UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

IMPACT OF STARVATION ON FAT AND MICROBIAL LOAD IN THE HOUSE CRICKET (*ACHETA DOMESTICUS*) USED FOR FOOD

ANA CAROLINA DA MOTA RODRIGUES ALVES INÁCIO

ORIENTADOR: Professor Ivar Vågsholm

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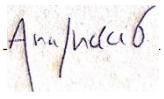
Assinatura: 

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Resumo

Impacto do jejum na matéria gorda e teor microbiano presente no grilo doméstico (*Acheta domesticus*) utilizado para alimentação humana

O consumo de insetos tem vindo a aumentar globalmente, particularmente em países industrializados. Ingredientes à base de insetos são considerados novos alimentos na Europa, o que suscita alguma preocupação em matéria de segurança dos alimentos destes ingredientes. O esvaziamento do trato gastrointestinal através de jejum antes do abate é visto como uma prática eficaz na redução do teor microbiano presente nos insetos, mas pode, no entanto, levar a perda de peso considerável e, consequentemente, à redução de lucro para os produtores. O objetivo deste estudo foi avaliar a perda de gordura no grilo doméstico (*Acheta domesticus*) quando submetido a períodos de jejum de 0h, 24h e 48h, e correspondentes teores microbianos (Aeróbios totais (AT) e *Enterobacteriaceae*). Foram ainda feitas colorações de Gram a partir das unidades formadoras de colónia (UFC) das placas de AT. O efeito do sexo na carga microbiana foi avaliado, não tendo sido encontradas diferenças significativas ($p=0.72$ e $p=0.46$ para AT e *Enterobacteriaceae*, respetivamente). A contagem de AT aumentou ($p=0.002$) em cerca de 1 log UFC/g no grupo de jejum de 48h. Apenas o grupo submetido a jejum por 24h mostrou um decréscimo significativo ($p=0.004$) nas contagens de *Enterobacteriaceae* na ordem de 1 log UFC/g. As colorações de Gram mostraram alteração da composição microbiana das amostras colhidas às 24 e às 48h, com predominância de cocos Gram-positivos às 24h e redução às 48h (de 68 para 48%). Foram detetados bacilos apenas no grupo 24h (8%). Não houve redução significativa do teor de gordura nem às 24h ($p=0.13$ em machos e $p=0.13$ em fêmeas) nem às 48h ($p=0.57$ e $p=0.98$ em machos e em fêmeas, respetivamente). A aplicação de um período de jejum de 24h foi eficaz na redução da carga microbiana de grilos crus sem redução de gordura significativa. Um período de jejum mais prolongado promoveu um teor microbiano mais elevado, possivelmente devido à modulação da diversidade microbiana.

Palavras-chave: Insetos edíveis, segurança dos alimentos, jejum, contagem de placas, teor em matéria gorda.

Abstract

Impact of starvation on fat and microbial load in the house cricket (*Acheta domesticus*) used for food

Insect consumption has been increasing worldwide, particularly in industrialized countries. Insect-based ingredients are considered novel foods in Europe, which raises some concern regarding the food safety of these products. Gut emptying by starvation prior to killing is perceived as an effective practice in the reduction of the microbial load of insects but can lead to weight loss and consequently a profit reduction to the farmers. The purpose of this study was to evaluate the fat loss of crickets (*Acheta domesticus*) starved for 0h, 24h and 48h, and their corresponding microbial loads (total aerobic counts (TAC) and *Enterobacteriaceae*). Gram stains were also performed for the colony-forming units (CFU) from TAC. The effect of sex on the microbial numbers was assessed, having not been found significant differences ($p=0.72$ and $p=0.46$ for TAC and *Enterobacteriaceae*, respectively). TAC increased ($p=0.002$) by almost 1 log CFU/g in the 48h starvation group. Only the 24h starvation group showed a significant decrease ($p=0.004$) in *Enterobacteriaceae* counts of 1 log CFU/g. The Gram stains showed changes in the microbiological composition of samples collected at 24 and 48h. Gram-positive cocci predominated at 24h but decreased at 48h (from 68 to 48%). Bacilli were only detected at 24h (8%). The fat content did not decrease significantly, neither at 24h ($p=0.13$ for males and $p=0.13$ for females) nor at 48h ($p=0.57$ and $p=0.98$ for males and females, respectively). Starvation for 24h was efficient in reducing the microbial load of raw crickets without significant fat loss. A longer starvation period promoted a higher microbial load, possibly due to modulation observed in the microbial diversity.

Keywords: Insects as food, food safety, starvation, hygiene indicators, lipid content.

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List of Abbreviations

AKH- Adipokinetic hormones

CCP- Critical control points

DM- Dry matter

EC- European Commission

EFSA- European Food Safety Authority

EU- European Union

FAO- Food and Agriculture Organization

FCR- Feed conversion ratio

GFP- Good farming practices

GHG- Greenhouse gases

GHP- Good hygiene practices

GMP- Good manufacturing practices

HACCP- Hazard Analysis and Critical Control Points

LD- Light and dark

MDG- Millennium Development Goals

NMKL- Nordic Committee on Food Analysis

RH- Relative humidity

SD- Standard Deviation

SDG- Sustainable Development Goals

TAC- Total aerobic counts

CFU- Colony-Forming Unit

VRBG- Violet Red Bile Glucose

WHO- World Health Organization

Internship report

As part of the Integrated Master's Degree in Veterinary Medicine from the Faculty of Veterinary Medicine, University of Lisbon, I completed an internship at the Faculty of Veterinary Medicine and Animal Sciences- Swedish University of Agricultural Sciences, SLU, at the department of Biomedical Sciences and Veterinary Public Health.

The internship had the duration of six months, starting on the 1st of October 2019 and ending on the 17th of March 2020.

My supervisor was Professor Ivar Vågsholm and my co-supervisor was Professor Maria João Fraqueza.

Along with Ivar Vågsholm, Anna Jansson, Erica Roman, Merko Vaga and Sofia Boqvist, I completed a study within the scope of food safety of the house cricket (*Acheta domesticus)* as food. The goal was to learn about food safety and the welfare of edible insects, in particular the house cricket. In addition, I learned about crickets' behavior and rearing conditions.

The study included the rearing of crickets up until adulthood, as well as microbiological analyses which were performed by me in the microbiology laboratory, and fat analyses that were performed by a laboratory technician.

During the length of the internship, I attended several seminars lectured by PhD student, namely:

- " Food safety hazards and risks in Cambodian meat value chain, with special emphasis on bacteria and AMR"- October 16th, 2019.
- "Exploring benzimidazole resistance in *Haemonchus contortus* by next generation sequencing and droplet digital PCR"- October 17th, 2019.
- "Improving sow welfare in group housing systems"- December 19th, 2019.
- "Crossing the line- Tracking small ruminant diseases in trade and across international borders in Zambia and Tanzania"- January 9th, 2020.
- "Models and scenarios for climate change"- January 15th, 2020.
- "The use of a box with an adjustable ceiling to afford safe prevention of too premature rising attempts during the post-anesthetic period", January 13th, 2020.

Due to the outbreak of the current COVID-19 pandemic, the internship was terminated two weeks before scheduled, as the predicted ending date was the 31st of March.

Introduction

For the past few decades, human population has been growing at an incredibly fast rate, and by 2050 it is estimated to come close to 10 billion (United Nations 2017). Facing this scenario, food security is thus becoming a challenge for humankind (Gahukar 2011) where undernourished people already constitute a high proportion of world population (FAO 2013). Earth resources are already being overly exploited (Halloran et al. 2018) and in order to provide food for an ever-growing population, food production will need to almost double (FAO 2013). The livestock sector is one of the most developed food industries and plays an important role in food security, accounting for 40 percent of agricultural gross domestic product. However, this sector is also responsible for a considerably negative environmental impact, being the biggest land user of all human activities (Steinfeld et al. 2006). Thus it has become imperative to find new innovative alternatives to production methods and novel food sources (van Huis 2015), aiming to secure food availability while producing more efficiently and consequently reducing the environmental impact (Tripathi et al. 2019).

