1 **Microbial quality of edible grasshoppers *Ruspolia differens* (Orthoptera: Tettigoniidae):**
2 **from wild harvesting to fork in the Kagera Region, Tanzania**

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Abstract

In Tanzania, edible *Ruspolia differens* are still harvested from the natural environments. In this perspective, little is known about the microbiological quality of wild harvested *R. differens*. This study was conducted to assess the microbiological quality of wild harvested *R. differens* and evaluate the efficacy of conventional processing methods in reducing microbial load. Two districts (Bukoba rural and Muleba) within the Kagera region were purposively selected for the study. Sampling was done from the same batches along the *R. differens* food chain as follows: (1) at harvest points in the villages, (2) after transportation to the market and plucking of wings and legs, (3) after rinsing with potable tap water and (4) after processing using conventional methods.

Generally, high microbial counts, i.e., total viable aerobic count (TVC), *Enterobacteriaceae*, lactic acid bacteria (LAB), bacterial endospores, and yeasts and moulds were observed in raw *R. differens* samples. A significant increase in microbial counts after transportation and plucking was only observed for TVC, bacterial endospores, and yeasts and moulds. A statistically significant reduction in all types of counts, with the exception of bacterial endospores, was observed after processing. All processed samples analyzed were devoid of salmonellae, *Listeria monocytogenes*, and *Escherichia coli*.

Practical applications

Although commonly used processing methods of *R. differens* were effective in reducing microbial load, bacterial endospores were hardly eliminated and could pose a health hazard to consumers; thus, improved handling of *R. differens* along the food chain could reduce such risks.

Keywords: entomophagy, microbial counts, processing methods, bacterial endospores, handling

1. INTRODUCTION

With the rapid growth of the world population, currently at 2.3% per year in Sub-Saharan Africa (FAO, 2013), and a global surge in demand for affordable, alternative and sustainable protein sources, there is need to re-evaluate other sources of food and how they are produced. In this perspective, the Food and Agriculture Organization of the United Nations (FAO) proposed a global initiative to increase use of insects as food and feed to ensure future food security (Van Huis et al., 2013). Although consumer acceptance of edible insects and insect derived products is still limited in Western societies, in many regions of Africa, Asia, and America, the culture of eating insects (entomophagy) by various ethnic groups (Christensen et al., 2006; Hoare, 2007; Van Huis et al., 2013) is well documented. Globally, over 2,000 species are known to be edible, and consumed by approximately 2 billion people (Van Huis et al., 2013). The choice of insects as food source is justified by the fact that their nutritional value (relative amount of proteins, fat, vitamins, and calories) compares favourably with that of meat and fish (Belluco et al., 2013; Van Huis et al., 2013; Xiaoming, Ying, Hong & Zhiyong, 2010).

In Africa, the majority of the consumed insects are still collected from the wild environments. Therefore, utilization is hampered by regional and seasonal availability, sustainability issues, pathogenic risks, and high perishability (Nonaka, 2009). In East Africa, local communities have developed the skills and techniques of harvesting, preparing, and preserving edible insects. For instance, harvesting and consumption of the locally occurring *R. differens*, locally known as *senene* by various tribes in E. Africa (Haya in Tanzania, Luo in Kenya, and Baganda in Uganda) form a major part of food culture and constitutes about 5-10% of protein intake of the rural and urban population (Ayieko, Kinyuru, Ndong'a & Kenji 2012; Kinyuru, Kenji, Muhoho & Ayieko, 2011). During certain times of the year, usually in the periods of March-May and October-December, huge swarms of *R. differens* are observed within E. Africa coinciding with rainy seasons (Mmari, Kinyuru, Laswai & Okoth, 2017). Most of the swarms are usually concentrated on streetlights in urban areas (they are attracted to the light in the evenings), on grasses and shrubberies with no apparent damage (Mmari et al., 2017). During swarming seasons, these insects are collected, prepared and consumed at the households' level contributing to nutrient intake. In addition, they also contribute to

households' sources of income where the surplus is traded at the nearby markets (Agea, Biryomumaisho, Buyinza & Nabanoga, 2008).

