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Can farm weeds improve the growth and microbiological quality of crickets (*Gryllus bimaculatus*)? --Manuscript Draft--

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Abstract:	<p>Farming of edible insects has been proposed as a means to reduce current practices of harvesting from the wild. While farming could relieve the pressure on wild populations, as well as on their natural habitats, and generate a continuous supply of edible insects to the consumers, the high cost of commercial chicken feeds is still a challenge to many farmers. The aim of this study was to investigate whether the partial replacement of commercial chicken feed with the farm weed, Wandering Jew (<i>Commelina sinensis</i>), would have an impact on weight gain and microbial quality of farmed field crickets (<i>Gryllus bimaculatus</i>). Therefore, we have experimentally compared two types of feed: (1) Starter commercial chicken feed only (SO), and (2) Starter commercial chicken feed supplemented with fresh Wandering Jew weeds (S+W). After a feeding period of thirty days, the final body weight differed significantly ($P = 0.026$) between crickets fed with SO and with S+W, which averaged at 1.11 g and 1.39 g, respectively. Generally, high counts of total aerobic, Enterobacteriaceae, lactic acid bacteria, bacterial endospores, yeasts and moulds were observed in both experimental groups. However, bacterial endospore counts of S+W fed crickets ($2.7 \log \text{ cfu/g}$) were significantly lower ($P = 0.021$) than these of the SO fed ones ($3.9 \log \text{ cfu/g}$). Metagenetic analyses indicated that Proteobacteria, Firmicutes and Tenericutes were the most abundant phyla, while members of the family Coxiellaceae, and the genera <i>Lactobacillus</i>, and <i>Spiroplasma</i> were the most abundant Operational Taxonomic Units (OTUs). Concerning the food safety, a few OTUs could be associated with potential food pathogens, such as Clostridiaceae, Staphylococcus and Enterobacteriaceae. In summary, the inclusion of fresh Wandering Jew weeds in commercial starter chicken feed produced crickets with increased body weight and improved microbial quality.</p>

Microbial quality of farmed crickets

Can farm weeds improve the growth and microbiological quality of crickets (*Gryllus bimaculatus*)?

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Abstract

Farming of edible insects has been proposed as a means to reduce current practices of harvesting from the wild. While farming could relieve the pressure on wild populations, as well as on their natural habitats, and generate a continuous supply of edible insects to the consumers, the high cost of commercial chicken feeds is still a challenge to many farmers. The aim of this study was to investigate whether the partial replacement of commercial chicken feed with the farm weed, Wandering Jew (*Commelina sinensis*), would have an impact on weight gain and microbial quality of farmed field crickets (*Gryllus bimaculatus*). Therefore, we have experimentally compared two types of feed: (1) Starter commercial chicken feed only (SO), and (2) Starter commercial chicken feed supplemented with fresh Wandering Jew weeds (S+W). After a feeding period of thirty days, the final body weight differed significantly ($P = 0.026$) between crickets fed with SO and with S+W, which averaged at 1.11 g and 1.39 g, respectively. Generally, high counts of total aerobic, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, yeasts and moulds were observed in both experimental groups. However, bacterial endospore counts of S+W fed crickets (2.7 log cfu/g) were significantly lower ($P = 0.021$) than these of the SO fed ones (3.9 log cfu/g). Metagenetic analyses indicated that Proteobacteria, Firmicutes and Tenericutes were the most abundant phyla, while members of the family Coxiellaceae, and the genera *Lactobacillus*, and *Spiroplasma* were the most abundant Operational Taxonomic Units (OTUs). Concerning the food safety, a few OTUs could be associated with potential food pathogens, such as *Clostridiaceae*, *Staphylococcus* and *Enterobacteriaceae*. In summary, the inclusion of fresh Wandering Jew weeds in commercial starter chicken feed produced crickets with increased body weight and improved microbial quality.

Keywords: Edible insects, food safety, microbial numbers, Next-generation sequencing, weight gain

1. Introduction

Over the past several years, there has been an increased interest of using edible insects as food and feed. Edible insects are considered as suitable alternatives to animal sources of food, such as chicken and beef (Belluco *et al.*, 2013; Nowak *et al.*, 2016). In this perspective, the potential of edible insects to address food and nutrition security is on the increase (van Huis, 2013). Although the use of insects as food and feed is gaining interest in western countries (Caparros Megido *et al.*, 2014; Mancini *et al.*, 2019; Orsi *et al.*, 2019), in most regions of Africa and Asia, edible insects already are part of the traditional food systems and diets (Christensen *et al.*, 2006; Kelemu *et al.*, 2015; van Huis, 2013). For instance, in Kenya, different kinds of insects such as termites, crickets, grasshoppers, ants, lake flies, collected from different natural environments have been consumed by various ethnic groups for decades (Ayieko *et al.*, 2012; Kelemu *et al.*, 2015).

However, the consumption of these insects is usually hampered by regional and seasonal availability, sustainability issues, high perishability, and pathogenic risks (Ng'ang'a *et al.*, 2018; Ssepuuya *et al.*, 2019). In this context, the development of insect farming has been proposed to (i) reduce the collection of insects from the wild and, thus, relieve the pressure on natural populations and habitats, (ii) generate a continuous supply of edible insects, and consequently, (iii) increase households' protein consumption (Caparros Megido *et al.*, 2015; Kinyuru and Kipkoech, 2018). A greater attention has been put on a handful of insects which can be easily domesticated and reared with significantly shorter life cycles. Some of these insects belong to the Gryllidae (cricket) family (Halloran *et al.*, 2016). For example, the house cricket (*Acheta domesticus*) and the field cricket (*Gryllus bimaculatus*) can be reared and harvested after 60 to 75 days under natural conditions (Caparros Megido *et al.*, 2015; Kinyuru and Kipkoech, 2018).

