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Omics and molecular technologies for the analysis of complex microbial communities

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INTRODUCTION

By 2050, the demand for food of animal origin is expected to increase by 58 to 70%. This will inevitably lead to increased demand for animal feed sources and strong competition between the feed and the human food fields. All this will be accentuated by ongoing climate change and land use for industrial crops for energy production. The growth of the population and the change in lifestyles will lead to a greater demand for protein sources and to date it seems possible that the edible insect can be a viable alternative to soya for the production of feedingstuffs for livestock, but also for food intended for human consumption (Van Huis A., 2013). The possibility to rear Black Soldier Fly (BSF) on diverse waste streams in a biorefinery approach requires attention to safety issues, both chemical and microbiological. Hygiene issues of edible insects can derive from the substrate but also from the gut microbial community, and may be affected by processing steps linking farming and consumption. However, little attention has been paid to microbial dynamics associated to intrinsic parameters, such as the rearing temperature.

EXPERIMENTAL DESIGN

Black Soldier Fly larvae (L), prepupae (PP), and frass (F) samples were grown at three different temperatures (20, 27 and 33°C) and processed for microbiological and metagenome analyses.

Plate enumeration

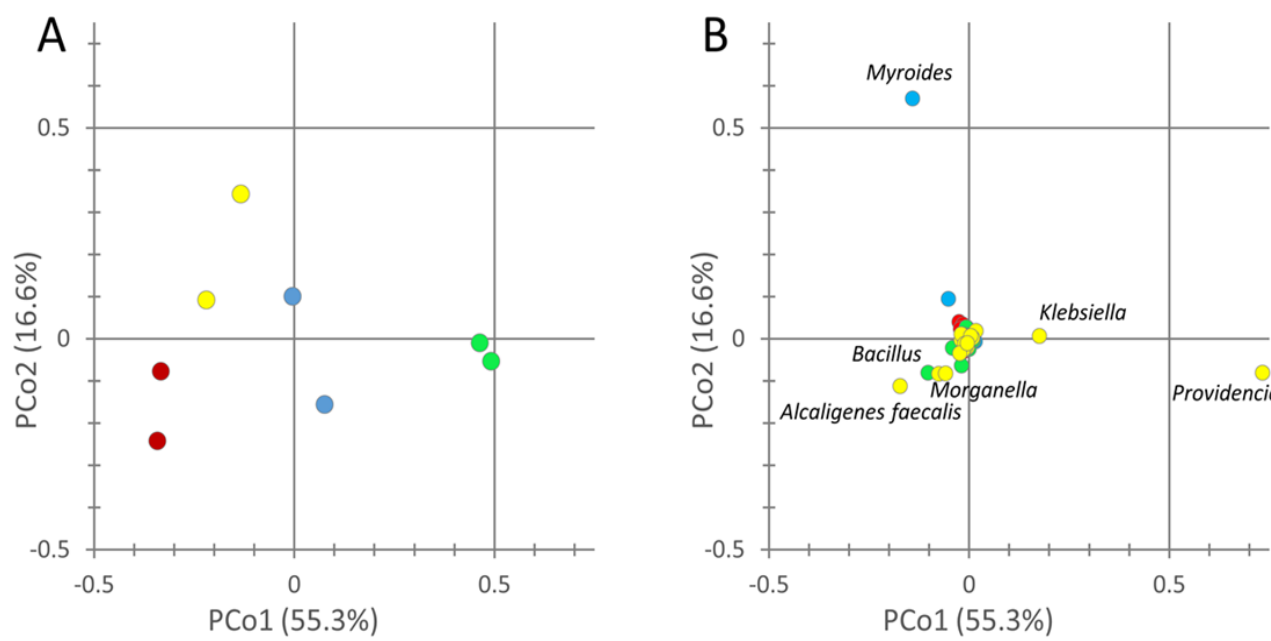
Three different samples, from two independent experiment (n=6) of BSFL, PP and F were assessed. Total mesophilic aerobes (PCA), spore-forming aerobic bacteria (PCA + starch), LAB (MRS), Enterobacteriaceae (VRBG), Yeasts and molds (DRBC) were detected. The presence of food pathogens in PP was assessed according to the following ISO methods:

<i>Campylobacter</i> spp. ISO 10272:2017	<i>Listeria monocytogenes</i> and <i>Listeria</i> spp. ISO 11290-2:2017
<i>Clostridium perfringens</i> ISO 7937:2004	<i>Salmonella</i> spp. ISO 6579-1:2017 and ISO/TS 6579-2:2012
Coagulase-positive staphylococci ISO 6888-1:2018	<i>Bacillus cereus</i> ISO 7932:2004

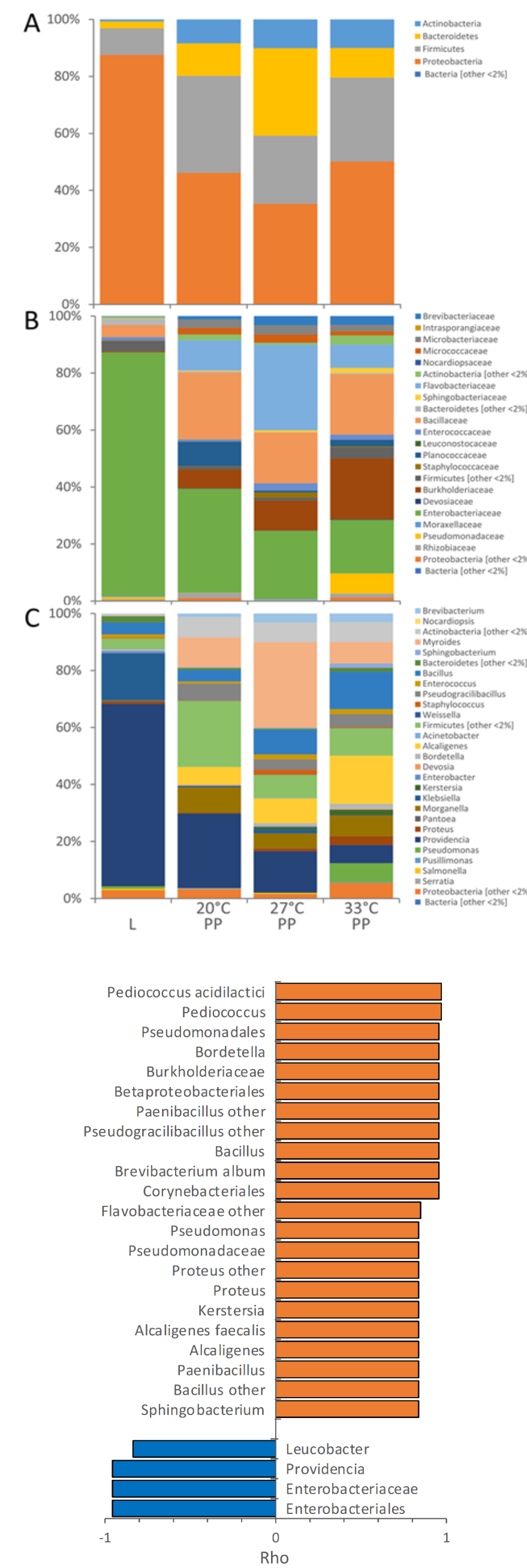
	PP			F		
	20°C	27°C	33°C	20°C	27°C	33°C
Total mesophilic aerobes	7.4 ± 0.9 ^b	8.5 ± 0.4 ^a	8.7 ± 0.3 ^a	8.7 ± 0.7 ^b	9.7 ± 0.4 ^a	9.8 ± 0.4 ^a
Spore-forming bacteria	6.8 ± 1.3 ^a	7.9 ± 0.5 ^a	7.9 ± 0.2 ^a	8.7 ± 0.8 ^b	9.7 ± 0.4 ^a	9.9 ± 0.6 ^a
Lactic acid bacteria	6.6 ± 0.4 ^a	6.9 ± 0.7 ^a	6.2 ± 0.5 ^a	7.6 ± 0.6 ^a	7.3 ± 0.4 ^a	7.0 ± 0.4 ^a
Enterobacteriaceae	3.7 ± 0.3 ^b	5.2 ± 0.6 ^a	4.8 ± 1.3 ^{ab}	6.2 ± 0.6 ^a	5.4 ± 0.8 ^{ab}	4.8 ± 0.9 ^b
Yeasts and molds	0.7 ± 0.9 ^a	1.2 ± 1.0 ^a	0.9 ± 0.8 ^a	2.9 ± 0.8 ^a	2.7 ± 0.7 ^a	2.8 ± 0.6 ^a
Bacillus cereus	2.3 ± 0.9 ^a	3.2 ± 0.7 ^a	2.3 ± 0.7 ^a			
Campylobacter spp.	3.2 ± 0.3 ^c	4.2 ± 0.4 ^b	4.7 ± 0.2 ^a			
Clostridium perfringens	0.8 ± 0.7 ^a	1.6 ± 1.3 ^a	1.0 ± 0.8 ^a			
Coagulase-positive staphylococci	3.9 ± 0.7 ^a	4.2 ± 0.7 ^a	3.7 ± 0.9 ^a			
Listeriaceae	4.8 ± 0.4 ^b	5.5 ± 0.3 ^a	5.8 ± 0.4 ^a			
Salmonella spp. #	2/6	1/6	4/6			

