most susceptible groups, are mainly

infected by inhalation of conidia, causing allergic bronchopulmonary aspergillosis, aspergillomas, and invasive aspergillosis (IA) [16]. Treatment for aspergillosis has improved greatly in the past twenty years, thanks mostly to new antifungal drugs in combination with anti-inflammatory therapy [17]. However, although the mortality rate for IA has decreased from >90% to <50%, this is still unacceptably high, and resistance to antifungals is increasing [18, 19]. The ubiquity of *A. flavus* conidia [20, 21] means that exposure to inoculum cannot be eliminated. A previous study showed that *A. flavus* isolates from different substrates are capable of virulence during infection in the model organism *D. melanogaster* [13] it appears that any environmental strain is a potential pathogen in a susceptible host [20, 22]. New approaches to prevent and treat aspergillosis are needed.

***Drosophila* as a model system for host-pathogen interactions:** Recently *Drosophila melanogaster* has been recognized as a model system to study microbial pathogenicity [12, 13, 23–26]. Its fast growth, short life cycle, ease of manipulation, low cost and simplicity of ethical and regulatory issues, make it an attractive host for such studies, and its immune system is similar in some ways to the mammalian innate immune system [12, 23, 24, 27, 28].

Here we use *Drosophila* as a model to ask whether oral probiotics may offer protection against opportunistic infection by *A. flavus*, a novel and previously unexplored approach. Microorganisms isolated from wild *Drosophila* and other potential probiotics were fed to flies in artificial diets prior to inoculation with the opportunistic pathogen *A. flavus*. The hypothesis was that some of these microorganisms can decrease mortality caused by *A. flavus*, effectively serving as probiotics.

METHODS:

Probiotics and inoculation of flies: The bacteria *Klebsiella* sp., *Bacillus* sp., and the yeasts *Candida inconspicua* and *Issatchenkia hanoiensis* were isolated from the gut of wild *Drosophila*, removed whole, macerated and streaked on agar. They were chosen for probiotic experiments due to their high frequency of isolation and ease of culture (data not shown). Also included were the following bacteria from the American Type Culture Collection (ATCC): *Bacillus subtilis* (ATCC 6633, original substrate unknown) and *B. cereus* (ATCC 13061, original substrate unknown, and ATCC 21768, originally isolated from turkey and chicken manure). They were chosen due to reports that they are potential probiotics for prevention or treatment of a number of diseases, and can compete with *A. flavus in vitro* [9, 29–31]. The identification of fungal strains was performed using the nuclear ribosomal Internal Transcribed Spacer (ITS) and a section of the 16S ribosomal gene for bacteria (unpublished data). Gram staining and morphology supported identification based on ITS and 16S sequences.

Fungi were grown on Yeast Agar Glucose (YAG) supplemented with 0.01 M $MgSO_4.7H_2O$ [24] and bacteria on Tryptic Soy Agar (TSA) at 37 ºC for 48 hours. Fungi and bacteria were suspended in sterilized water at a concentration of ~ 1.6 x 10^9 CFU/mL and stored at 8 ºC. The suspensions were used within one month and were tested for viability by plating on agar.

Twenty-five µL of the probiotic suspensions were seeded in tubes containing 10 mL of 1:1 Nutrifly fly food (Genesee Scientific, San Diego, CA, prepared according to manufacturer's instructions but without tegosept and propionic acid) and YAG or TSA (for fungi and bacteria respectively) and were incubated as mentioned above. Four-day-old females of *D. melanogaster* strain Oregon R were left in empty plastic tubes for 1-2 hours to induce hunger and then moved to tubes containing Nutrifly and probiotic. We did not determine the number of CFUs consumed by flies. Flies were left feeding for ~ 20 hours before infection with *Aspergillus*.

To confirm that colonization of flies fed with probiotics was successful, microorganisms were re-isolated from the intestine of inoculated flies as follows: Flies were washed with Tween 80 (0.01%) and vortexed for one minute to release microbial cells from body surfaces. Clean flies were surface-sterilized by immersion in 70 % ethanol for 1 min and rinsed in sterile water before plating on YAG and TSA media. Plates were incubated at 28 ºC for seven days. Similarly, flies infected with *A. flavus* but without probiotics were plated on YAG to ensure that infection was successful [13]. As an additional control, flies were fed heat-killed microorganisms (autoclaved 15 min at 15 psi) prior to infection with *A. flavus* to determine whether

protection was due to a nutritional effect rather than an active probiotic effect. Experiments were repeated three times on different dates, with five replicate tubes of ~ 36 flies per date.