In Tanzania, *R. differens* are traditionally consumed as toasted, deep-fried or smoked products, depending on the season. However, the pathogenic risks can be high for raw consumed grasshoppers as compared to the cooked ones (Vega & Kaya, 2012). In addition, Klunder, Wolkers-Rooijackers, Korpela and Nout (2012) reported that the risks could be higher when consuming wild harvested insects, when compared to those that are cultivated in a controlled environment. For instance, Banjo, Lawal and Adeyemi (2006a) reported the presence of the pathogenic bacteria *Staphylococcus aureus* Rosenbach, *Pseudomonas aeruginosa* (Schroeter) Migula, and *Bacillus cereus* Frankland & Frankland in edible rhinoceros beetle species in West Africa, whose occurrence is a risk factor for food-borne diseases. In a different study, Musundire, Osuga, Cheseto, Irungu and Torto (2016) identified the human carcinogen mycotoxin (aflatoxin B1) associated with fungal contamination, at low levels in edible stink bugs (*Encosternum delegorguei* Spinola) that were wild harvested in Zimbabwe. Similarly, Schabel (2008) reported some cases of botulism and other food-borne illnesses linked with the consumption of wild harvested insects that had been stored in poorly hygienic conditions in Africa.

Up to date, nutritional composition of various insects has been studied and reported (e.g Banjo, Lawal & Songonuga, 2006b; Finke, 2002; Fombong, Van Der Borgh & Vanden Broeck, 2017; Kinyuru et al., 2011; Rumpold & Schlüter, 2013; Ssepuuya, Mukisa & Nakimbugwe, 2016; Van Huis et al., 2013). However, microbiological data are scarcely available, as indicated in the European Food Safety Authority opinion report (EFSA Scientific Committee, 2015). Only a few studies are available containing microbiological data of fresh edible insects and processed edible insect products (EFSA Scientific Committee, 2015). In addition, the scientific evidence assessing the microbial quality of edible insects along the food chain is weak, sporadic, or missing; searching the literature reveals the scarcity of the data.

In this perspective, the microbiological quality of edible insects needs to be assessed along the entire farm-to-fork food chain (production, harvesting, handling, processing, distribution, sale, and consumption). Therefore, this study was conducted to assess the microbial load and bacterial pathogens during harvesting, handling (transportation to the market, plucking of

wings, legs, ovipositors, appendages) and the efficacy of conventional processing methods along the food chain of wild harvested *R. differens*.

2. MATERIALS AND METHODS

2.1 Study sites and samples collection

The study was conducted in the northwestern corner of Tanzania in the Kagera region, situated between 1°45'S and 32°40'E. The Kagera region (one of Tanzania's 30 administrative regions) shares borders with Uganda to the North, Rwanda, and Burundi to the West and Lake Victoria to the East. Two districts within the Kagera region (Bukoba Rural and Muleba) were purposively selected during the months of March-May in 2017, when *R. differens* swarms were observed.

During the swarming season, *R. differens* appear at streets circling the lights soon after the dark. To simulate this in the villages, locals have developed their own harvesting techniques. Typically, the lamps/bulbs are lit outside the houses. The areas where lamps/bulbs are located are secured with corrugated iron sheet folded into cone shape leading to baskets, small drums where falling *R. differens* are collected (Mmari et al., 2017). Using this technique large quantities of grasshoppers are harvested within short time when they are attracted to light. Elsewhere, where *R. differens* swarms are less abundant, harvesting is done early in the morning between 6.00 am and 7.00 am, when they are inactive and, therefore, could not fly (Kinyuru et al., 2011). After the locals harvested *R. differens* using the above techniques, the sampling at harvest points was done as follows: random sampling within 4-5 villages in each district was conducted. In total, 27 unplucked samples of *R. differens* (Bukoba Rural =14 and Muleba =13) of approximately 0.5 kg each were sampled (Figure 1).