The viable count of total mesophilic aerobes in PP was lower (P < 0.05) at the rearing temperature of 20 °C (7.4 cfu/g) than at both 27 and 33 °C by approx. one magnitude. Spore-forming bacteria were less abundant than total aerobes, at all the temperatures (P < 0.05). Lactic bacteria and Enterobacteriaceae ranged between 6.2 and 6.9 log cfu/g and between 3.7 and 5.2 log cfu/g, respectively, without any difference associated to temperature. A low load of cultivable yeasts and molds was observed, between 0.7 and 1.2 log cfu/g, without any significant influence of temperature. *Bacillus cereus*, *Campylobacter* spp., *Clostridium perfringens*, coagulase-positive staphylococci, and Listeriaceae were detected in PP grown at all the tested temperatures. The amount of *Campylobacter* and Listeriaceae was positively correlated to temperature. The former passed from 3.2 to 4.7 log cfu/g and the latter from 4.8 to 5.8 log cfu/g increasing the rearing temperature from 20 to 33 °C. Coagulase-positive staphylococci, *Bacillus cereus*, and *Clostridium perfringens* (laying in the ranges of 3.7-4.2, 2.3-3.2, and 0.8-1.6 log cfu/g, respectively) were not affected by the temperature (P > 0.05). *Salmonella* was found in 7 out of 18 samples. Among the positive samples, only one (PP grown at 20 °C) allowed the recovery of colonies by direct plating, at a concentration of 1.1 log cfu/g. As a whole, all the searched pathogens were detected in the whole set of samples. Where significant differences among the diverse temperatures were detected (i.e. *Campylobacter* and listeriaceae), counts get higher with the increasing temperature, according to the optimal growth temperature of these mesophilic bacteria.