Infection with *Aspergillus flavus*: Following overnight feeding with probiotics, flies were infected with the opportunistic pathogen *Aspergillus flavus* using a previously described rolling assay [13, 24]. We used the rolling assay instead of ingestion or injection methods because it is reliable, repeatable, a more natural form of infection, and less laborious [13]. An isolate of *A. flavus* previously shown to be highly virulent on *Drosophila* was used (ABPMA1) [13, 20]. Flies were shaken in a plate culture containing a lawn of *A. flavus* conidia for ~ 1 min (**Fig. 1A**). After infection, flies were left in empty tubes 1-2 hours and then moved to tubes containing fly food at ~ 28 °C and with constant light, conditions that may maximize *Drosophila*'s susceptibility to *Aspergillus* infections [13, 24]. Inoculated flies had 1 - 4 × 10^5 conidia on their bodies; the number of conidia germinating and colonizing the internal tissues was not determined. Fly survival was recorded daily for eight days, the period in which survival is usually measured [13].

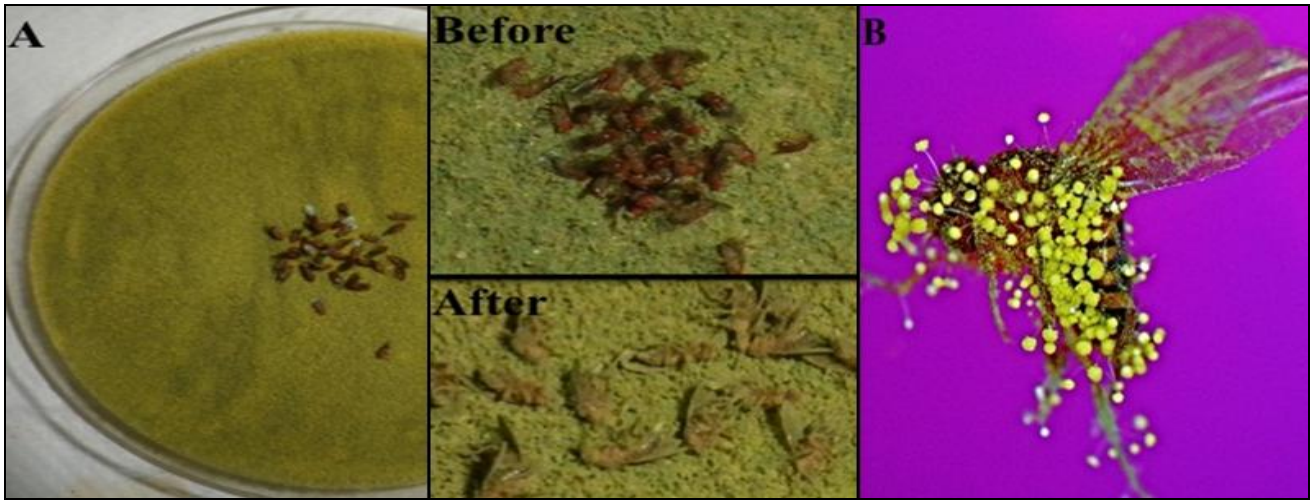

FIG. 1: METHOD OF INFECTION OF *DROSOPHILA MELANOGASTER* WITH *ASPERGILLUS FLAVUS*. A: *ASPERGILLUS FLAVUS* COLONY IN YAG AGAR PRE/POST SHAKING OF ANESTHETIZED FLIES. B: SURFACE-STERILIZED DEAD FLY PLACED ON AGAR SHOWING SPORULATING *A. FLAVUS*, EVIDENCE THAT INFECTION WAS SUCCESSFUL

To confirm that dead flies had been successfully infected by *A. flavus*, the flies were surface-sterilized as previously described [13]. These flies were put on water agar supplemented with dichloran-rose bengal [32] and observed for growth of *A. flavus* (**Fig. 1B**). The location and extent of fungal colonization was not determined.