Edible insects are perceived as an alternative source of protein for humans as well as for animals, and large-scale production has the potential to be environmentally friendlier when compared to other animal protein sources (Halloran et al. 2018; Sogari et al. 2019). However, although the consumption of insects is an ancient practice, predominantly in developing countries (van Huis et al. 2013), it is a novelty in most Western societies and food neophobia represents a challenge to the industry (Rumpold and Langen 2019). Moreover, being considered novel foods in the European Union (EU; European Comission 2018), knowledge regarding food safety of insects is still lacking (Garofalo et al. 2019) and so the European Food Safety Authority (EFSA) has adopted a conservative approach towards their production and consumption (EFSA 2015). Despite the EU recommendations, some European countries such as Germany and the Netherlands have already started commercializing insects.

Currently, there are more than 2,000 species known to be safe for human consumption (Jongema 2017), but not all can adapt to captivity, hence not all are suitable for mass rearing (Olzer et al. 2019). The house cricket (*Acheta domesticus*) is considered to be one of the species with the biggest potential for production in the world (EFSA 2015) due to its relatively short life cycle, easy manipulation, high reproductive rate and resistance to diseases (Patton 1978). Furthermore, crickets are a great source of protein and unsaturated fats, as well as vitamins and minerals, whilst being considered a delicious food (Homann et al. 2017).

Cricket-based food products can be presented in various formats such as whole crickets, powders, pastes and oils (Dossey et al. 2016). Processed cricket products are usually made from the whole insect, including the gastrointestinal tract, which increases their microbial

load and may affect food quality and shelf-life, by the action of spoilage microbes, as well as food safety if pathogens are present. Therefore, adequate processing and storing methods must be applied (Klunder et al. 2012) and the producers can also apply a starvation period before killing with the aim of emptying the gut and thus reducing crickets' microbial load (FAO 2013; EFSA 2015; Megido et al. 2017).

Regarding food safety, crickets can present a health risk to consumers. Possible hazards are of chemical (heavy metals, mycotoxins, insects' toxins, veterinary drugs and contaminants) and microbiological origin (bacteria, viruses, parasites, fungi and prions), as well as allergens and environmental contaminants, like pesticides (EFSA 2015).

When facing stressful conditions such as starvation, crickets may respond with a series of behavioral and physiological adaptations, and the most common is entering diapause, a hormonally mediated process characterized by a decrease in metabolism (Zhang et al. 2019). Despite the attempt to maintain the body composition in the absence of food, crickets start using body reserves from the fat body, mostly glycogen and fat (Schooley et al. 2012). Starvation may induce significant weight loss and affect the crickets' nutritional composition, resulting in a reduction of profit to the producers.

This study aimed to evaluate the fat loss of starved crickets (*Acheta domesticus*) for different periods of time and their corresponding microbial loads. Total aerobic counts (TAC) at 30 ºC and *Enterobacteriaceae* were the microbial indicators that were analyzed. Fat loss was assessed by determination of the total fat content.

I Literature review

1. Food security

1.1. Food security challenges in the world

Every year, human population increases by 83 million. The current population is over 7.6 billion and by 2050 it is expected to reach 9.8 billion (United Nations 2017). Even though approximately one third of the food produced for human consumption (1.3 billion tons per year) goes to waste (Varelas 2019), the Food and Agriculture Organization (FAO) (FAO 2019) estimates that almost 820 million people mainly in developed countries are chronically undernourished, meaning that the number of hungry people in the world remains unacceptably high.

The World Food Summit (1996) on World Food Security defined food security as the status where "all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and

healthy life". In 2015 the World Health Organization (WHO) published a new set of 17 goals, the Sustainable Development Goals (SDG), to substitute the Millennium Development Goals (MDG). These goals seek to fight poverty and hunger on a global scale, while promoting sustainable agriculture and adequate nutrition, ensuring availability of clean water, land, energy and combating climate changes and its impacts (WHO 2015).

With the increasing purchasing power, rapid economic growth and urbanization seen in developing countries, particularly in Asia, food preferences start to shift towards a more globalized diet. This often translates into a higher meat, fat and sugar consumption (Raheem et al. 2019). On the contrary, developed countries have been suffering a significant slowdown in meat consumption, as in the past decades high levels have already been reached (Wu et al. 2014).

In response to the increased demand for higher quality food products, food operators have been, for the past several decades, investing in more industrialized mass-production systems (King et al. 2017). The food industry is responsible for a considerable environmental impact, and the livestock sector is to blame the most (van Huis 2015), as it is accountable for 18% of total greenhouse gases (GHG) emissions (CO_2 equivalent), a higher share than the transport sector (Steinfeld et al. 2006; FAO 2013), and uses 80% of all agricultural land (Weindl et al. 2017). Along with many other factors contributing to the negative environmental impact of livestock, such as excessive water use, both for livestock and for livestock feed, the decrease in soil quality and the adverse effect on biodiversity (Tullo et al. 2019), are putting a strain on earth´s resources. Thus, it is predicted that the production of animal-based foods will become insufficient to meet the demand worldwide (Wu et al. 2014).

Furthermore, instability in food prices and subsequent concerns over food security (Durst et al. 2010), environmental sustainability, health concerns and animal welfare are receiving a great deal of attention which forces the food industry to consider alternative food sources, especially protein (Durst et al. 2010; Van Huis 2016). Novel foods, such as insects, may thus play an important role in achieving the United Nations SDG (WHO 2015), by representing an eco-friendlier protein source since insects have higher conversion feed rates than other livestock (Varelas 2019).

1.2. EU legislation regarding novel foods and edible insects

The European Commission (EC) defines novel food as "food that had not been consumed to a significant degree by humans in the EU before 15 May 1997", and includes new foods or food ingredients, food products obtained by new technological processes and

foods that are part of the traditional diet of human populations outside the EU (European Comission 2019).

The new Regulation (EU) 2015/2283, applied from the 1st of January 2018, on novel foods came to replace Regulation (EC) No 258/97 and Regulation (EC) No 1852/2001 which ceased on the 31st of December 2017 (European Comission 2018).

Presently, insects are considered as novel foods and according to the new Regulation, in order for a new food, ingredient or production method to be approved by EC and be allowed to be sold in the EU, EFSA is requested to perform a risk assessment. This includes the evaluation of all hazards regarding human health and characterization of the food, based on the dossier delivered by the food business operator who requests authorization for their product. The characterization of the food product includes its origin, production methods, compositional analysis, including nutrients and their bioavailability and possible contaminants, known toxins and anti-nutrients. If the proposed novel food is known to be part of the traditional diet of human populations from countries outside the EU for at least 25 years, proving safe use, its authorization in EU markets should be facilitated. However, that does not automatically guarantee the approval of the product (European Comission 2015).

Novel foods from animal origin are much less common than those from plants (Belluco et al. 2017). Besides insects, other examples of approved novel animal-derived products are phosphatidylserine from fish phospholipids and krill oil (European Comission 2017).

The marketing of insects as food for humans in European countries is currently in debate and, since insects are not a traditional ingredient in Western diets, regulation on this matter is still unclear due to the lack of information available in terms of food safety (Raheem et al. 2019). Presently, since there are no specific regulations regarding the production of insects in Europe, the recommendations are an extrapolation of the general food production principles and thus Regulation (EC) No 178/2002 (the general food law principles and requirements) and Regulation (EC) No 852/2004 (food hygiene of foodstuffs) are applicable (van Huis 2019). Nonetheless, despite the recommendations of the EU, some member states such as the Netherlands, Belgium, United Kingdom, Finland and Denmark allow the production and selling of insects as food (Bugsolutely 2018). According to Regulation (EC) No 178/2002, food operators have the responsibility of ensuring that their products are safe for the consumers and so they must perform a risk analysis for each foodstuff or ingredient. Also, the label is required to include information about the common name of the insect and the species in Latin and information about the correct use or preparation of the insect, if necessary.

2. Insects as food

2.1. Consumption patterns worldwide

Insects have been included in human diets for thousands of years, whether as an emergency food, a habitual food or as a delicacy (Durst et al. 2010), and are consumed at all stages of development such as eggs, larvae, pupae and adults (Verkerk et al. 2007). Worldwide, eaten by approximately 2 billion people, mainly in Asia and Africa (Premalatha et al. 2011; van Huis et al. 2013), insects are an important source of protein, fat, minerals and vitamins (Durst et al. 2010). It is estimated that over 2000 species of insects are consumed by humans and in total, 2,111 species are considered edible (Jongema 2017). The most consumed species are from the orders Orthoptera (crickets, locusts and katydids), Hymenoptera (bees, wasps and ants), Coleoptera (weevils and longhorn beetles), Lepidoptera (butterflies and moths), Blattodea (termites) and Hemiptera (water bugs) (ANSES 2015).