Cricket farming has been successfully implemented in Thailand (Halloran *et al.*, 2016) and Kenya (Kinyuru and Kipkoech, 2018), while gaining interest in Cambodia, Lao People's Democratic Republic, and the Democratic Republic of the Congo (Halloran *et al.*, 2018). Although cricket farming has shown its potential for large-scale production, the relatively high cost of the local

commercial feeds, such as commercial chicken feeds, is still a challenge to farmers (Caparros Megido *et al.*, 2015; Durst and Hanboonsong, 2015). However, previous studies have demonstrated that crickets can be farmed by replacing the commercial feeds with locally available agricultural by-products (Caparros Megido *et al.*, 2015; Magara *et al.*, 2019; Orinda *et al.*, 2017), or agricultural farm weeds and wastes vegetable (Choo *et al.*, 2017; Kinyuru and Kipkoech, 2018; Miech *et al.*, 2016). Although the nutritional compositions of such farmed insects were mostly reported, studies on their microbial safety are scarce. Furthermore, it is worth noting that insect feeds and farming environments are important factors that eventually will influence the insects' microbial composition (Dillon and Dillon, 2004; EFSA, 2015). In this regard, the microbial dynamics of edible insects meant for human consumption depends on the rearing process (Wynants *et al.*, 2018).

In Kenya, a country that heavily relies on agriculture, farm weeds are common in farmlands, as well as in some natural environments. Following the study by Kinyuru and Kipkoech (2018), who assessed the level of the acceptance of five common farm weeds by *A. domesticus*, (level of acceptance was categorized into high, medium or low. Consumption of at least 75% of the farm weed was considered high acceptance), the Wandering Jew (*Commelina sinensis*) had highest acceptability score, over 75%. From this perspective, an experiment was designed to assess whether a partial replacement of commercial chicken feed with farm weeds (Wandering Jew) would have an impact on the weight gain and microbial quality of farmed field crickets (*G. bimaculatus*).

2. Materials and methods

Study site, rearing containers and experimental treatments

Cricket rearing was done at the Jomo Kenyatta University of Agriculture and Technology (JKUAT) insect farm (1°08'S, 37°02'E, 1519 m.a.s.l). The experiment was designed as an exact replica of what many small-scale farmers rearing crickets in Kenya are currently practicing. In order to obtain the pinheads for the rearing trial, the eggs were collected from the stock colony of *G. bimaculatus* using moist cotton balls which were replaced after every 2 days. The cotton

balls containing the eggs were thinly spread-out and placed in sterilized 2 L transparent rectangular plastic containers (21×14×15 cm) (Kenpoly Manufacturers Ltd., Nairobi, Kenya) and incubated in a controlled environmental chamber maintained at 30 °C, 70% relative humidity, and a photoperiod of 12 hours :12 hours light: dark until hatched. The emerging nymphs were transferred into 50 L plastic containers, and fed until when they were 30 days old.

The rearing was carried out in 50 L plastic containers with a top diameter of 56.4 cm, a bottom diameter of 48.5 cm and a height of 33.4 cm (Kenpoly Manufacturers Ltd., Nairobi, Kenya). Cardboard egg cartons (29 × 29 cm) were placed horizontally inside the plastic containers to increase the insect living areas and to provide the shelter. In the experiment, two types of feeds were used as experimental treatments: (1) Starter commercial chicken feed only (SO), and (2) Starter commercial chicken feed + fresh Wandering Jew weeds (S+W). Eight rearing plastic containers containing cardboard egg cartons were arranged randomly in the rearing room, and 40 nymphs, 30 days old were added in each of them. Then, for each feed (n = 4 replications) was administered randomly as follows: SO = 20 g/rearing container/2 days while S+W = S (10 g/rearing container/2 days) + W (10 g/container/2 days). Wet cotton wools were placed on 16 cm diameter saucers and placed in each rearing container to provide water; they were changed after every 2 days. The rearing containers were cleaned after every 2 days to remove the substrates, faeces and dead crickets. The rearing containers were covered by a 2 mm net to keep the insects inside and prevent entry of predators. The rearing was carried out for 30 days during the months of June and July, 2018.

Weight gain and mortality

The weight gain was determined at the onset of the experiment, as well as after 15 and 30 days; while the mortality rate was assessed at 15 and 30 days of rearing. Thirty crickets per experimental feed condition were randomly selected during each sampling period and used for weight gain determination. After 15 days, the rearing containers for each experimental feed condition were randomly selected, and two cardboard egg cartons per rearing container were lifted and shaken into a clean container, and the homogenized samples obtained used for weight gain determination. Thereafter, all cardboard egg cartons were removed, and live and

dead crickets recorded for each feed condition. Similarly, at the end of 30 days of rearing, the live and dead crickets were recorded. Then, four homogenized samples per feed condition consisting only of live crickets were obtained. One homogenized sample per feed condition was used for weight gain determination. Separate weight gains for both female and male crickets were also recorded. The remaining samples for each feed condition were refrigerated for 24 hours to sedate the insects, and samples for microbial counts were withdrawn. Subsequently, remaining samples were stored at -18° C as whole insects awaiting metagenetics analysis.