Statistical analysis: Survival of infected flies inoculated with probiotics, heat-killed probiotics, and three types of controls (uninfected flies, flies fed with probiotics but without *Aspergillus*, and flies infected with *Aspergillus flavus* without probiotics) was calculated using Kaplan–Meier analysis, with the following parameters: the number of days' survival was defined as the time to event and flies still alive on day 8 after infection as censored observations [33]. Flies that died within 3 hours of rolling were discarded from the analysis. Differences in survival of infected flies fed different probiotics and controls were compared on day 8 using Log - Rank tests. All statistical analysis was performed using JMP, version 8.

RESULTS: *Bacillus cereus* (ATCC 13061), *Candida inconspicua*, *Issatchenkia hanoiensis*, and *Klebsiella* sp. significantly increased survival of flies challenged with *A. flavus* ($P < 0.001$; **Table 1**, **Fig. 2**) at eight days post infection. Increase in survival was noted after two days post infection in some cases (**Fig. 2**). The remaining microorganisms tested [*B. cereus* (ATCC 21768), *Bacillus* sp., and *B. subtilis* (ATCC 6633)] did not significantly enhance survival compared to flies inoculated with *A. flavus* alone ($P > 0.05$ in all cases; **Fig. 3**).

TABLE 1: SURVIVAL ANALYSIS OF *DROSOPHILA MELANOGASTER* FED WITH MICROORGANISMS PRIOR TO INFECTION WITH *ASPERGILLUS FLAVUS*; LOG-RANK TESTS SHOW DIFFERENCES BETWEEN EXPERIMENTAL GROUPS VS CONTROL FLIES INFECTED WITH *A. FLAVUS* ALONE

Experimental groups	χ^2	P
Treatments: Flies fed with probiotics prior to infection with *A. flavus*		
Bacillus cereus (ATCC 13061)	27.618	< 0.0001
Candida inconspicua	29.71	< 0.0001
Issatchenkia hanoiensis	48.576	< 0.0001
Klebsiella sp.	18.429	< 0.0001
Bacillus subtilis (ATCC 6633)	2.045	0.1527
Bacillus cereus (ATCC 21768)	3.761	0.0525
Bacillus sp.	0.862	0.3532
Bacillus cereus (ATCC 13061), heat-killed control	1.519	0.2177
Issatchenkia hanoiensis, heat-killed control	3.105	0.0781
Negative controls: Flies fed with probiotics but not infected with *A. flavus*		
Untreated flies	933.502	< 0.0001
Bacillus cereus (ATCC 13061)	969.547	< 0.0001
Candida inconspicua	970.597	< 0.0001
Issatchenkia hanoiensis	965.325	< 0.0001
Klebsiella sp.	968.991	< 0.0001
Bacillus subtilis (ATCC 6633)	972.582	< 0.0001
Bacillus cereus (ATCC 21768)	987.548	< 0.0001
Bacillus sp.	963.774	< 0.0001

The bacteria and yeasts alone did not cause mortality: flies fed with these microorganisms but not infected with *Aspergillus* showed no significant reduction in survival compared to controls (uninfected flies without probiotics) (P = 1, **Fig. 2, 3)**.