Furthermore, the commercialization of insects, both harvested from nature and large-scale rearing, acts as an important source of income for farmers, especially for local communities in developing countries (Durst et al. 2010).

Currently, one obstacle presented to the development of insect industries in developed countries is the low rate of acceptance by Western consumers (Belluco et al. 2017). Mostly for environmental reasons and with increased media coverage, this preconception is being revolutionized (Kauppi et al. 2019).

2.2. Consumers perceptions and acceptance

In Western countries insects have often been recognized as non-edible, percieved as unclean, repugnant and unsafe for consumption (Looy et al. 2014). However, with the approaching global food crisis, new food options have been brought to the table, and insects are now beginning to enter the food markets in these societies (La Barbera et al. 2018).

The biggest challenge presented to the insect industry in developed countries is the low acceptance by the consumer (Orsi et al. 2019). Many studies (Schösler et al. 2012; Vanhonacker et al. 2013; Hartmann et al. 2015; La Barbera et al. 2018; Onwezen et al. 2019; Van Thielen et al. 2019) have investigated the acceptance of insects as food. In general, it is expected that Western consumers will be more easily drawn to eating foods made from processed insects, in which their repulsive characteristics are not so apparent. Mass media counts as another factor infuencing consumers acceptance, since media coverage have always established a negative association to insects by creating or reinforcing fears and

phobias (Hartmann et al. 2015). This scenario is changing and social media are starting to engage in the promotion of entomophagy (Van Thielen et al. 2019).

The acceptance of a novel food is influenced by a combination of factors: the intrinsic traits of the food, the consumer´s cultural and geographical environment and the consumer's beliefs and personality traits (Hartmann and Siegrist 2016). In the especific case of insects, acceptance relyies predominantely on emotional and cultural aspects, being disgust and neophobia the main resposible factors for rejection (Orsi et al. 2019). Food safety, on the other hand, is often taken for granted by Western consumers (Poortvliet et al. 2019) and it does not seem to be a decising factor (Hartmann et al. 2018).

Consumers that are concerned about health and environmental issues are found to be the most likely to accept the consumption of processed insect-based foods. Young men represent the group that best accept insects, and men in general are two times more willing to try it than women (Verbeke 2015; Orsi et al. 2019; Palmieri et al. 2019). In a study comparing Northern and Central Europe consumer's acceptance, the results show that consumers from Northern European countries are more accepting of entomophagy (Piha et al. 2018).

2.3. Environmental impact of mass-production

According to Regulation (EC) No 1069/2009, insects are considered as 'farmed animals', and compared to other conventional livestock production systems, their large scale production is considered to be a more sustainable alternative (Oonincx et al. 2010). Moreover, these production systems, when optimized, require low-tech and low-capital investment (FAO 2013). Insects consume considerably less water (Miglietta et al. 2015) and feed (Collavo and Paoletti 2005), require less space but similar amounts of energy (EFSA 2015), create less waste (van Huis 2013), have higher fertility rates (Durst et al. 2010), emit less GHGs and produce lower levels of pollutants like ammonia than other types of livestock (Oonincx et al. 2010).

Although insects are perceived as efficient recyclers of organic waste into nutritionally valuable proteins (DeFoliart 1975), Regulation (EC) No 767/2009 prohibites the use of certain substrates as feed which include urban, domestic or catering waste, manure and separated digestive tract of other animals. This may restrain the full potencial of insects as a more sustainable option (Lundy and Parrella 2015; Belluco et al. 2017).

Nonetheless, comparing the feed conversion ratio (FCR) of different animal proteins, insects have considerably lower FCR (1.7kg) compared to beef (10kg), pork (5kg) and chicken (2.5kg) (Collavo and Paoletti 2005). The carcass yield for chicken and pork is 55% and for beef is 40%. In contrast, as crickets can be eaten as whole, their edible fraction can go up to 80%

(Amy Zhong 2017). The fact that insects are poikilothermic is a large contributor to their low FCR, since they do not require expendure of energy on body temperature regulation (van Huis et al. 2013).

With the rising global population and the shift in diet patterns, by 2050 it is expected an increase of 80% in global agricultural GHG emissions from the food sector (Tilman and Clark 2014). Insects are of great interest when it comes to environmental sustainability due to their lower GHG and NH_3 emissions (Oonincx et al. 2010; Testa et al. 2017).

It is however note-worthy to state that the claimed environmental advantages of rearing insects, including crickets, is largely dependent on the feed offered and the conditions of production applied (Lundy and Parrella 2015).

3. The house cricket (*Acheta domesticus*)

3.1. Life cycle and rearing conditions

The house cricket is a convenient species to rear, especially due to their short life cycle, easy manipulation, high reproductive rate and immunity from diseases and parasites (Patton 1978).

A crickets' life cycle is comprised of three life cycle stages: egg, nymph and adult (Figure 1). The eggs incubate for 10 to 14 days after which crickets hatch as nymphs and suffer between 6 and 12 molts in a period of 6 to 8 weeks, after which they become adults. Adults can live for approximately 2 months. Males and females can start mating at 2 to 3 days old and oviposition starts at around day 9 of adult stage. In the beginning of adulthood, females lay on average 95 eggs per day, gradually decreasing the oviposition rate until death. During their lifetime, females can lay up to 3,000 eggs (Clifford and Woodring 1990; Hanboonsong and Durst 2014; Miech 2018).

Environmental factors like temperature, relative humidity (RH) and light and dark (LD) cycle can greatly influence crickets' development and growth. According to previous studies, the optimal rearing temperature is 26-32 °C but survival and development is possible between 25 °C and 35 ºC (Busvine 1955; Clifford and Woodring 1990). RH can range from 40 and 70% (Weber et al. 1987; Booth and Kiddell 2007; Miech 2018) and various LD cycles have been applied such as 12:12h, 8:16h, 16:8h and 24:0h (Patton 1967; Patton 1978; Weber et al. 1987; Kaufman et al. 1989; Booth and Kiddell 2007). Overcrowding is also a preponderant factor since it can lead to higher mortality rates. Patton (1978) suggested a minimum area of 2.5 cm^2 per cricket.

Protein requirements for this species range from 20 to 30% (McFarlane 1964; Patton 1967; Sorjonen et al. 2019), carbohydrates from 32% to 47% and fat from 3.2% to 5.2% (Patton

1967). Vitamins and minerals are also crucial dietary components for crickets which require most of the B-group vitamins (Ritchot and McFarlane 1961), sodium, potassium, calcium (Mcfarlane 1991), among others.

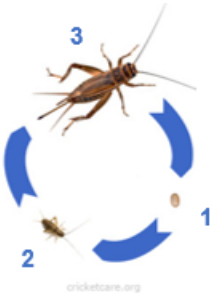

Figure 1. Life cycle of Acheta domesticus. Egg (1), nymph (2) and adult (3). Adapted from cricketcare.org (2020).

3.2. Nutritional composition and organoleptic features

Insects are known to have a very complete nutrient profile while being a delicious food source (XiaoMing et al. 2010), most frequently recognized as a protein-rich and fat-rich ingredient (van Huis 2018).

Individuals from order Orthoptera have approximately 60% protein (in percentage of dry matter (DM)) (Churchward-Venne et al. 2017; von Hackewitz 2018) and the house cricket, in particular, has a protein content between 44 to 70% DM.

The body composition of house crickets, varies with sex and developmental stages (Finke 2002). Kulma et al. (2019) observed that males contained higher protein (66.3–69.6 vs 61.2–64.9%) and lower fat contents (12.9–16.1 vs 18.3–21.7%) than females. Table 1 shows the nutritional composition of the house cricket. Significant differences in the protein and fat contents, as well as in the mineral and vitamin composition (Table 2), can be atributed to the use of different extraction methods and to different feeds provided. The total protein content is usually extrapolated from the determination of nitrogenous compounds (Adámek et al. 2019) and chitin, a nitrogen-containing polysaccharide that constitutes the exosqueleton (Parajulee et al. 1993), is included in this fraction. This polysacharide is not digestible (Poelaert et al. 2016), therefore when determining the nitrogen content, the real protein value is overestimated (Churchward-Venne et al. 2017). Protein digestibility is also influenced by processing technologies, especially thermal treatments, and Adámek et al. (2019) found that after roasting the digestibility of mealworms (*Tenebrio molitor*) was higher compared to not applying any thermal treatment. Poelaert et al. (2016) obtained similar results and observed that mealworms had higher digestibility compared to house crickets.