Culture dependent microbiological analysis

Microbial counts were done at the end of 30 days rearing period. After 24 hours of sedation, the crickets were homogenized per feed condition from which 20 g of the homogenate was pulverized prior to analysis as described by Stoops *et al.* (2016). Then, an aliquot of 10 g of pulverized insect sample was aseptically transferred into a sterile stomacher bag (Bagmixer® 400 W, Interscience, St Nom, France) and 90 ml of sterile peptone physiological salt solution (0.85% (w/v) NaCl, 0.1% (w/v) peptone, was added. The mixture was homogenized for 1 minute in a stomacher. Then, using standardized ISO methods for microbial analyses of food described by Dijk *et al.* (2015), a ten-fold serial dilution series was plated on different media using the pour plate method except for moulds and yeasts.

Total viable aerobic counts (TVC) were determined on Plate Count Agar (PCA, Biokar Diagnostic) incubated at 30 °C for 72 hours, *Enterobacteriaceae* on Violet Red Bile Glucose medium (VRBG, Biokar Diagnostic) with an overlay of the same medium and incubated at 37 °C for 24 hours, lactic acid bacteria (LAB) on de Man Rogosa Sharpe medium (MRS, Biokar Diagnostic) with an overlay of the same medium and incubated at 30 °C for 72 hours. For yeasts and moulds, 0.1 ml was plated using the spread plate method on Dichloran Rose-Bengal Chloramphenicol Agar (RBA, Biokar Diagnostic) and incubated at 25 °C for 120 hours. Bacterial endospores were determined by giving the 10⁻¹ dilution a heat shock treatment (10 minutes at 80 °C), followed by serial dilution, plating using the pour plate method onto PCA and incubation at 37 °C for 48 hours. For each experimental feed condition eight replications were conducted during plating, and microbial counts were expressed as log cfu/g.

Metagenetic analyses

To study the bacterial community, following the thawing process, 20 g of pulverized samples per feed condition were subjected to high-throughput 16S rRNA gene sequencing (V4 region, 250 bp) using the Illumina MiSeq platform. The microbial genomic DNA was extracted from each homogenized sample per feed condition (0.2 g) in duplicate following the protocol of the PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, California, USA). DNA quality and concentrations were assessed using a Nanodrop Spectrophotometer (mySPEC, VWR), and all extracted DNA were stored at -20°C for further downstream processing.

PCR amplification was performed using the dual index strategy as described by Kozich *et al.* (2013). Briefly, DNA extracts were subjected to a PCR assay using barcode-labelled versions of the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 805R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso *et al.*, 2011), amplifying the V4 region (250 bp) of the 16S rRNA gene. The PCR amplification (20 µl) contained 5 ng / µl genomic DNA, 1.5 µl of dNTP mix (0.15 mM each), 0.5 µl of each primer (0.5 µM), 2 µl of Titanium Taq PCR buffer, 0.4 µl of Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France), and 14.1 µl of sterile milli-Q water. The PCR amplification protocol consisted of an initial denaturation at 95 °C for 2 minutes, then, 30 cycles of denaturation at 95 °C for 45 seconds, followed by primer annealing at 59 °C for 45 seconds, and a final extension step at 72°C for 10 minutes.

Following the amplification, the PCR products were purified by Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified using the Qubit fluorometer (HS reaction kit, Invitrogen, Carlsbad, CA, USA), combined into a library in equimolar concentrations and subjected to an ethanol precipitation. As a final quality control, the pooled library was subjected to electrophoresis on a agarose gel (1.5 %), after which amplicons were cut out and purified with the NucleoSpin gel and PCR cleanup kit (Machery-Nagel, Düren, Germany). Finally, the library was diluted to 2 nM concentrations and sequenced at the Center of Medical Genetics Antwerp (University of Antwerp, Belgium).

Thereafter, sequences were received in the format of a de-multiplexed FASTQ file. Paired-end reads were merged with a maximum of five mismatches using USEARCH (v.9.2.64) (Edgar, 2013) to form consensus sequences and truncated at the 250th base. Shorter reads or reads with a total expected error threshold above 0.10 for all bases after truncation were discarded. Next, the “classify.seqs” and “remove.lineage” commands in Mothur (v1.39.3; Edgar, 2013) and the Silva database (v123; Gurevich *et al.*, 2013) were used to identify and remove potential mitochondrial, chloroplast, archaeal and eukaryote contaminants. Samples that yielded too few sequences were discarded from further analysis. Remaining sequences were grouped into operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the UPARSE greedy algorithm in USEARCH, during which chimeric sequences were also removed (Edgar, 2013), as the global singletons (i.e. OTUs representing only a single sequence in the entire dataset), minimizing the risk of retaining sequences from sequencing errors (Brown *et al.*, 2015; Waud *et al.*, 2014). The taxonomic origin of each OTU was determined with the SINTAX algorithm implemented in USEARCH (Edgar, 2016) based on the Silva database v123 (LTP v123). Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.80.

Statistical analysis

Statistical analyses were performed using Stata SE version 12 (StataCorp LP, TX). Data are presented as averages with their standard deviations. Microbial counts were expressed as log cfu/g. Chao1 and Shannon-Wiener diversity indices were calculated using the R-packages Vegan (v.2.5-5) (R Development Core Team, 2013). Independent t-test was performed to determine significant differences in individual microbial counts (Total aerobic viable counts, *Enterobacteriaceae*, lactic acid bacteria, bacterial endospores, and yeasts and moulds), the weight gain, mortality, and alpha diversity indices (OTUs richness, Chao1, Shannon-Wiener, coverage, and evenness) between the two feeds in the experiment. All tests considered 0.05% significance level.