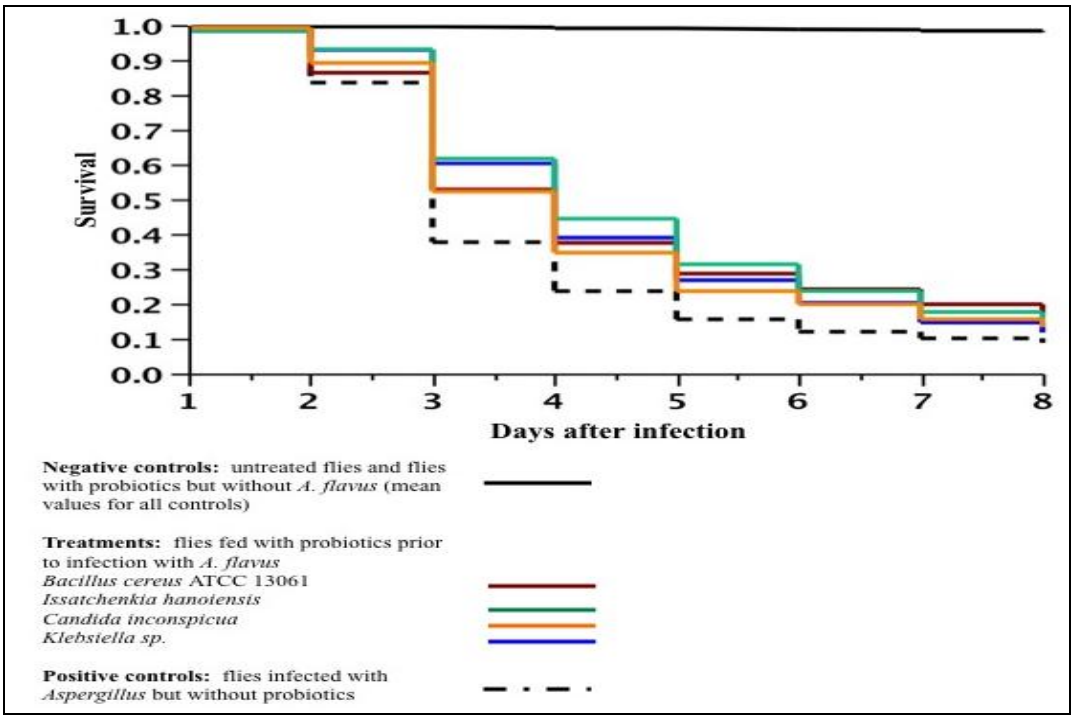

FIG. 2: SURVIVAL OF *DROSOPHILA MELANOGASTER* FED WITH MICROORGANISMS BEFORE INFECTION WITH THE PATHOGENIC FUNGUS *ASPERGILLUS FLAVUS:* ORGANISMS PROVIDING SIGNIFICANT PROTECTION THE Y-AXIS SHOWS PROPORTION OF FLIES SURVIVING. THE BLACK HORIZONTAL LINE AT TOP SHOWS MEAN SURVIVAL OF UNINOCULATED FLIES AND FLIES FED WITH PROBIOTICS BUT NOT INOCULATED WITH *A. FLAVUS*, POOLED FOR EASE OF VISUALIZATION. THE PROBIOTIC ORGANISMS SHOWN, *BACILLUS CEREUS* ATCC 13061, *CANDIDA INCONSPICUA*, *ISSATCHENKIA HANOIENSIS* AND *KLEBSIELLA* SP., INCREASED SURVIVAL SIGNIFICANTLY COMPARED TO CONTROLS. THE DASHED LINE SHOWS SURVIVAL OF FLIES INOCULATED WITH *A. FLAVUS* WITHOUT PREVIOUS INOCULATION BY ANOTHER MICROORGANISM (POSITIVE CONTROLS).