The surplus *R. differens* after home consumption are usually sold in the local markets, and then processed immediately due to their high perishability (Figure 1). Transportation of *R. differens* to the markets, locals usually use traditionally woven baskets and used woven grain bags. Plucking of wings, legs and appendages, is usually done on bare ground or on old woven polypropylene bags laid on the ground. After transportation to the local markets, plucking is done first, a job traditionally assigned to women (Mmari et al., 2017).

In the local markets, where surplus *R. differens* from the same batches that were previously sampled at harvest points were traded, random sampling was done as follows: after

transportation and plucking process (Figure 1), in total 21 plucked samples of *R. differens* (Bukoba Rural =11 and Muleba =10). Then, immediately after rinsing using potable tap water (Figure 1), in total 21 rinsed samples, (Bukoba Rural =11 and Muleba =10) were collected. Cleaning involved washing with portable tap water and draining.

The efficacy of conventional processing methods (i.e. deep frying – rinsed raw *senene* were placed in boiling cooking oil for ten min until they turned deep-brown in color; toasting - rinsed raw *senene* were placed on a hot pan, stirred for ten min until they turned deep-brown; smoking – involved a brief boiling step of raw *senene* for five min, draining them, and rolling boiled *senene* in a fresh banana leaves and placed on metal sieves where smoke was produced by burning woods) as guided by Mmari et al. (2017) was evaluated. Sampling was done from the previously sampled batches as follows: Bukoba Rural (deep fried =8, toasted =6 and smoked =6) and Muleba (deep fried =10, toasted =7 and smoked =5) each weighing about 0.5 kg (Figure 1).

The samples were then packed in cool boxes and transported to the Food microbiology laboratory at Jomo Kenyatta University of Agriculture and Technology, for laboratory analysis which commenced within 15 hr of collection.

2.2 Classical bacterial counts

Standardized ISO methods for microbial analyses of food were used to determine bacterial counts (Dijk et al., 2007). Briefly, five gram of insect sample was aseptically transferred into a sterile stomacher bag (Bagmixer® 400 W, Interscience, St Nom, France) and 45 ml of sterile peptone physiological salt solution (0.85% (w/v) NaCl, 0.1% (w/v) peptone, was added. The mixture was homogenised for one min in a stomacher. A ten-fold serial dilution series of 1 ml 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 hr, *Enterobacteriaceae* on Violet Red Bile Glucose medium (VRBG, Biokar Diagnostic) with an overlay of the same medium and incubated at 37 °C for 24 hr, 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 hr. For yeasts and moulds, 0.1 ml was plated using the spread plate method on Dichrolan Rose-Bengal Chloramphenicol Agar (RBA, Biokar Diagnostic) and incubated at 25 °C for 120 hr. Bacterial endospores were determined by giving the 10⁻¹ dilution a heat shock

treatment (10 min at 80 °C), followed by serial dilution, plating using the pour plate method onto PCA and incubation at 37 °C for 48 hr. All microbial counts were expressed as log cfu/g.

2.3 Pathogens detection

Common food-borne pathogens were detected according to ISO standards: salmonellae (in 25 g; ISO 6579; ISO, 2002), *Listeria monocytogenes* (in 25 g; ISO 11290-1; ISO, 1996A1:2004), and *Escherichia coli* (ISO 7251:2005). The detection was only assessed on processed (deep-fried, toasted, and smoked) *R. differens* samples.

2.4 Statistical analysis

All microbial counts were expressed as log cfu/g. Statistical analyses were performed using Stata SE version 12 (StataCorp LP, Texas, USA). One-way ANOVA was performed to determine significant differences in individual microbial counts at harvest points (unplucked samples), after transportation and plucking (plucked samples), and after rinsing (rinsed samples); and among different conventional processing methods under investigation. For all analyses, means were separated using Bonferroni adjustment at 95 % confidence level.