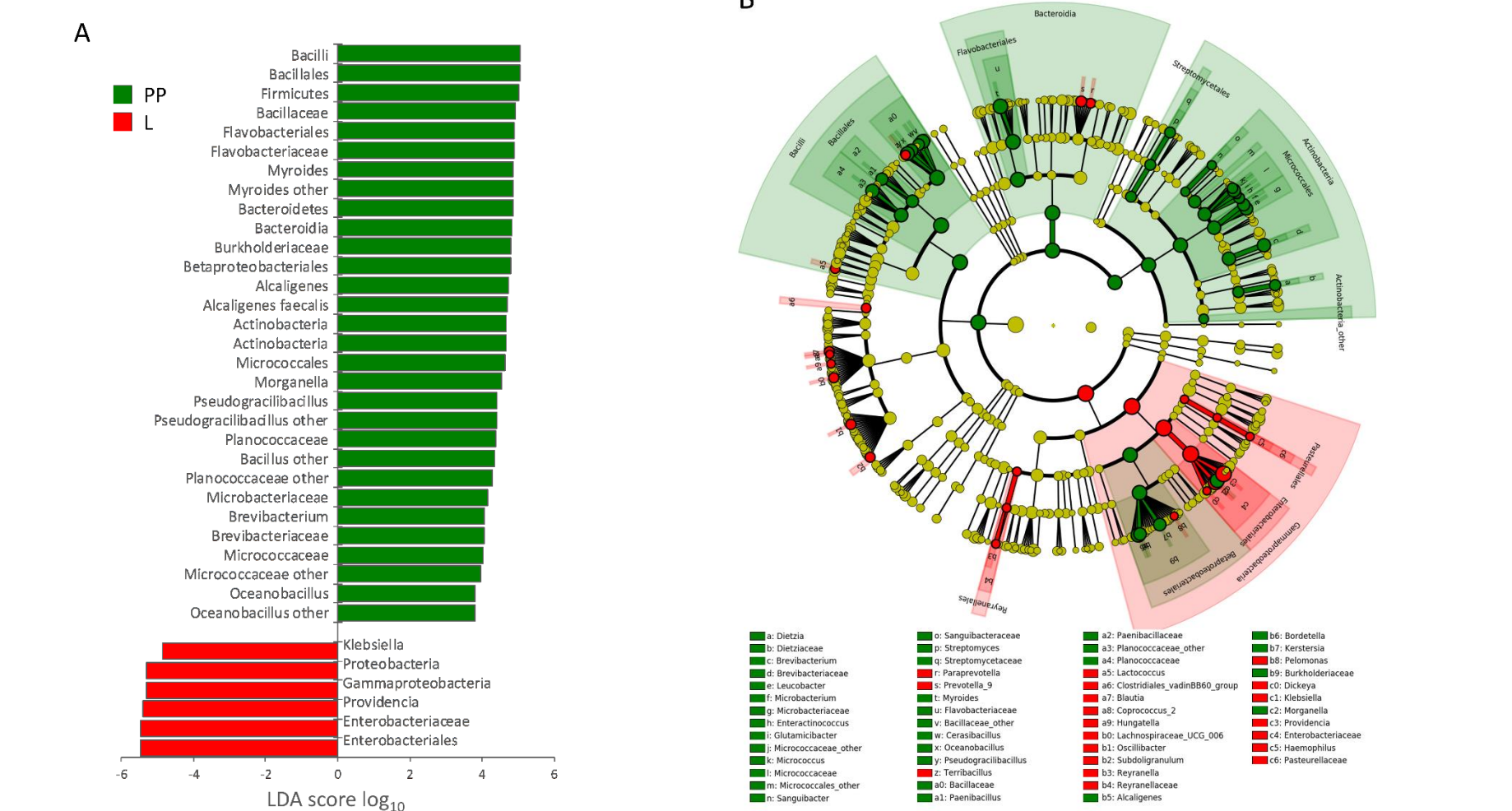
16S rRNA gene profiling



The microbiota of L and PP clustered in distinct groups based on Bray-Curtis distance, that had different centroids (PERMANOVA, P = 0.001), and intra-group permutational similarity significantly greater than the inter-group one (ANOSIM, P = 0.001). Grouping was evident in the PCo1-PCo2 plot (A), where the first coordinate was the most informative (55.3% of the total variance) and clearly separated L and PP samples. PP lay at progressively lower PCo1 with the increase of the rearing temperature. Positive contribution to PCo1 came from *Providencia* and, at lower extent, *Klebsiella*, while negative contribution came from *Alcaligenes faecalis*, *Bacillus*, *Morganella*, and *Myroides* (B).