3. Results and Discussion

Weight gain and mortality

Table 1 summarizes the crickets' body weight, weight gain and the mortality within 30 days rearing period. During the onset of the experiment, the cricket nymphs' weight per experimental feed condition did not differ significantly $t(58) = 0.372$, $P = 0.626$. Initial weights ranged from 0.24 to 0.53 g, with mean weights of 0.39 g and 0.37 g for SO and S+W, respectively. However, a steady increase in the weight gain was observed between the two feeds during 30 days of rearing, as shown in Table 1. Similar trends were reported by Caparros Megido *et al.* (2015) on oriental ground crickets (*Teleogryllus testaceus*) that were fed on agricultural side streams and by Kinyuru and Kipkoech (2018) on house crickets (*A. domesticus*) reared using farm weeds. Although there was small variation in average weight gain within the first 15 days of rearing, the mean weights between the two experimental feeds did not differ significantly $t(58) = 0.211$, $P = 0.715$. Previous studies (Kinyuru and Kipkoech, 2018; Orinda *et al.*, 2017), demonstrated that young cricket nymphs' have little preference to agricultural side streams and agro-byproducts as compared to the adult crickets, and this was also observed in this study.

Table 1. Body weight, weight gain and mortality of *G. bimaculatus* fed Starter chicken feed only (SO) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Duration (d)	Body weight (g)		Mortality (%)	
	SO	S+W	SO	S+W
0	0.39±0.1 ^a	0.37±0.1 ^a	-	-
15	0.88±0.1 ^a	0.83±0.2 ^a	5.62±1.9 ^a	7.50±1.6 ^a
30	1.11±0.2 ^a	1.39±0.2 ^b	17.50±2.9 ^a	15.00±2.6 ^a
Weight gain (g)				
0-15	0.49	0.46	-	-
15-30	0.23	0.56	-	-

Data are the means ± standard deviations

^{a,b}, Means with the same superscript within the same row do not differ significantly ($P > 0.05$).

The final weights of adult *G. bimaculatus* ranged from 0.89 g to 1.5 g and the average weight gain at the end of the experiment differed significantly between the two experimental feed conditions (Table 1). In a different study, Orinda *et al.* (2017) reported slightly lower body

weights (0.68 g – 0.99 g) of adult *G. bimaculatus* fed with agro-byproducts, while Caparros Megido *et al.* (2015) recorded higher body weights (1.8 – 3.1 g) of *T. testaceus* reared with agricultural side streams. In this study, the weight of adult *G. bimaculatus* fed with S+W was the highest (average 1.39 g), and as demonstrated previously (Kinyuru and Kipkoech, 2018), the fresh farm weeds, such as Wandering Jew weeds, are more palatable, and tend to increase the crickets' feed consumption capability, and hence, their high weight gain. In addition, crickets in this experimental feed (S+W) condition demanded little water, since they were able to obtain more moisture from fresh farm weeds. This finding has demonstrated that small-scale farmers rearing crickets can partially replace the commonly used commercial chicken feeds with naturally occurring farm weeds and even produce crickets with higher sellable weight. Similarly, when native broiler finisher was substituted with agricultural side streams and fed on *T. testaceus*, high quality crickets were produced and the cost of production was low (Caparros Megido *et al.*, 2015).

The body weight and weight gain of both male and female *G. bimaculatus* during the 30 days rearing period are presented in Table 2. At the onset of the experiment, it was difficult to differentiate between sexes; hence, the weight of males and females was not determined at this age. After 15 days of rearing, a steady increase in the weight gain in both feed conditions was observed, with mean weights ranging 0.76 – 0.78 g (male) and 0.86 – 0.89 g (female), but no significant difference was observed (Table 2).

Table 2. Body weight and weight gain of male and female *G. bimaculatus* fed Starter chicken feed only (S0) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Duration (d)	Body weight (g)			
	S0		S+W	
	Male	Female	Male	Female
15	0.77±0.1 ^a	0.87±0.1 ^a	0.78±0.1 ^a	0.86±0.1 ^a
30	1.12±0.1 ^a	1.42±0.1 ^b	1.14±0.2 ^a	1.44±0.1 ^b
Weight gain (g)				
15-30	0.37	0.55	0.36	0.58

Data are the means ± standard deviations

^{a,b}, Means with the same superscript within the same row do not differ significantly ($p > 0.05$)

At the end of the rearing period, the body weight of female crickets was significantly higher than that of male crickets in both feed conditions (Table 2). Normally, at this age the female crickets are adults, have developed ovipositors, and are feeding voraciously to meet the protein demand for egg development, which could be associated with the observed increase in weight gain, as compared to their male counterparts.

At the end of rearing trial, the mortality averaged 17.50% and 15.00% for SO and S+W, respectively, and no significant difference was observed between these conditions (Table 1). Researchers have demonstrated that the type and quantity of feed provided to the crickets profusely influenced the mortality. For instance, when *T. testaceus* were fed young cashew leaf flour only, or a mixture of young cashew leaf flour and brown rice flour, the mortality ranged between 81.11 – 94.44%. However, when young cassava leaf flour only or native broiler finisher only or a mixture of young cassava leaf flour and brown rice flour were provided as feeds to *T. testaceus*, the mortality rate was less than 13.13% (Caparros Megido *et al.*, 2015). In a separate study, Kinyuru and Kipkoech (2018) reported mortality below 2.00% mortality when *A. domesticus* were fed with agricultural side streams and farm weeds. In the above findings (Caparros Megido *et al.*, 2015; Kinyuru and Kipkoech, 2018), the mortality was mostly associated with unpalatable feeds, cannibalism, and predation. However, in the current study, a number of whole crickets were found dead and the cause of their death could not be stated with certainty. In this regard, further research is proposed to determine the cause(s) of mortality, such as assessing the parasitoids associated with the crickets' life cycle.