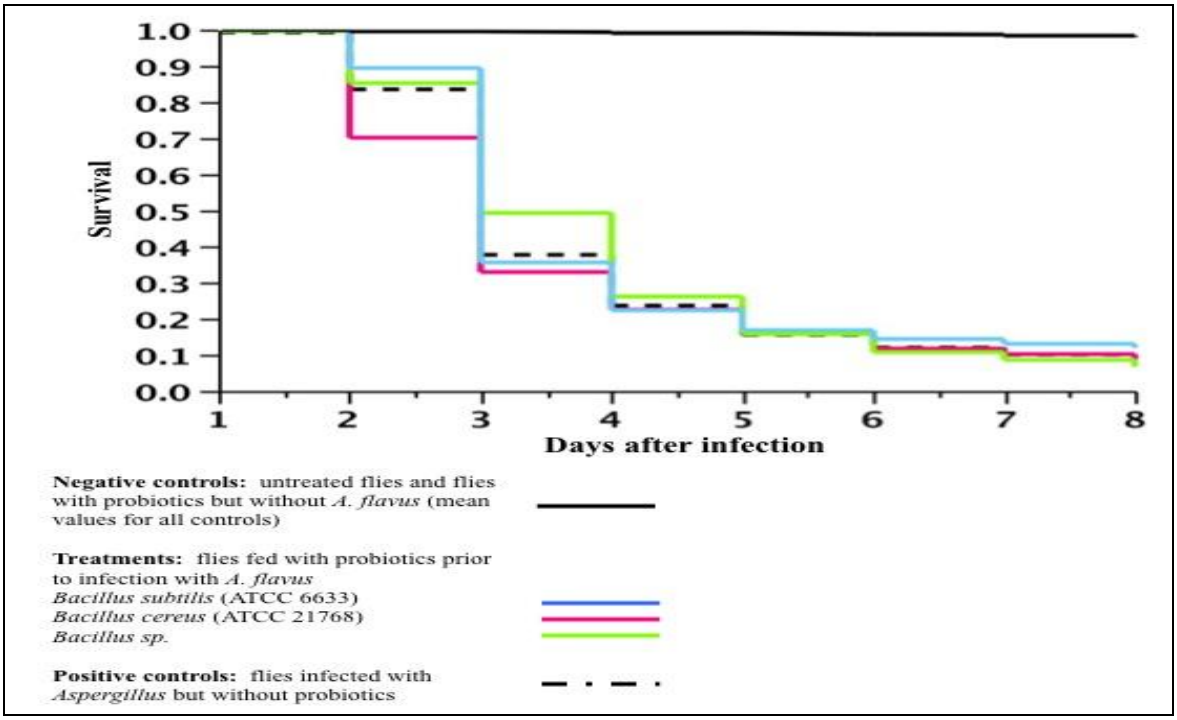

FIG. 3: SURVIVAL OF *DROSOPHILA MELANOGASTER* INOCULATED WITH MICROORGANISMS BEFORE INOCULATION WITH THE PATHOGENIC FUNGUS *ASPERGILLUS FLAVUS*: ORGANISMS NOT PROVIDING SIGNIFICANT PROTECTION. SEE FIG. 2 FOR DESCRIPTION.

Flies inoculated with killed cells/spores of *B. cereus* (ATCC 13061) and *I. hanoiensis* received no protection ($P > 0.05$) at 8 days post infection with *A. flavus* (**Fig. 4**), implying that the protection provided by these microorganisms was not due to the nutritional content of the cells.