3. RESULTS AND DISCUSSION

3.1 Microbial quality of raw *R. differens* samples

Table 1 summarizes the results of microbial counts at harvest points (unplucked samples), after transportation and plucking (plucked samples) and rinsing (rinsed samples) of *R. differens*. Generally, culture-dependent microbial counts, including the TVC, *Enterobacteriaceae*, LAB, bacterial endospores, and yeasts and moulds, showed high numbers ranging from 7.1 - 8.5, 5.4 - 5.6, 6.1 - 7.0, 3.0 - 3.3, and 4.7 - 5.0 log cfu/g respectively for the unplucked samples investigated. However, no statistical differences for individual counts were observed between the two districts for the unplucked samples analyzed (all p-values > 0.05). Up to date, there are no specific microbiological criteria for insects used as human food. However, according to the Superior Health Council (SHC) and the Federal Agency for the Safety of the Food Chain (FASFC), process hygiene criteria for minced meat described in European Union Regulation (EC) No. 1441/2007 can be used for edible insects (SHC and FASFC, 2014). The TVC of the minced meat according to the guideline ranges between 5.7 – 6.7 log cfu/g. In this study, the numbers obtained by far exceeded this value.

However, the values obtained compare closely to numbers found for grasshoppers (*Locusta migratoria migratorioides*) bought from an eco-shop in Belgium (Stoops et al., 2016). Similarly, previous studies (Banjo, Lawal & Adeyemi, 2006a; Klunder et al., 2012; Mpuchane et al., 2000; Vega & Kaya, 2012) reported that insects collected in the wild might be highly contaminated with microorganisms, when compared to insects that are farmed in a controlled environment.

In this study, a significant increase in microbial counts (TVC, bacterial endospores, and yeasts and moulds) after transportation to the local markets and plucking of wings and legs (plucked samples) was observed (Table 1). Traditionally, *senene* appendages, ovipositors and wings are not generally consumed and are usually plucked off and discarded (Mmari et al., 2017). However, exposure of plucked insects in direct contact with the soil or use of previously used bags to store insects could have led to further contamination (Banjo, Lawal & Adeyemi, 2006a; Klunder et al., 2012). This observation is supported by a study carried out by Mmari et al. (2017) in Tanzania regarding *R. differens* food chain. The authors indicated that after wild harvesting and transportation (usually done using containers and sacks previously used to store other produce such as maize), plucking (done on bare ground in contact with the soil) could lead to a further contamination, as also observed in the present study. In a different study, Musundire et al. (2016) reported the growth of fungi and accumulation of aflatoxin B1 in edible stink bugs (*Encosternum delegorguei* Spinola) that were wild harvested, and transported in traditionally woven baskets and gunny bags used to store maize as compared to those transported in clean zip lock bags in Zimbabwe.

No significant increase was observed in the microbial counts of *Enterobacteriaceae* and LAB after transportation and plucking (plucked samples) although the log counts increased slightly in both districts (Table 1). This could be explained by the fact that the two classes of bacteria are strongly associated with the feed, and mostly concentrated in the gastrointestinal tract (Klunder et al., 2012; Stoops et al., 2017). However, the slight increase in these two microbial counts could possibly be due to unhygienic handling environments, improperly cleaned hands and handling equipment (Mmari et al., 2017).

In most food industries, washing is a technique usually applied to products before further processing to aid in the decontamination process (Huffman, 2002). For edible insects, just like any other food products, washing is a crucial procedure before processing. In Tanzania, the standard requirements of drinking water (piped) and bottled drinking water are specified by

Tanzania Bureau of Standards (TSZ 789:2003). Although the water quality was not ascertained in the current study, majority of the sampled markets had access to piped water. Following the analysis, a significant reduction in individual microbial counts (TVC, bacterial endospores, and yeasts and moulds) was observed while log counts of *Enterobacteriaceae* and LAB did not change after the rinsing process in both districts (Table 1). These findings are supported by Wynants et al. (2017), who assessed the quality of the water before and after rinsing mealworm larvae. A significant reduction in TVC after rinsing was observed, indicating that the procedure removes a considerable amount of microorganisms (Wynants et al., 2017).