Crickets are also a good source of minerals (Table 2) such as calcium, phosphurum, mangnesium, iron and zinc (Michaelsen et al. 2009; Finke 2015), and vitamins (Barker et al. 1998).

NUTRIENT COMPOSITION (%)					
	DM		Fresh Weight		
Stage	Protein (%)	Fat (%)	Protein (%)	Fat (%)	Reference
Adults	47.1	25.8	14.9	4.1	(Ayieko and Orinda 2020)
	64.1	24.0			(Ramos-Elorduy Blásquez et al. 2012)
	63.3	17.3			(Makkar et al. 2014)
			18.6	6.0	(Yhoung-aree 2010)
			20.5	6.8	(Finke 2002)
			20.1	5.06	(Payne et al. 2016a)
			15.6	4.56	(Payne et al. 2016b)
Cricket Powder	44.2	25.5			(Montowska et al. 2019)
	70.6	17.7			(Bosch et al. 2014)

Table 1. Fat and protein concentration (% DM and % fresh weight) of the house cricket in different stages of production according to various references.

MINERAL AND VITAMIN COMPOSITION (µg/g)												
Stage	Na	Fe	Zn	Ca	Mg	P	Vit A	Vit C	Vit D	Vit E	B12	Reference
Adults (DM)	8502.3	51.8	21.79	3147.7		331.3	0.35			331.3		(Ayieko and Orinda 2020)
		112.3	186.4	2100	800	7800	0.24	97.4		54.27		(Barker et al. 1998)
											0.174	(Finke and Oonincx 2017)
Adults (Fresh weight)		19.3	67.1	410	300	3000						(Finke et al. 2020)
	1520	54.6		1040			65.3	30				(Payne et al. 2016a)
	1630	61.1	110	996	551	4960	0.144	30	6.4	22.6	0.0537	(Payne et al. 2016b)
	1340	19.3	67.1	407	337	2950	<0.3	30		3.2	0.0537	(Finke 2002)
Cricket powder	2860	47	160	1733	1130							(Montowska et al. 2019)

Table 2. Mineral and vitamin composition (µg/g) of the house cricket in various stages of development according to various references.

3.3. Fat body, fat and carbohydrates mobilization

In insects, energy reserves are stored in the trophocytes, the main cells of the fat body. These cells are analogous to adipocytes in vertebrates but instead of only storing fat, the main forms of energy reserves in trophocytes are glycogen and fat, mostly triglycerides (Schooley et al. 2012). The fat body is a tissue situated in the hemocoel surrounding the organs and is of great importance in the insect´s life. Besides fat and carbohydrates storage and metabolism, it also has other metabolic functions - it is responsible for protein synthesis and amino acid and nitrogen metabolism (Oliveira and Cruz-Landim 2006). For its functions, the fat body is considered as equivalent to both adipose tissue and liver in vertebrates (Canavoso et al. 2001).

In order for the fat body to be involved in these metabolic pathways, it must be able to receive signaling from other organs, which is mostly achieved via hormonal regulation. The adipokinetic hormones (AKH) are synthesized and released from the *corpus cardiacum* and are responsible for the mobilization of energy from the fat body in occasions of higher energy demands such as reproduction, starvation and stress (Arrese and Soulages 2010; Mochanová et al. 2018).

The AKH responses to these factors can vary with the insect species (Arrese and Soulages 2010). In turn, octopamine, the invertebrate analogue of dopamine, acts as a neurotransmitter, modulating the release of AKH (Meyer-Fernandes et al. 2000). For *Acheta domesticus,* Woodring et al. (1989) reported that the hyperlipidemic effect in response of handling was negligible, but starvation for 48h lowered the blood lipid level (from 21.2 ± 1.6 mg/ml in fed crickets to 17.1 ± 1.2 mg/ml).

Besides glycogen, trehalose, a disaccharide consisting of two monomers of glucose, is also present in the hemolymph as the main sugar in insects. Alternative sources of trehalose are glycogen breakdown from the fat body and gluconeogenesis. Hemolymph sugars are used as energy sources during starvation (Becker et al. 1996; Thompson 2003).

3.3.1. Stress response to starvation

Food availability is a dynamic factor of most habitats (Johnson and White 2009). Starvation stress leads to a series of adaptations, including behavioral and physiological changes. When starvation occurs in nature, crickets can alter their behavior by entering diapause (a decrease in the metabolic rate), enduring cannibalism and even migrating. In captivity however, migration is not possible, thus leaving diapause and cannibalism to be the most relevant adaptations (Zhang et al. 2019).

During diapause, insects rely mostly on their fat reserves, but also on other stored energy sources, to maintain homeostasis (Hahn and Denlinger 2007). Trehalose is readily accessible in the hemolymph, being the first source of energy used by the starved insect. Glycogen stored in the fat body is then converted into trehalose and released into the hemolymph, under the control of AKH. Fat reserves are the most important energy resource in long term starvation (Mariano et al. 2009; Zhang et al. 2017; Yamada et al. 2018; Jiang et al. 2019; Zhang et al. 2019).

Starvation induces an initial increase in physical activity as a strategy to search for food. Longer periods of starvation lead to decreased levels of activity, including the reduction or pausing of reproduction. This adaptative behavior is intended to conserve energy and enable survival. In addition, starvation can augment the tendency for cannibalism, described as intraspecific predation, but food shortage is not an obligatory condition for this behavior. Other factors contributing to the occurrence of cannibalism are overcrowding and size differences between predator and prey (Fox 1975; Scharf 2016).

4. Food safety aspects of *Acheta domesticus*

Despite the well-recognized benefits of entomophagy, mass-reared house crickets can also pose a health risk to the consumers. Like for other farmed species, the control of microbiological (bacteria, viruses, parasites, fungi, prions), and chemical hazards (heavy metals, toxins, veterinary drugs, hormones) is dependent on the implementation of good hygiene practices (GHP), good manufacturing practices (GMP) and good farming practices (GFP) (Fernandez-Cassi et al. 2019). Hazards can be introduced during all stages of production including rearing, growth, harvesting and processing as well as in the feed, which can also be a source of environmental contaminants (EFSA 2015).

4.1. Microbiological hazards

Insects are phylogenetically very different from mammals, thus pathogenic agents in insects are usually not pathogenic to animals and humans. Insects' microbiota comprises commensal intestinal microbiota and those on their surface, the latter acquired during primary production, processing and storage (ANSES 2015).

High counts of total viable bacteria and *Enterobacteriaceae* have been reported on fresh crickets (Klunder et al. 2012) but after processing, such as freezing, freeze-drying or boiling the microbial load is substantially reduced (Fernandez-Cassi et al. 2019). Important food pathogens like *Salmonella* spp. and *Escherichia coli* have been identified but not *Listeria*

monocytogenes (Van Der Fels-Klerx et al. 2018). Other documented species include *Yersinia* spp., *Citrobacter* spp., *Fusobacterium* spp., *Campylobacter spp.* and *Bacteroides* spp. In a study evaluating the microbiota of ready-to-eat crickets, Milanovic et al. (2019) detected by DNA extraction and sequencing, *Clostridium* spp. and *Staphylococcus* spp.. *Clostridium* spp. is a family of spore-forming bacteria with pathogenic potential due to their resistance to thermal treatments and ability to produce toxins. Important species of this genus include *C. botulinum, C. difficile* and *C. perfringens*. *Bacillus cereus*, another sporulating bacteria, was also identified in the house cricket (Fasolato et al. 2018).