Classical microbiological analyses of *G. bimaculatus*

Microbial counts of freshly farmed adult *G. bimaculatus* are presented in Table 3. Normally, plate counts are used as indicators of good hygienic conditions or assessment of the quality of the food products (Stoops *et al.*, 2016). On average, microbial counts were generally high, as shown in Table 3. The total aerobic counts averaged 7.4 and 7.5 log cfu/g for SO and S+W, respectively, and did not differ significantly $t(14) = 0.738$, $P = 0.473$. Although these numbers were above 6.7 log cfu/g, the recommended high hygiene criterion for raw minced meat (Grabowski and Klein, 2017a), they were slightly lower than 8 log cfu/g reported by Caparros

Megido *et al.* (2017), on *A. domesticus* bought from rearing company, and 8.2 – 8.4 log cfu/g for reared tropical house crickets *Gryllodes sigillatus* (Vandeweyer *et al.*, 2018), and this may suggest an improved handling and or quality of feed during rearing. However, the aerobic viable counts in this study compare closely to numbers found for *A. domesticus* purchased from a rearing company (Klunder *et al.*, 2012), as well as to samples of *A. domesticus* and *G. bimaculatus* that were bought from pet shops (Grabowski and Klein, 2017a).

Similarly, no significant differences were observed between crickets from both feed conditions for counts of *Enterobacteriaceae* $t(14) = 0.599$, $P = 0.558$, and yeasts and moulds $t(14) = 1.483$, $P = 0.160$, although they were above recommended limits of raw minced meat (*Enterobacteriaceae* <2.0 and yeasts & moulds <4.0 log cfu/g) (Grabowski and Klein, 2017a). In other studies, Grabowski and Klein (2017a) reported high counts of *Enterobacteriaceae* (7 log cfu/g) for both *A. domesticus* and *G. bimaculatus*, while Vandeweyer *et al.* (2018) found counts between 7.2 – 7.5 log cfu/g for farmed *G. sigillatus*. In contrast, Klunder *et al.* (2012) reported significantly lower value (4 log cfu/g) for *A. domesticus* purchased from a local rearing company. It could be that for some of the rearing companies, from where samples were taken, the insects were not intended for human consumption, possibly explaining the huge variation. However, the counts for yeasts and moulds in this study are in line with the ones reported in literature for *A. domesticus* (Caparros Megido *et al.*, 2017) and for *G. bimaculatus* (Grabowski and Klein, 2017a).

Table 3. Microbial counts (log cfu/g) of *G. bimaculatus* fed Starter chicken feed only (S0) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Microbial counts	Type of feeds	
	S0	S+W
Total aerobic viable counts	7.4±0.1 ^a	7.5±0.3 ^a
Enterobacteriaceae	6.4±0.4 ^a	6.2±0.8 ^a
Lactic acid bacteria	6.8±0.3 ^a	6.1±0.1 ^b
Bacterial endospores	3.9±0.1 ^a	2.7±0.2 ^b
Yeasts and moulds	5.2±0.2 ^a	4.8±0.6 ^a

Data are the means ± standard deviations

^{a,b}, Means with the same superscript within the same row do not differ significantly ($p > 0.05$)

On the other hand, a significant difference was observed for lactic acid bacteria $t(14) = 5.697$, $P = 0.001$, and bacterial endospores $t(14) = 4.429$, $P = 0.021$, between the two feed conditions. The bacterial endospores averaged at 3.9 log cfu/g and 2.7 log cfu/g for feeds SO and S+W, respectively. Klunder *et al.* (2012) reported bacterial endospores (3.6 log cfu/g) of *A. domesticus*, which is comparable to crickets fed with SO, but slightly higher than these fed with S+W. This finding may suggest that by partially replacing the commercially available chicken feeds with Wandering Jew, there is a possibility of reducing the risks posed by bacterial endospores. Previously, Wandering Jew leave extracts have indeed shown promising antibacterial activities against common bacterial pathogens, such as *Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus* (Dash *et al.*, 2017), although this cannot be stated with certainty as the main reason for low endospores counts in crickets fed with feed S+W. Moreover, bacterial endospores could be acquired when insects come into contact with the soil or through feed (Klunder *et al.*, 2012). Although the bacterial endospores in both feeds were not determined in this study, high ratios of commercial chicken feeds, such as SO, usually consist of grains that are normally spread-dried on the floor and that could act as points of contamination.

The high microbial counts can imply a risk for food safety, and therefore, these results support the assumption that a processing step is required to avoid or minimize the risks involved with the consumption of edible insects (Klunder *et al.*, 2012). Common processing methods have demonstrated the ability to eliminate or reduce the risks (Caparros Megido *et al.*, 2018; Gatheru *et al.*, 2019; Grabowski and Klein, 2017b; Klunder *et al.*, 2012; Ng'ang'a *et al.*, 2018; Vandeweyer *et al.*, 2017a), but not sufficient enough to eliminate the bacterial endospores (Klunder *et al.*, 2012; Ng'ang'a *et al.*, 2018; Vandeweyer *et al.*, 2017a). In this regard, the risks caused by spore-forming bacteria could be minimized by optimizing a combination of factors, such as the substrates used, the farming environment, and good agricultural and handling practices.