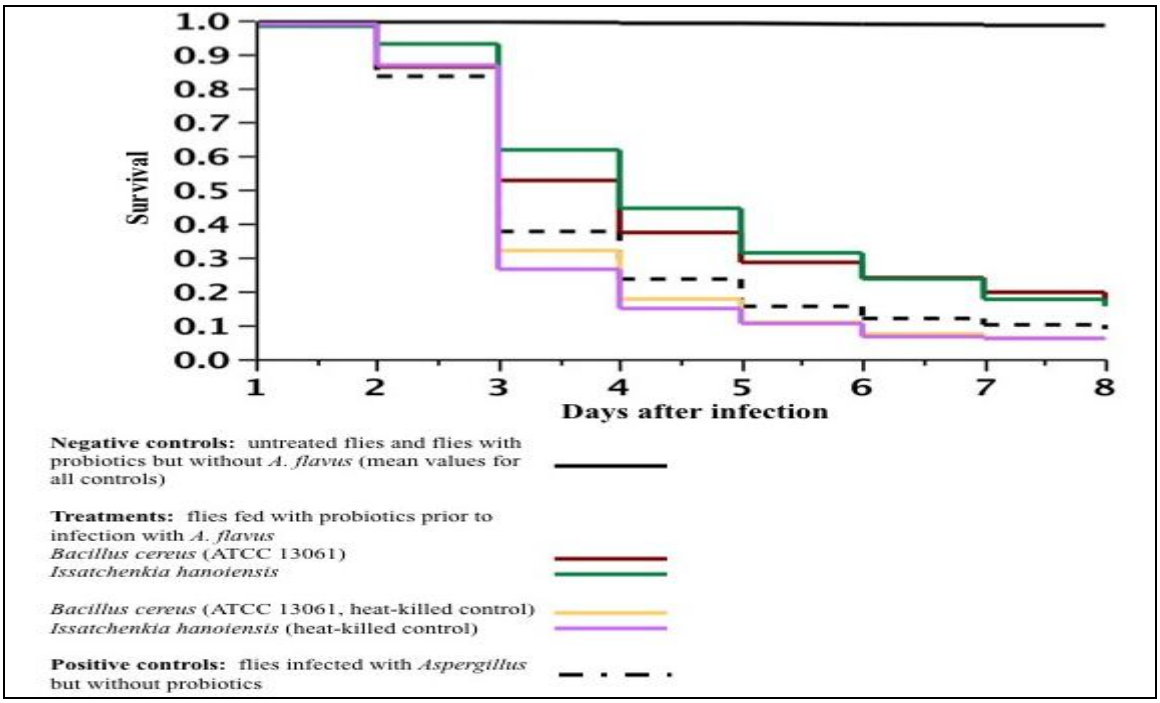

FIG. 4: SURVIVAL OF *DROSOPHILA MELANOGASTER* INOCULATED WITH HEAT-KILLED PROBIOTICS VS. LIVE PROBIOTICS BEFORE INOCULATION WITH THE PATHOGENIC FUNGUS *ASPERGILLUS FLAVUS*. THE Y-AXIS SHOWS PROPORTION OF FLIES SURVIVING. CONTROL LINES IN THE UPPER GRAPH SHOWS SURVIVAL AVERAGE OF UNINOCULATED FLIES AND FLIES FED WITH PROBIOTICS, POOLED FOR EASE OF VISUALIZATION. THE DASHED LINE SHOWS SURVIVAL OF FLIES INOCULATED WITH *A. FLAVUS* WITHOUT PREVIOUS INOCULATION BY ANOTHER MICROORGANISM (POSITIVE CONTROLS)

DISCUSSION:

Protective roles of bacteria and fungi against infection by *Aspergillus flavus:* Flies fed with *Bacillus cereus* (ATCC 13061), *Candida inconspicua*, *Issatchenkia hanoiensis*, and *Klebsiella sp*. showed protection 8 days after infection with *A. flavus* (**Table 1, Fig. 2**). This indicates that these microorganisms increase resistance of flies to *A. flavus*, probably due to stimulation of the immune system of the host [9, 31]. The immune system in flies can be stimulated through enhanced production of cytokines, plasmatocytes (cells like macrophages) and immunoglobulins [3].Other mechanisms of protection are also possible. In mice, intra-gastric doses of *B. subtilis* sporulated quickly and formed robust biofilms [31] the same may have happened in the intestinal tract of flies. However, mechanisms of protection were not examined in this study.

A. flavus produces severe systemic infection in flies, probably affecting internal organs and others parts of the body [13]. Fungal dissemination throughout different organs begins with invasion or inhalation of *A. flavus* spores, which may involve particular virulence routes such as interaction of the fungus with epithelial receptors and colonization of the digestive tract [23]. *A. flavus* is not host-specific, and is capable of causing disease in humans, plants, and insects [34]. *A. flavus* strains differed significantly in virulence on *D. melanogaster*, implying variability in virulence factors [13]. A similar explanation was given for differences in virulence during infection of immunocompromised *Drosophila* by *A. fumigatus* and *A. terreus* [35].

Previous studies on *Aspergillus* infections in *Drosophila* have used immunosuppressed flies, for two reasons [12, 24, 35]. First, in most studies the pathogen tested was *A. fumigatus*, which is not very virulent against *Drosophila*, so that immunosuppression is necessary for infection; in contrast, *A. flavus* is more virulent, so immunosuppressed flies are not necessary [13]. Second, since human aspergillosis is mostly of concern in immunosuppressed patients, immunosuppressed *Drosophila* is considered a better model [27]. Because the present study is an initial proof-of-concept of the use of probiotics against opportunistic fungal infections, immunosuppression would add an additional level of complexity. Also, stimulation of immune responses is one of the main mechanisms of protection by probiotics, and an immunosuppressed host would not be a good model as regards this line of defense.