Viral infections in crickets can mean considerable production losses for the farmers due to high mortality rates. The most important pathogenic viruses in crickets are cricket paralysis virus (Dicistroviridae) and cricket densovirus (Parvoviridae). Although these viruses' families include pathogens to humans, there is evidence that they cannot replicate in human cells. Arboviruses (viruses that replicate in invertebrates and are capable of infecting vertebrates), as well as food-borne viruses such as norovirus, hepatitis A and E viruses, have not been reported in crickets. Crickets can, however, act as mechanical vectors for human viruses when contaminated during production or processing and GHP constitute the most effective preventive measure (EFSA 2015; Fernandez-Cassi et al. 2018; Fernandez-Cassi et al. 2019).

Fungal species can affect crickets in both primary production and in the final product. During their life cycle, crickets can be infected by fungi, yeasts and molds, leading to higher mortality rates. Edible crickets can also be contaminated by mycotoxin-producing fungi *Aspergillus* spp., *Penincilium* spp. and *Fusarium* spp., which pose a risk to human health. Mycotoxins are exceedingly difficult to eliminate from foods and feeds and are resistant to heat treatments. Moreover, high counts for yeast and molds in insect-based foods can compromise food quality by promoting fast deterioration (Fernandez-Cassi et al. 2018; Fernandez-Cassi et al. 2019).

Parasites pathogenic to humans have not been reported in crickets (Fernandez-Cassi et al. 2018). There is however evidence suggesting that crickets can act as intermediate hosts for *Abbreviata antartica*, a nematode pathogenic to lizards. This parasite could infect humans, although further research is needed to assess the pathogenicity to humans (King et al. 2013). Nevertheless, potential parasites in crickets can easily be destroyed by thermal treatment, either heating or freezing, making parasites a low risk hazard in this species (Fernandez-Cassi et al. 2019).

Another concerning hazard category in foods of animal origin is prions. Insects do not encode prion proteins but can act as mechanical vectors due to the high stability of prions in the environment, which can remain infective after ingestion by insects (Finke et al. 2015). Hence, the feed provided to mass-reared crickets must comply with Annex III of Regulation

(EC) No 767/2009 listing prohibited feed materials and Regulation (EU) No 1148/2014 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies.

4.1.1. Total Aerobic Counts and *Enterobacteriaceae*

Currently, there are no specific hygiene criteria concerning food safety of edible insects in the EU (Megido et al. 2017; Fernandez-Cassi et al. 2019). Thus, it has been suggested the use of the same hygiene indicators for other foodstuffs containing animal protein (Grabowski and Klein 2017; Vandeweyer et al. 2017), such as TAC and *Enterobacteriaceae* counts (European Comission 2005). *Enterobacteriaceae* are usually an indicator of fecal contamination but in crickets they are originated from the gastrointestinal tract and, even though some measures like fasting for 24 to 48 hours before killing can be applied (Kooh et al. 2000; Fernandez-Cassi et al. 2019), reducing the microbial load to acceptable values is a challenge (Grabowski and Klein 2017). Although the presence of non-pathogenic microorganisms in edible insects is acceptable, high log CFU/g values accelerate deterioration, therefore reducing the shelf-life of the product (Klunder et al. 2012).

In this study, these hygiene indicators were analyzed with the intent of evaluating the efficacy of starvation on reducing the microbial load of frozen house crickets without any other processing step.

4.2. Allergens, chemical and physical hazards

Allergic reactions to crickets are considered rare and mild when compared to other allergies (Pener 2016). Arginine kinase, an ATP phosphotransferase found in invertebrates (Downs et al. 2016) acts as the most relevant allergen in crickets. Other allergens include hexamerin B1, a storage and transport protein (Goodman and Cusson 2012) and glyceraldehyde 3-phosphate dehydrogenase (EFSA 2015). Crickets' allergens can also present cross reactivity with allergens from other species like shrimps (Srinroch et al. 2015) and locusts. Allergies to crickets are usually induced by inhalation or contact but can also be due to ingestion and may cause asthma and rhino-conjunctivitis, dermatitis and angioedema (EFSA 2015; Pener 2016).

Since crickets do not produce natural toxins nor do they have reported antinutritive compounds, chemical hazards in this species derive from the environment and feed. Bioaccumulation of pesticides (organophosphorus pirimiphos-methyl), organic pollutants (dioxins, organochloride compounds, flame retardants and polycyclic aromatic hydrocarbons)

and heavy metals (cadmium, mercury and arsenic) can occur (Fernandez-Cassi et al. 2018; Fernandez-Cassi et al. 2019). Hence, in mass-production farms, the feed and housing material must be carefully selected and monitored. Likewise, veterinary drugs ought to be used with prudence (ANSES 2015).

Regarding physical hazards, some parts of the cricket's body like the wings and legs, may cause intestinal constipation (FAO 2013). During production and processing, the product can also become contaminated by foreign bodies (ANSES 2015). Physical hazards, however, are ranked as low risk since adequate measures during processing and commercialization (appropriate product labelling) can be applied in order to reduce the risk (Fernandez-Cassi et al. 2018).

5. Good production practices to ensure a safe product

The insect production sector can grossly be divided into two stages: primary production and processing. The primary production generally includes (1) feeding of insects with selected substrates, (2) growth phase, (3) harvesting and (4) pre-treatment. Feeding of insects must be in accordance with Regulation (EC) No 183/2005 on feed hygiene, and GMP are advisable to reduce the risk of feed contamination. During the growth phase, four factors are of high importance to avoid contamination by microorganisms and chemicals which are greatly dependent on the species reared: temperature, humidity, ventilation, and adequate enclosure facilities. During harvesting, workers must guarantee the complete separation of insects from the substrate and feces, as these can act as a source of spoilage microbiota, as well as dead animals or foreign bodies. Pre-treatment refers to the step prior to killing and processing. Chilling is the commonly preferred method as a way of immobilizing the animals and therefore facilitate storing and transport (van Huis 2019).

Killing is accomplished by applying a thermal treatment, either very high or very low temperatures. When blanching is applied, which refers to the dipping of the insects in hot water, the water used is a possible source of contamination by spore-forming bacteria, hence the importance of monitoring and properly treating the water (van Huis 2019). After killing, various processing technologies, further described in section 6, may be employed with different purposes (van Huis 2019).

5.1. Hazard Analysis and Critical Control Point system

The Hazard Analysis and Critical Control Points (HACCP) system was developed to identify hazards (biological, chemical, and physical) during the production of foods and

implement control measures to guarantee their safety. Prior to the implementation of HACCP, a series of pre-requirements must be assured, such as GHP, GMP, GFP, facility design and pest control (WHO and FAO 2009; FAO 2014). This system is intended to prevent defects and irregularities based on evidenced health risks for each foodstuff, through the monitorization of various parameters, including critical control points (CCP) along the production chain. Corrective measures are applied whenever a deviation from the critical limits defined for a CCP is detected (Blaauboer et al. 2016; Jo and Lee 2016; Marshall et al. 2016).

The implementation of HACCP is not obligatory in primary production of foodstuffs, including insect production, but according to Regulation (EC) No 852/2004, it is of the producers' interest to establish an HACCP plan, since this is a systematic and science based system designed to reach higher standards of food safety (WHO and FAO 2009). Consumer confidence is increasingly dependent on food safety, especially in the case of insects, since they belong to a new group of animal products. Hence HACCP is key to ensure edible insects are produced under high safety standards (van Huis 2019).

Despite the vast amount of literature regarding food safety of edible insects, concrete information concerning specific hazards is still lacking. This constitutes a limitation to the execution of a hazard analysis by the HACCP team (Fraqueza and Patarata 2017).

6. Processing technologies

Processing is critical in some food industries, since it destroys potential hazards like pathogenic agents, as well as spoilage organisms, therefore extending the products´ shelf life. It can also improve the palatability and visual presentation, making it more appealing to the consumer (Dossey et al. 2016). Killing is the first processing step of edible insects. The most common methods are freeze-drying, sun-drying and boiling (Baiano 2020). After killing, conservation and storage of insect-based foods can be achieved by the maintenance of low temperatures (freezing) and low water percentage (drying by lyophilization or roasting), the addition of chemical preservatives or by the use of modified atmosphere packaging (Dossey et al. 2016; Marshall et al. 2016).

Insects are commonly eaten whole and popular cooking methods include steaming, boiling, roasting, toasting, frying, smoking, drying and stewing (Marshall et al. 2016; Nyangena et al. 2020). Other forms of presentation are powders and pastes, as well as extracts like protein, fat or chitin, which can be incorporated as ingredients in other processed foods (Klunder et al. 2012; Melgar-Lalanne et al. 2019).