Metagenetics analyses

The bacterial community composition of the *G. bimaculatus* was characterized using high-throughput 16S rRNA. A total of 1976 Operational Taxonomic Units (OTUs) were obtained

(Table S1). The *G. bimaculatus* that were reared using SO were dominated by three bacterial phyla ascribed to Proteobacteria (55.56 - 96.90%; *Coxiellaceae*), Firmicutes (0.25 - 26.15%; *Lactobacillus*), Tenericutes (0.02 - 8.12%; *Spiroplasma*), and also, those reared using S+W; Proteobacteria (53.56 - 98.20%; *Coxiellaceae*), Firmicutes (0.15 - 26.41%; *Lactobacillus*) and Tenericutes (0.03 - 8.97%; *Spiroplasma*) were also the dominant phyla (Figure 1). Similarly, *A. domesticus* and powdered crickets were dominated by Proteobacteria (42.6% and 28.4%, respectively) and Firmicutes (34% and 54%, respectively), while Tenericutes (0.7% and 1.6%, respectively) was detected in low abundance (Garofalo *et al.*, 2017).

However, comparing these results to those obtained by Vandeweyer *et al.* (2017b), on average, *A. domesticus* and *G. sigillatus* were dominated by Bacteroidetes (43%) and Firmicutes (35%) while Proteobacteria was low in abundance. Similarly, during rearing and processing of *G. sigillatus*, the most abundant phyla were Bacteroidetes ranging from 35.8 to 71.2%, Firmicutes (5.4 - 56.6%) and Proteobacteria ranging from 4.9 to 16.0% (Vandeweyer *et al.*, 2018). In other similar studies, Firmicutes and Proteobacteria were also the dominant phyla in *Tenebrio molitor* larvae (Wynants *et al.*, 2018) and *Locusta migratoria* (Stoops *et al.*, 2017), while Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria were dominant phyla in *Ruspolia differens* (Ssepuyya *et al.*, 2019).

When the bacterial community of *T. molitor* larvae from three rearing cycles within the same company was characterized, one cycle was dominated by the phyla *Spiroplasma* (80%) (Vandeweyer *et al.*, 2017b). In this study, however, the bacterial family *Coxiellaceae* (OTUs 7) dominated, representing 70.58% of the total sequences (SO) and 79.98% of the total sequences (S+W), and the bacterial pattern seems to be less different between the two experimental feeds (Figure 1). The dominance of the phyla Proteobacteria, Firmicutes and Bacteroidetes in this study, and what is reported in the literature demonstrate that different edible insect species can be colonized by similar bacterial communities, although with significant variations. Normally, these phyla represent a group of bacteria that are gut-associated with insects and highly influenced by the diets and the hosts' intestinal bacterial community composition (Colman *et al.*, 2012).

The diversity indices were averaged for replicate samples per experimental feed condition and presented in Table 4. The total average coverage, based on Chao1 calculation was $74.42 \pm 1.60\%$ and $73.81 \pm 1.30\%$ for SO and S+W, respectively, indicating that most abundant bacterial communities in *G. bimaculatus* were recovered. These values are slightly lower compared with those reported in literature for *G. sigillatus* (Vandeweyer *et al.*, 2018) and *A. domesticus* (Vandeweyer *et al.*, 2017b). The observed richness, Chao1, Shannon-Wiener, and evenness diversity indices did not differ significantly between the two experimental feed conditions (Table 4). Although it maybe hypothesized that the addition of the Wandering Jew weeds in the commercial starter chicken feed may increase the bacterial diversity, the Shannon-Wiener diversity index, which denotes the diversity based on both richness and abundance, did not differ significantly, but it was slightly higher for S+W than that of SO (Table 4).

Table 4. Microbial community diversity indices of the *G. bimaculatus* fed Starter chicken feed only (SO) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Type of feed	Alpha diversity index				
	OTUs richness	Chao1	Coverage	Shannon-Wiener	Evenness
SO	32 ± 5.20^a	43 ± 7.80^a	74.42 ± 1.61^a	1.32 ± 0.07^a	0.12 ± 0.01^a
S+W	33 ± 1.73^a	44 ± 7.13^a	73.81 ± 1.30^a	1.43 ± 0.05^a	0.14 ± 0.01^a

Sequences were grouped into Operational Taxonomic Units (OTUs) defined by 97% sequence identity at the 16S rRNA gene (V4 region, 250 bp). Values are the mean $\pm$ standard deviation of analyses performed on two replicate samples, with two technical replicates per sample ($n = 2 \times 2$), Richness = the number of observed OTUs; Chao1 = the species richness estimator (DeLong, 2013); Coverage = observed richness/Chao1 estimate $\times 100$ (Vandeweyer *et al.*, 2017); Shannon-Wiener index = an indication of species diversity, a combined measure of the number of species in a community (richness) and the number of individuals per species (Di Bitetti, 2017); Evenness = the relative frequency of species in a community (Di Bitetti, 2017).