3.2 Microbial quality of processed *R. differens* samples

Table 2 summarizes and compares the microbiological quality of ready-to-cook (rinsed) and ready-to-eat (after processing i.e., deep-frying, toasting, and smoking). Processing *R. differens* resulted in a statistically significant reduction of all types of microbial counts except bacterial endospores. For the total TVC, and yeasts and moulds, reductions of about 6.1 and 2.6 log cfu/g respectively were observed after heat treatment (Table 2). Similarly, Klunder et al. (2012) found low TVC for boiled house crickets with significant reductions of about 3.1 log cfu/g. Elsewhere, Caparros Megido et al. (2017) reported similar findings after assessing blanched mealworms, house crickets, and smoked termites.

Enterobacteriaceae and LAB were completely eliminated or reduced to a level below the detection limit when *R. differens* samples were processed using any of the three methods. These groups of bacteria are usually heat sensitive (Klunder et al., 2012), and that fact is supported by this study, where any of the three methods under investigation involved heating. In a different study, Vandeweyer, Lenaerts, Callens and Van Campenhout (2017) reported similar findings after blanching mealworm larvae.

Bacterial endospores were reduced slightly indicating that the conventional processing methods are incapable of eliminating them entirely. Similarly, Vandeweyer et al. (2017) found no significant change on bacterial endospores counts after blanching mealworm larvae for ten, twenty, and forty min. Other researchers (Klunder et al., 2012; Ter Beek & Brul, 2010) have demonstrated that spore-forming bacterial species, such as *Bacillus* spp. are often introduced from soil and are not entirely inactivated despite the heat treatment; and thus could cause spoilage or health risks on foods. The poor handling of insects in direct contact with soil or use of poorly stored bags during transport/storage could have a profound effect on

bacterial endospores and thus increase the health risks. However, SHC and FASFC (2014) recommended that, the source of insects' feedstuff and the farming environments could have a great impact on the insects' microflora. For instance, Klunder et al. (2012) reported higher numbers of spore-producing bacteria in house crickets (*Acheta domesticus*) that were being cultivated in containers filled with soil than in mealworm larvae (*Tenebrio molitor*), which were cultivated in wheat flour.

3.3 Major food-borne pathogens

In this study, the preference of major pathogens analyzed was based on international standards used to describe food quality and food safety of foodstuffs containing animal protein (Grabowski and Klein, 2017). Traditionally, within the Haya tribe in Tanzania, *senene* are not consumed raw whilst they are processed using different conventional methods (Figure 1) before consumption (Mmari et al., 2017). In this context, the current study focused on the safety of processed *senene* in the markets ready for consumption. All processed samples analyzed were free of salmonellae, *L. monocytogenes*, and *E. coli*. In insects, the majorities of these bacteria originates from the gastrointestinal tract and are usually highly sensitive to heat (Klunder et al., 2012). The absence of these pathogens in ready-to-eat samples may suggest that the identified methods of processing *R. differens* (deep-frying, toasting and smoking) are effective, and processed *senene* may not pose health risks to consumers. Similar results were reported by Grabowski and Klein (2017) after deep frying processed edible insects' products. Although there are no specific microbiological criteria for insects used as human food, food safety criteria for minced meat that include salmonellae (absence in 25 g), *L. monocytogenes* (<2.0 log cfu/g), *E. coli* (most probable number < 230/100 g) (Grabowski and Klein, 2017) could give useful indication. In a recently updated circular from the Belgian FASFC (2016), products available on the market should be periodically tested for the presence of *Salmonella* and *Listeria monocytogenes* pathogens to assess the effectiveness of handling. In this study, comparing the obtained results with the current recommendations showed that food hygiene criteria for major food-borne pathogens of processed *R. differens* were met.