Despite their nutritional value, insects offered whole are often rejected by the westerner consumer. To solve this problematic the industry is betting farther on foods that incorporate

ingredients originated from insects. Some examples include biscuits, breads, protein powders and bars, fermented foods and burgers (Kewuyemi et al. 2020; Mishyna et al. 2020).

II Objectives

The main purpose of this study was to evaluate the effect of starvation on the microbial load and fat content of the house cricket.

III Materials and methods

1. Sample preparation

In September 2017, wild crickets (*Acheta domesticus*) were caught in their natural habitat near Uppsala and reared in a laboratory at the Swedish University of Agricultural Sciences under climate-controlled conditions. Controlled breeding was applied to promote genetic variability, where males and females from different groups were coupled for every new generation.

For this study, breeding was achieved by mixing selected adult crickets (males and females) in plastic boxes (16.5W x 14D x 14H cm) with small cups filled with humid sand for oviposition. Mating occurred for a period of 3 days, after which the sand cups were removed and incubated at 32 ºC in darkness. After 10 to 12 days of incubation, the sand cups with newly hatched crickets were transferred to new plastic boxes, where they were reared for the experiment. The breeding stage took place between the 4th and the 27th of October. In total, 22 females and 13 males were used for breeding and the resultant colony was composed of about 500 to 600 individuals.

During the growth phase, the crickets were housed in plastic boxes (16.5W x 14D x 14H cm and 28W x 20D x 28H cm) adapted to fit a thin steel net in one of the sides to enable ventilation, which were enriched with hiding units made of black water piping tubes (L6 x Ø 2.5 cm) (Vaga et al. 2018) and straws (L5 x Ø 0.53 cm and L5 x Ø 0.8 cm). The room was kept at controlled temperature of 30 ± 1 ºC and relative humidity of 45-55% with a 12h lighting regime. Feed was provided *ad libitum* and consisted of a pelleted feed mixture with the composition shown in Table 3. Water was given in plastic tubes (L10 x Ø 1.2 cm) that were refilled every 5 to 10 days. The nymphalid stage lasted about 8 weeks.

FEED INGREDIENTS (G/KG DM)	
Oat bran	296
Wheat bran	308
Wheat meal (kernels)	224
Premix vaga[1]	4
Rapeseed meal[2]	150
Limestone	18

Table 3. Ingredient list of the feed. [1] Vitamin mixture. [2] ExPro-00SF from AKA Ltd. (Malmö, Sweden).

2. Sample collection

In this experiment, three groups of crickets were compared: a control group fed *ad libitum* that was euthanized by freezing at -20 °C at the start of the experiment, and a second and third group initially fed the same diet but starved for 24h and 48h. Crickets from the two study groups were euthanized immediately after completion of the experiment as described above. In order to monitor mortality and cannibalism during starvation, crickets were separated in small plastic boxes (11W x 7.5D x 4H cm and 12.5W x 12.5D x 5H cm), one male and one female in each, without food. Only water and shelter tubes were provided.

Starvation was executed at two different times, that is, the first group was starved for 24h and two weeks later the second group was starved for 48h. This allowed the remaining crickets to become adults. The separation of crickets was always done at the same time, from 7 to 8 am and euthanasia by freezing at 8 am. Each group was composed of 100 crickets, 50 females and 50 males, which were separated in 50 small boxes for starvation. The control group was not separated, having been immediately euthanized by freezing at -20 ºC.

Simultaneously, a behavioral study was conducted with crickets from the same family. The purpose of this study was to quantify glycogen of starved crickets. The same separation method was applied for two other groups of crickets (24h and 48h starvation), and a control group (no starvation) was also included. Each group was composed of 48 crickets, separated in 24 small plastic boxes (11W x 7.5D x 4H cm and 12.5W x 12.5D x 5H cm). In each group, 8 boxes (12.5W x 12.5D x 5H cm) were filmed separately for 8 minutes. Euthanasia was done by freezing at -80 ºC. Both studies were conducted at the same time.

After 24h in the freezer, the samples were separated in plastic bags, according to their intent: microbiological analysis, fat and glycogen quantification. For microbiological analysis there were 8 replicates per group, each sample containing between 7 and 9 crickets, both females and males. For fat quantification, 3 replicates with 6 crickets each were made for each sex. The analysis and results of glycogen quantification and behavior observation are outside the scope of this thesis.

2.1. Evaluation of sex on microbial load

To assess whether there is a significant effect of sex on the microbial load of starved crickets, a pilot study was conducted. Crickets were starved for 24h and euthanized by freezing at -20 ºC. 3 replicates of each sex (a pool of 10 crickets each) were analyzed for Total Aerobic Counts (TAC) and *Enterobacteriaceae*. The weight of the samples varied between 2.46 g and 5.07 g. For each sample, whole adult crickets without prior processing/treatment were crushed

with a mortar and homogenized with buffered peptone water (BPW) (1:9) in a Stomacher (easyMIX Lab Blender, AESChemunex, Weber Scientific, Hamilton, NJ) for 2 min. Serial dilutions were performed according to Nordic Committee on Food Analysis (NMKL) method nr. 86, 5th edition 2013 for TAC (equivalent to ISO 4833-1:2013) and NMKL method nr. 144, 3rd edition 2005 for *Enterobacteriaceae* (equivalent to ISO 21528-2:2017).

The data were analysed on Microsoft Office Excel for Windows where an independent t-test was done to calculate the statistical significance of sex on the microbial load (p=0.05), using the log CFU/g values of TAC and *Enterobacteriaceae* counts.

3. Microbiological analyses

The samples were taken out of the freezer 30 min before starting the analyses, to defrost. They were further transferred to a Stomacher bag and crushed with a mortar. After weighing (weights were between 1.96 g and 3.58 g), the crushed material was suspended in sterile buffered peptone water (1:9) and homogenized in a Stomacher for 2 min. Tenfold dilution series were prepared using a Dilicup (Dilushaker III Digital 6 rows LED 21).

3.1. Total aerobic counts at 30 ºC

From the previous serial dilutions, 1ml aliquots of dilutions 10^{-5} to 10^{-9} were pour-plated into standard plate count agar (Oxoid, Basingstoke, UK) and homogenized. A thin overlayer of the same medium was added after solidification. The plates were incubated at 30 ± 1 ºC for 72±6h. After the incubation period, the plates with 25 to 250 colonies were counted using a colony counter (BZG 30, Gerber Instruments, Switzerland). The results were expressed in log CFU/g. The methodology used was according to the NMKL method nr. 86, 5th edition 2013, equivalent to ISO 4833-1:2013.

3.1.1. Gram staining

With the intend of characterizing the bacterial communities present in the cricket samples, Gram stains were completed for isolates from the groups 24h and 48h. For practical reasons, no Gram stains were done for the control group. From 5 out of the 8 samples inoculated for TAC, 5 colonies were sub-cultured on blood-agar plates (SVA, National Veterinary Institute), a non-selective enrichment medium, and incubated at 30 ºC for 20h ± 2h. In total, 25 colonies were analyzed for each group. After the incubation period, a Gram stain

was done. Observation of the Gram-stained slides was made with a light microscope on the 100x objective lens (Leitz).

3.2. *Enterobacteriaceae* counts

The enumeration of *Enterobacteriaceae* was executed according to the NMKL method nr. 144, 3rd edition 2005, equivalent to ISO 21528-2:2017. From the serial dilutions, 1 ml aliquots of dilutions 10^{-4} to 10^{-7} were cultured by pour-plating in Violet Red Bile Glucose (VRBG) medium (Becton, Dickson and Company, Sparks Glencoe, USA) and homogenized. A thin overlayer of the same medium was added after solidification. The plates were incubated at 37 ºC for 24h ± 3h. The colonies were then enumerated in the plates that had between 15 and 150 colonies.

For biochemical confirmation, 5 colonies from each plate were sub-cultured on blood-agar medium and subjected to oxidase reaction test (Becton, Dickinson and Company, Sparks, USA), after incubation at 37 ± 1 ºC for 24 ± 3h.

The data were statistically evaluated on Microsoft Office Excel for Windows using a Wilcoxon Signed Rank test with a significance level of 5%.