Generally, except the *Coxiellaceae* (OTU 7), *Lactobacillus* (OTU 5), and *Spiroplasma* (OTU 4), other OTUs were found at relative abundance lower than 1% (Table S1). The occurrence of *Coxiellaceae* (OTU 7) and *Spiroplasma* (OTU 4) in relatively high abundance plus other OTUs

with less than 1% abundance that included *Staphylococcus*, *Porphyromonadaceae*, *Acetobacter*, *Pseudomonas*, *Rikenellaceae*, and *Morganella* in both experimental feed conditions could have contributed to high total viable counts (Table 3). On average, 17.4% of all bacterial sequences (SO) and 11.5% of all bacterial sequences (S+W) belonged to members of the genus *Lactobacillus* (OTUs 5 and 1; Figure 1), corresponding to high counts of lactic acid bacteria (SO: 6.8 log cfu/g and S+W: 6.1 log cfu/g). These counts differed significantly between the two feeds (Table 3). In addition, *Pediococcus* and *Enterococcus* were also detected in both experimental feed conditions. Although for some species of the genera *Lactobacillus*, *Pediococcus* and *Enterococcus* probiotic properties have been claimed when ingested in right amount (Fijan, 2014), it is also worth noting that some species of the genera *Lactobacillus*, *Leuconostoc*, and *Lactococcus* also act as food spoilage organisms particularly in refrigerated meat products (Nieminen *et al.*, 2011). In this regard, it is possible that lactic acid bacteria cause spoilage in edible insects when poorly preserved since they contain high protein content just like conventional meats or meat products.

Although the occurrence of pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* spp., was not detected after metagenetic analysis, these pathogens can't be completely ruled out. *Escherichia coli* and *Salmonella* can still be within the family *Enterobacteriaceae* which occurred at relative low abundance (<1%; Figure 1) between the two experimental feed conditions. *Enterobacteriaceae* are normally associated with human and animal faeces and are frequently used as indicators of good handling or good processing practices (Stoops *et al.*, 2016). In a similar study, after pyrosequencing, powdered crickets contained a low abundance (below 2%) of *Listeria* spp., while other major pathogens were not detected (Garofalo *et al.*, 2017). Furthermore, the occurrence of the family *Clostridiaceae* and the genus *Staphylococcus* in very low abundance (<1%; Figure 1) in crickets from both feeding conditions could represent the presence of pathogenic species as well. Recently, *Clostridium* spp. were characterized from *A. domesticus* and powdered crickets (Garofalo *et al.*, 2017). Normally, these classes of bacterial endospores have a potential to cause food borne diseases, and represent a challenge in the production and processing of edible insects. An important source of contamination is when insects get into contact with the soil during rearing, via the

feed containing spores or by unhygienic handling during production (Klunder *et al.*, 2012). As a result, good sourcing and preparation of feeds, as well as good handling and processing practices, are crucial during the entire production process of insects intended for human consumption.

4. Conclusion

Our study provides an overview of the weight gain and microbial quality of raw farmed crickets intended for human consumption. The finding has demonstrated that the commercial starter chicken feed, which is usually expensive for local farmers, can be partially replaced with natural farm weeds (Wandering Jew) and this results in crickets with higher body mass. Although most microbial counts observed exceeded the recommended process hygiene criteria of comparable food matrices, such as minced meat, the bacterial endospore counts in crickets fed with S+W diet were significantly lower and thus promising, and could reduce the risks associated with ready-to-eat insects. However, studies on antimicrobial properties of Wandering Jew and reason (s) for higher preference are recommended.

The culture-independent results have shown that the crickets cultivated on the two types of feed can harbor similar bacterial communities with some minor variations. With regard to food safety, a few possible risks were identified such as OTUs related to the genus *Staphylococcus*, and the families *Enterobacteriaceae* and *Clostridiaceae*. These may contain pathogenic species, and involve the risk of food borne diseases. However, culture-dependent assays, such as presence-absence tests or specific counts of pathogens are needed for a full evaluation of the microbiological safety of the crickets reared on the alternative substrate.

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Microbial quality of farmed crickets

Declarations of interest

None

Supplementary data (Table S1)

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Figure 1. Relative abundance (%) of Operational Taxonomic Units (OTUs) present in samples of raw *G. bimaculatus* fed Starter chicken feed only (S0) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 days

Manuscript: Can farm weeds improve the growth and microbiological quality of fa**Table S1: Bacterial community composition and relative abundance of the *G. bimaculatus* fed Sta**

OTUs	Bacterial community	S0	S+W
		Relative a	Relative abundance (%)
7	Coxiellaceae	69.71594	79.11877
5	Lactobacillus sp.	16.93584	11.15115
4	Spiroplasma sp.	4.926489	3.439687
2	Lactobacillus sp.	1.409242	0.977117
9	Enterobacteriaceae	0.946331	0.618206
11	Enterobacteriaceae	0.8599	0.556678
10	Enterobacteriaceae	0.782261	0.52738
12	Enterobacteriaceae	0.57278	0.38821
14	Enterobacteriaceae	0.309097	0.191907
60	Porphyromonadaceae	0.240242	0.325203
78	Porphyromonadaceae	0.169927	0.098149
35	Morganella sp.	0.128915	0.007324
90	Porphyromonadaceae	0.137699	0.067384
1	Lactobacillus sp.	0.16993	0.127449
3	Pediococcus sp.	0.177254	0.124519
105	Porphyromonadaceae	0.112796	0.045411
103	Porphyromonadaceae	0.093752	0.062992
36	Acetobacter sp.	0.136236	0.080572
29	Clostridiaceae	0.096684	0.074712
136	Alistipes sp.	0.020508	0.084966
28	Enterobacteriaceae	0.067386	0.065922
129	Porphyromonadaceae	0.070314	0.027833
17	Enterococcus sp.	0.111332	0.109869
111	Porphyromonadaceae	0.036623	0.118656
138	Lachnospiraceae	0.064455	0.020509
160	Porphyromonadaceae	0.054201	0.021973
96	Enterobacteriaceae	0.06006	0.082033
26	Pseudomonas sp.	0.061527	0.016114
25	Enterobacteriaceae	0.060062	0.051273
31	Clostridiaceae	0.067386	0.032229
156	Rikenellaceae	0.048341	0.023438
142	Porphyromonadaceae	0.014649	0.051272
34	Staphylococcus sp.	0.041017	0.019044
133	Porphyromonadaceae	0.033692	0.067384
	Others	0.012671	0.012247

irmed crickets (*Gryllus bimaculatus*)?