The microbiota of L was dominated by Proteobacteria (87.4%), with remarkably high amounts of *Providencia* (64.1%) and *Klebsiella* (16.4%), followed by Firmicutes (9.3%), especially *Bacillus* (4.3%). PP were poorer than L in Proteobacteria and richer in Bacteroidia, Actinobacteria, and Bacilli. *Providencia* remained abundant in PP (from 6.4 to 26.1%, depending on the temperature), although it significantly decreased compared to L, likewise *Klebsiella* and other minor Enterobacteriaceae. The genera *Morganella*, *Alcaligenes*, *Bordetella*, and *Kerstersia* behaved in contrast to most Proteobacteria and got significantly enriched in PP. *Morganella* and *Alcaligenes*, in particular, reached remarkable levels (8.9 and 16.9%, respectively). The significant increase of Bacteroidia in PP was mainly due to *Myroides*, a Flavobacteriaceae that reached the 30%. Bacilli also included several biomarkers characterizing PP, such as Planococcaceae, Paenibacillaceae, *Pseudogracilibacillus*, *Oceanobacillus*, and unclassified members of the genus *Bacillus*. Planococcaceae, *Pseudogracilibacillus* and unclassified *Bacillus* reached the 8.5, 5.9, and 13.1%, respectively. Within Actinobacteria, the main biomarkers characterizing PP were Micrococcales belonging to *Brevibacterium*, which increased up to 3.3%, and several minor genera. The increase of incubation temperature negatively correlated with the level of Enterobacteriaceae in PP, mostly due to the decrease of *Providencia*. Unlike *Providencia*, other Proteobacteria positively correlated with temperature, such as *Kerstersia*, *Proteus*, *Pseudomonas*, *Alcaligenes*, and *Bordetella*. *Pseudomonas* was abundant only in PP reared at 33 °C (6.7%), whereas at the lower temperatures it accounted for less than 0.1%. *Bacillus* was also positively associated to the increasing temperature and reached the 13.1% at 33 °C. Increasing temperatures also favored the populations of other Actinobacteria (*Brevibacterium*), Bacilli (e.g. *Enterococcus*, *Pediococcus*, *Paenibacillus*, *Pseudogracilibacillus*) and Bacteroidetes (*Sphingobacterium*), although occurring in low amounts. The correlation of taxa with temperature is in agreement with their contribution in the PCoA biplot, resulting in PP samples being located at lower PCo1 values with the increase of the rearing temperature



DISCUSSION

In the present study, the larvae of *H. illucens* were reared at three different temperatures (20, 27, and 33 °C), utilizing a standard vegetal substrate, until reaching the prepupae stadium. The larvae efficiently grew across a wide range of temperatures, although with differences in the development rate, confirming the robustness of *H. illucens*. The flexibility towards the rearing temperature offers the opportunity to apply these process also in developing countries, with minimal investment in energy consuming devices for cooling or heating. The rearing temperature slightly affected the microbial load of PP, with only total mesophilic aerobes and enterobacteriaceae significantly lower at 20 °C than at the higher temperatures. Metagenome analysis revealed a significant effect of the rearing temperature on the microbiota composition. The rearing temperature was negatively associated to the amount of *Providencia*, that was the lowest at 33°C. On the contrary, temperature was positively associated to a variety of other genera, such as *Alcaligenes*, *Pseudogracilibacillus*, *Bacillus*, *Proteus*, *Enterococcus*, *Pediococcus*, *Bordetella*, *Pseudomonas*, and *Kerstersia*. Thus, temperature has to be included among the main factors affecting the microbiota of prepupae and may have contributed, together with the rearing substrate, to the wide differences in the structure of the microbial community associated to *H. illucens* reported in literature.