4. Fat quantification

4.1. Sample preparation

To quantify the fat content of the crickets being studied, the samples were freeze-dried after the individuals having been cut transversally in 3 or 4 pieces and weighed in an analytical 4 decimal place balance. The samples were then frozen at -80 ºC and later grinded with a mortar. To keep the samples frozen, the mortar was used inside a thermal box with dry ice during grinding. Between samples, the mortar was cleaned with ethanol to remove fat residues of the previous sample.

Initially, it was planned that each group were to have 6 replicates of 3 individuals for each sex. However, after freeze-drying, the mean sample weight was 0.3988g. Hence, in order to reduce the error associated with an insufficient sample weight, the samples were paired two by two, being tested in total 3 replicates of 6 individuals.

4.2. Determination of total fat by Soxhlet extraction

The determination of the total fat was executed by Soxhlet extraction using Soxtec/Hydrotec™ 8000 Total Fat Solution (FOSS, Denmark), consisting of a hydrolysis unit "Hydrotec 8000" (Figure 2A), an extraction unit "Soxtec 8000" (Figure 2B) and patented filters "Hydrocaps" (Figure 3). The hydrolysis unit allows the hydrolysis of 12 samples at a time while the extraction unit only allows 6.

Figure 2. A- Hydrolysis unit "Hydrotec 8000"; B- Extraction unit "Soxtec 8000" (FOSS 2014).

Figure 3. Filters "Hydrocap" (Fisher Scienfitic 2020).

Before performing the analyses, samples were left at room temperature. One hour prior to hydrolysis, 6 extraction cups were dried for 1h at 103 ºC which were then cooled in a desiccator. The samples were weighed in an analytical 4 decimal place balance into the hydrocaps. These were put in the beaker of the hydrolysis unit which was automatically filled with hydrochloric acid 3 M (HCl) and boiled for 2h. The hydrolysis step was intended to separate fat from other components of the sample. After hydrolysis was completed, the hydrocaps were removed from the beaker. The hydrolyzed samples were covered with a layer of cotton to remove any residue of acid and put in an oven at 60 ºC overnight.

For the extraction step, the hydrocaps, as well as the extraction cups were attached to the extraction unit and the cups were filled with solvent (petroleum ether) using an external

solvent dispenser attached to the system. Extraction was performed at 90 ºC and consisted of three steps: boiling (20 min), rinsing (40 min) and solvent recovery (8 min). Finalized this stage, the extraction cups were dried at 103 ºC for 30 min and then put in a desiccator for cooling until room temperature. Finally, the ups were weighted.

The results were obtained using the following equation:

Fat (g/kg DM) = 100 x (V_2 – V_1) / sample weight (g)

V_1 = Weight of empty cup (g)

V_2 = Weight of empty cup + extracted fat (g)

For total fat content, the statistical analysis was performed in R using a factorial analysis of variance (ANOVA) and Tukey's post-hoc analyses with a significance level of 5%.

IV Results

1. Evaluation of sex on the microbial load

For the pilot study, Table 4 shows the log CFU/g values of *Enterobacteriaceae* and TAC at 30 ºC in male and female individuals of *Acheta domesticus* starved for 24 hours. The mean values for *Enterobacteriaceae* in female crickets was 6.5 log CFU/g and in males was 6.8 log CFU/g. The mean TAC values in females and males were 7.8 log CFU/g and 7.6 log CFU/g for females and males, respectively. The results show that there was no significant difference on the microbial load of female and male crickets. For this reason, the samples used for microbiological analyses in the experiment comprised both females and males.

TOTAL AEROBIC COUNTS AT 30 ºC (log CFU/g)			***ENTEROBACTERIACEAE* (log CFU/g)**		
Sample (n = 10)	Female	Male	Sample (n = 10)	Female	Male
1	8.4	7.8	1	6.7	6.2
2	7.7	7.8	2	6.3	7.5
3	7.2	7.3	3	6.4	6.7
MEAN (±SD)	**6.5±0.2**	**6.8±0.7**	**MEAN (±SD)**	**7.8±0.6**	**7.6±0.3**

Table 4. Results of the pilot study on the microbial load of crickets (Acheta domesticus) starved for 24 hours.

2. Effect of starvation on the microbial load and total fat content

Based on the results of the pilot study, the samples used for the determination of TAC at 30 ºC as well as *Enterobacteriaceae* were composed of both male and female individuals, each sample being a pool of 7 to 8 crickets (Table 5). As presented in Table 5 and illustrated in Figure 4, the starvation groups had crescent mean TAC values over time: 7.3 log CFU/g at 0h (T0), 7.8 log CFU/g at 24h (T24) and at 48 (T48) 8.2 log CFU/g.

TOTAL AEROBIC COUNTS AT 30 ºC (LOG CFU/G)						
Sample	n T0	T0	n T24	T24	n T48	T48
1	8	7.5	8	7.5	7	7.9
2	8	7.4	8	8.9	7	8
3	8	7.1	8	7.4	7	8
4	8	7	8	7.8	7	7.8
5	8	7.6	8	7.8	7	7.7
6	8	7.2	8	6.8	7	8.3
7	8	7.8	8	7.7	7	8.8
8	9	6.8	8	8.2	7	8.7
MEAN±SD		**7.3±0.3**		**7.8±0.6**		**8.2±0.4**

Table 5. Results of the total aerobic counts at 30 ºC (log CFU/g) of crickets (Acheta domesticus) after different periods of starvation.

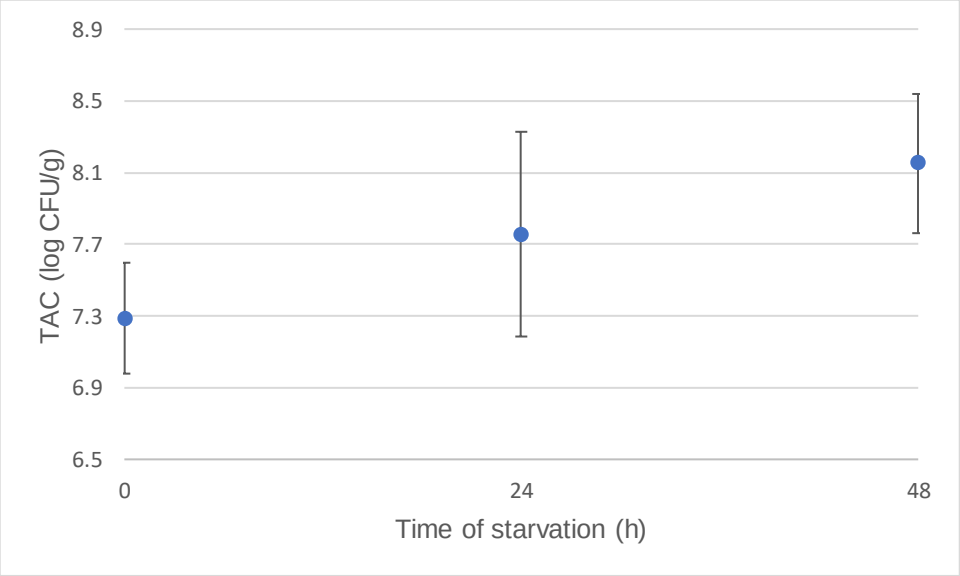

Figure 4. Mean values and SD of TAC at 30 ºC (log CFU/g) of crickets after different periods of starvation.

In Table 6 is presented the colony composition of the TAC plates in the 24h and 48h starvation groups, in percentage (%) of each cell type. There was a variation between the 24h and the 48h groups, having been registered a decrease in Gram-positive cocci (from 68 to 48%) and an increase in Gram-negative cocci (from 24 to 48%). Bacilliform colonies were not detected in the 48h groups but were present in small numbers (8%) in the 24h group.

GRAM STAIN		
Gram reaction and cell morphology	24h (%)	48h (%)
Gram-positive bacilli	4	0
Gram-negative bacilli	4	0
Gram-positive cocci	68	48
Gram-negative cocci	24	48
Fungi	0	4

Table 6. Results of the Gram stains from the TAC plates of the 24 and 48h starvation groups. In total, 25 isolates were tested for each group.