arter chicken feed only (S0) and Starter chicken feed + Wandering Jew weeds (S+W) for 30 day

/s

Reviewer #1:	
Comment	Response
There is no where in the manuscript where the study sample size is indicated	<ul style="list-style-type: none"> ➤ The numbers of nymphs during onset of experiment have been indicated in line 103 ➤ The sample size for weight gain determination have been indicated in line 113 ➤ The sample size for classical microbial works is shown in lines 128-129 ➤ The sample size for metagenetic analysis is shown in lines 148-151
There were no replicates and since this work borders on food/feed quality and safety, how can we make conclusions without replicates? If there were, how many were they?	<ul style="list-style-type: none"> ➤ Replications during experimental set-up are shown in line 103 ➤ Replications for classical microbial works are shown in line 145 ➤ Replications for metagenetic analysis are shown in line 151
It would be important to discern whether the bacteria observed are as a result of handling/processing bias or if the trends are reproducible.	<ul style="list-style-type: none"> ➤ The authors would like to clarify that handling of crickets during rearing and sampling was minimal and hygiene aspects were observed particularly for samples meant for microbial works. Therefore, the bacteria contamination observed in this study could not be associated with handling/processing aspects
The analyses methods were sound though it would have been interesting if the bacterial endospores were determined in both feeds prior being used by the crickets and after the 30-day rearing period. This would have shaped the discussion and even the conclusion.	<ul style="list-style-type: none"> ➤ This comment is well noted and very important. Unfortunate this analysis was not done. However, a justification of probable sources of these bacterial endospores is indicated in lines 308-310. ➤ A recommendation for future research
Furthermore, the manuscript has a number of language issues and may require editorial services. Some corrections are in the main text in track changes.	<ul style="list-style-type: none"> ➤ All the comments/suggestions appended to the main text have been addressed accordingly. See whole text
Strides have been made in this aspect. The work by Gatheru and team 2019 addresses some of the microbial safety aspect. Have a look at that work.	<ul style="list-style-type: none"> ➤ This research article has been incorporated in discussion section on effect of processing on microbial hazards
Indicate the type of diet used in the 3 cycles. Were the processing methods similar to what is adapted for rearing crickets in the Kenyan farms? Comparing different rearing cycles on the same diet has more weight as compared to comparing rearing cycles alone.	<ul style="list-style-type: none"> ➤ The authors felt it is important to do the comparison with this research work. However, the diet(s) used during rearing is not indicated in the publication
Reviewer #2:	
Comment	Response
Line 68 Surprised that two publications were not mentioned:	<ul style="list-style-type: none"> ➤ The two publications have been included in lines 68-69

<p>* Miech P, Berggren Å, Lindberg JE, Chhay T, Khieu B, Jansson A. 2016. Growth and survival of reared Cambodian field crickets (<i>Teleogryllus testaceus</i>) fed weeds, agricultural and food industry by-products. <i>Journal of Insects as Food and Feed</i> 2: 285 - 92</p> <p>* Choo YR, Yan AT, Loh RHJ, Chin ZY, Jo CS, et al. 2017. Can waste vegetables and weeds be used to farm crickets and grasshoppers in Singapore? A laboratory experiment on the use of vegetables and weeds for farming crickets and grasshoppers. <i>UTAR AGRICULTURE SCIENCE JOURNAL</i> 3: 18-22.</p>	
Line 199 weight gain	➤ Addressed accordingly line 201
Line 207 Leave out: "It should be noted that" and start with "Although"	➤ Addressed accordingly line 209
Line 213 Table 1. The word average is often used. However, it is not clear how this was calculated. For example, what is average gain and why are there no standard deviations? If it is based on individual crickets there should be a standard deviation.	<p>➤ Average gain has been replaced with weight gain. The authors would like to clarify that only body weight was based on individual crickets.</p> <p>➤ Weight gain was determined by subtracting overall cumulative weights during sampling periods, that is why no standard deviations are indicated</p>
Line 219 (Table 1) is enough.	➤ Addressed accordingly line 221
Line 233 Why not body weight and weight gain. Why use average?	➤ Addressed accordingly line 235
Line 239 When it was not possible to differentiate between sexes, you can leave one row out in the table (Day 0). Why again no standard deviations with weight gain?	➤ The empty rows deleted line 242. Standard deviations for weight gain have not been indicated as explained above
Line 248 Use the same number of decimals. Besides two decimals is really too much for mortality (see the rest of the paragraph. See also table 1). In the same table for mortality there is 0 at day 0. To my opinion these should be hyphens as no mortality could be calculated at day 0.	<p>➤ Addressed accordingly lines 250, 258</p> <p>➤ Hyphens inserted in Table 1 on mortality</p>
Line 250 Would it not been better to use instead of previously to use: Caparros Megido et al. (2015) demonstrated? Now previously could also mean that the authors of the article had done an experiment.	➤ Addressed accordingly line 252
Line 308 Leave out "It is worth noting that". Leave that to the reader.	➤ Addressed accordingly line 311