Is the protective effect against *Aspergillus flavus* due to improved nutrition?: Inactivated probiotics have been shown to increase survival and growth in some studies; this is at least partly attributable to nutritional value of the microbial cells, including growth promoters [3]. To distinguish nutritional effects from active protective effects of probiotics, flies were fed with heat-killed cells of two of the four microbes that showed positive protective effects when alive, the bacterium *Bacillus cereus* (ATCC 13061) and the yeast *Issatchenkia hanoiensis* (**Table 1**, **Fig. 4**). *Drosophila* fed with heat-killed microorganisms did not show the increase in survival after challenge with *A. flavus* that was seen in flies fed live microorganisms. This suggests that the protection observed was not due to nutritional value of the microorganisms. The Nutrifly diet used is a commercial mixture derived from corn, soy and yeast, and is optimized for *Drosophila*, so additional nutrients would not be expected to stimulate growth.

Do probiotics have potential applications for aspergillosis and other systemic infections?*:* Probiotics are commonly used in poultry and livestock for disease prevention and nutritional benefits [3]. Aspergillosis is a problem in poultry, but the effect of probiotics in preventing the disease has not been studied. It would be interesting to know the prevalence of aspergillosis cases in poultry fed with probiotics vs. controls, but such data have not been published. In fact, *A. oryzae*, a domesticated form of *A. flavus*, is itself used in poultry as a nutritional probiotic [36] and could be expected to protect against aspergillosis by competing against related pathogenic organisms [37].

In humans, many aspergillosis patients have immunosuppression issues, which means that microorganisms in general have to be viewed as potential opportunistic pathogens, and their application is potentially dangerous. The main route for *Aspergillus* infection in human hosts is the respiratory tract and, therefore, the use orally

administered probiotics has not been proposed previously. Nevertheless, these patients have a core microbiota, the composition of which presumably affects susceptibility to disease, and could be optimized to reduce that susceptibility. Furthermore, there are other groups susceptible to *Aspergillus* infections, for example cystic fibrosis patients, who are not immunosuppressed and who might potentially benefit from probiotics. Given the very high mortality of aspergillosis, new strategies for prevention are clearly needed.

Candida inconspicua is a little known species that is occasionally reported as an opportunistic human pathogen, though much less common than *C. albicans* [38]. *Issatchenkia hanoiensis* was originally isolated from insect frass in Viet Nam [39] it has also been reported from various fermented foods [40–42]. *Bacillus cereus* is a common soil bacterium that sometimes causes foodborne illness; some strains have been used as probiotics in animal feeds to reduce the risk of *Salmonella* contamination [43]. Some *Klebsiella* species cause severe human infections [44–46] but identification of the species used in this study remains unclear. Of these organisms, only *B. cereus* has previously been used as a probiotic, to control diarrhoea and improve feed efficiency in pigs [47] the fact that some of these organisms have been reported as opportunistic pathogens is an argument against their use. However, they illustrate that microbial diversity for potential probiotics has been underexploited, and that procedures like this one are valuable tools for identifying potential candidate species.

CONCLUSIONS: The oral administration of *Bacillus cereus* (ATCC 13061), *Candida inconspicua*, *Issatchenkia hanoiensis*, and *Klebsiella* sp. can partially protect against infection by *A. flavus* in *Drosophila*. This is a novel and interesting finding. Clearly, more experiments are necessary. For instance, the success of probiotics experiments often depends on details of conditions and strains used [2, 3]. We show here that two strains of *Bacillus cereus* have very different results in *Drosophila*. The reason for this difference is undetermined and suggests that more strains should be tested. The convenience of *Drosophila* makes it a good model system for preliminary screening of species and isolates for use as probiotics. For example, *I. hanoiensis*, *C. inconspicua*, and *Klebsiella* sp., shown here to protect flies from *A. flavus*, have not previously been used as probiotics in animals or humans. The potential of probiotics as a therapeutic alternative to combat other opportunistic fungal pathogens should also be studied.

The expansion of probiotics in human and animal health has been so tremendous that it is likely that in the near future they will be applied to other uses, perhaps including protection against systemic infections. The present study, though it does not explain the mechanisms involved or why different microorganisms varied in effectiveness, is an important first step.

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CONFLICT OF INTEREST: P. Bayman is an officer of Atabei Ecosystems LLC, a company focused on microbial biocontrol of plant pests and pathogens.

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