4. CONCLUSION

Our study provides an overview of microbial counts of *R. differens* along the food chain. Although high microbial counts were observed for raw *R. differens* samples, the routinely applied processing techniques resulted in killing vegetative cells but hardly eliminated

bacterial endospores. Thus, when edible insects are processed, bacterial spores and their survival still need special attention in order to further reduce the risks of ready-to-eat insects.

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TABLE 1 Microbial counts (log cfu/g) of raw *R. differens* at harvest points (unplucked), after transportation and plucking (plucked), and rinsing (rinsed).

Microbial counts (log cfu/g)	Bukoba Rural			Muleba		
	Unplucked	Plucked	Rinsed	Unplucked	Plucked	Rinsed
Total viable aerobic counts	7.1±0.4 ^a	8.1±0.4 ^b	6.9±0.3 ^a	7.5±0.3 ^{ab}	8.5±0.4 ^b	7.0±0.3 ^a
Enterobacteriaceae	5.6±0.4 ^{ab}	6.1±0.2 ^{ac}	5.8±0.2 ^{ab}	5.4±0.2 ^{ab}	6.3±0.2 ^c	5.6±0.2 ^a
Lactic acid bacteria	6.1±0.4 ^a	6.8±0.5 ^a	6.5±0.3 ^a	7.0±0.5 ^a	6.3±0.2 ^a	6.9±0.3 ^a
Bacterial endospores	3.0±0.1 ^a	3.6±0.1 ^b	2.8±0.1 ^a	3.3±0.1 ^a	3.8±0.1 ^b	3.2±0.1 ^a
Yeasts and moulds	4.7±0.1 ^a	5.6±0.2 ^b	4.3±0.1 ^a	5.0±0.2 ^a	5.4±0.2 ^b	5.0±0.2 ^a

Data are the means with three replicates ± standard errors (Unplucked n= 11, Plucked n= 10, Rinsed n= 10 for each district)

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

395 TABLE 2 Microbial counts (log cfu/g) of ready-to-cook (rinsed) and ready-to-eat (processed) of *R. differens*.

	Ready-to-cook <i>R. differens</i>		Ready-to-eat <i>R. differens</i>					
	Bukoba Rural	Muleba	Bukoba Rural			Muleba		
Microbial counts (log cfu/g)	Rinsed	Rinsed	Deep-fried	Toasted	Smoked	Deep-fried	Toasted	Smoked
Total viable aerobic counts	6.9±0.3 ^a	7.0±0.3 ^a	2.1±0.1 ^b	2.0±0.1 ^b	2.2±0.1 ^b	2.4±0.3 ^b	2.2±0.1 ^b	2.4±0.2 ^b
Enterobacteriaceae	5.8±0.2 ^a	5.6±0.2 ^a	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b
Lactic acid bacteria	6.5±0.3 ^a	6.9±0.3 ^a	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b	<1±0.0 ^b
Bacterial endospores	2.8±0.1 ^a	3.2±0.1 ^a	2.2±0.1 ^b	2.0±0.2 ^b	1.9±0.1 ^b	2.1±0.1 ^b	2.7±0.2 ^a	2.3±0.2 ^b
Yeasts and moulds	4.3±0.1 ^a	5.0±0.2 ^a	2.0±0.2 ^b	2.1±0.1 ^b	2.6±0.2 ^c	2.1±0.2 ^b	2.0±0.1 ^b	2.8±0.2 ^c

396 Data are the means with three replicate ± standard errors (Rinsed n= 10, Deep-fried n= 8, Toasted n= 6, Smoked n= 5 for each district)

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

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FIGURE 1 A flowchart showing food chain of wild harvested *R. differens* and sampling points of this study indicated