In Table 7 are listed the samples included in each group (T0- 0h; T24- 24h; T48- 48h), with the corresponding *Enterobacteriaceae* values, mean and standard deviation (SD). As it can be observed in Figure 5, the *Enterobacteriaceae* counts did not suffer a linear decline over time with starvation, having been observed a decrease between 0h and 24h of starvation (from 6.2 to 5.2 log CFU/g) but similar values for the 0 and 48h groups (6.2 to 6.1 log CFU/g).

***ENTEROBACTERIACEAE* COUNTS (LOG CFU/G)**						
Sample	n T0	T0	n T24	T24	n T48	T48
1	8	6.2	8	5.2	7	6.3
2	8	5.5	8	4.7	7	5.9
3	8	6.3	8	5.7	7	4
4	8	6.6	8	5.5	7	5.6
5	8	5.8	8	5.8	7	5.5
6	8	6.3	8	5.9	7	5
7	8	6.5	8	4.2	7	8.5
8	9	6.1	8	4.7	7	7.9
MEAN±SD		**6.2±0.3**		**5.2±0.6**		**6.1±1.4**

Table 7. Results of the Enterobacteriaceae counts (log CFU/g) of crickets (Acheta domesticus) after starvation.

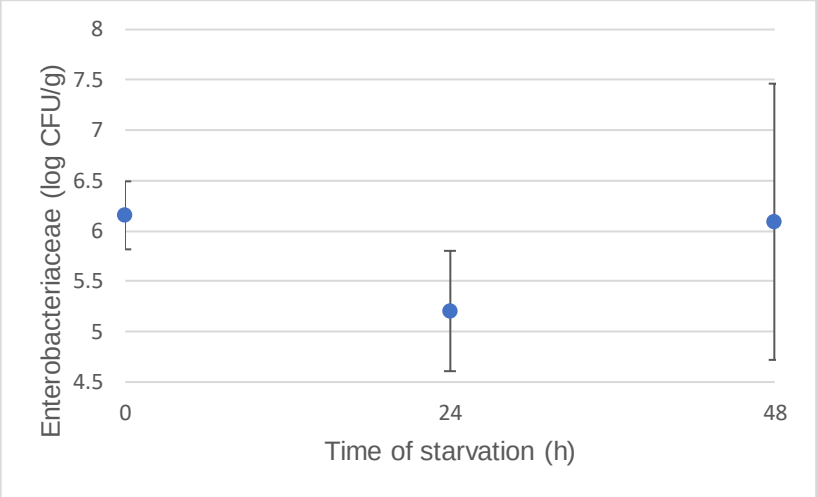

Figure 5. Mean values and SD of Enterobacteriaceae counts (log CFU/g) of crickets after different periods of starvation.

As shown in Table 8, there was a total of 3 samples for each group of crickets (T0- 0h; T24- 24h; T48- 48h) used to evaluate the fat content, and for each sex, with the corresponding fat contents (g/kg DM), means and SD. Figure 6 and Figure 7 illustrate the mean values and SD of the fat contents obtained for females and males, respectively. The results show a similar pattern for both sexes. When submitted to a starvation period of 24h, crickets had a reduced fat content, from 384.2 to 322.6 g/kg DM in males, and from 329.3 to 280.9 g/kg DM in females. With a starvation period of 48h, the fat content was reduced in males (384.2 to 355.9 g/kg DM) but slightly increased in females (329.3 to 333.3 g/kg DM).

FAT CONTENT (G/KG DM)							
Males				Females			
Sample (n=6)	T0	T24	T48	Sample (n=6)	T0	T24	T48
1	422.3	342.6	364.3	1	302.3	292.9	333.3
2	395.1	288.7	369.9	2	355.7	284	366.9
3	335.2	336.5	333.3	3	329.8	265.6	299.7
MEAN± SD	**384.2± 36.4**	**322.6± 24.1**	**355.9± 16.1**	**MEAN± SD**	**329.3± 21.8**	**280.9± 11.4**	**333.3± 27.4**

Table 8. Fat content (g/Kg DM) of male and female crickets starved for 0h, 24h and 48 h.

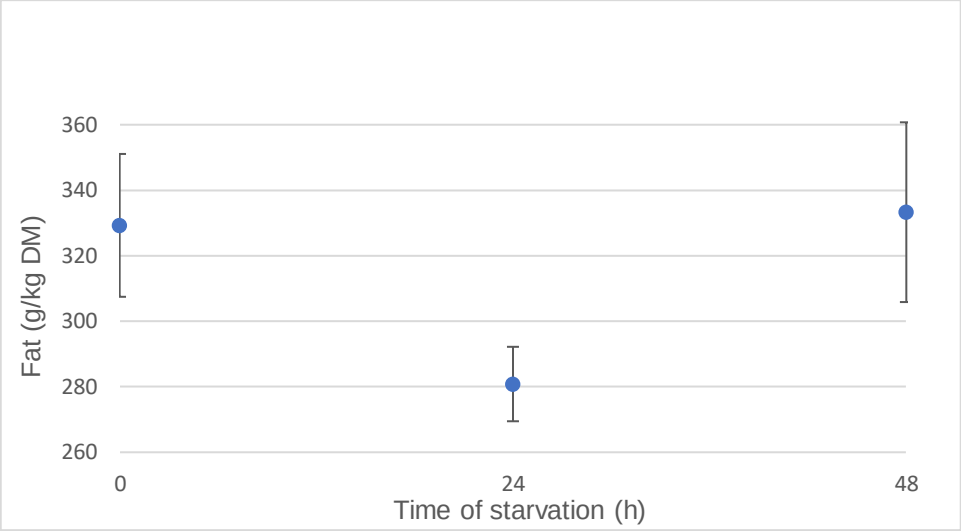

Figure 6. Fat content (g/kg DM) of female crickets after different periods of starvation.

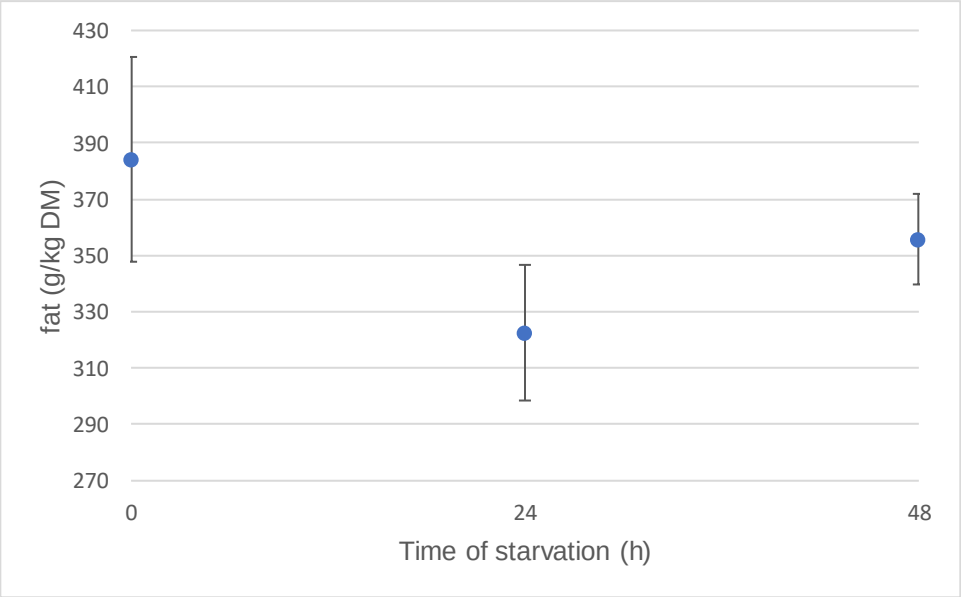

Figure 7. Fat content (g/kg DM) of male crickets after different periods of starvation.

V Discussion

In this study, no sex related differences were found for either TAC (p=0.72) nor *Enterobacteriaceae* (p=0.46). The results show a significant increase in TAC after 48h of starvation (p=0.002), not like after 24h (p=0.08). *Enterobacteriaceae* counts decreased after 24h of starvation (p=0.004) but not after 48h (p=0.5). Similarly to the findings of Wynants et al. (2017) in *Tenebrio molitor*, TAC in the house cricket were not significantly reduced with starvation after 24 and 48 hours. Likewise, Dillon and Charnley (2002) found a "larger population of bacteria" after starvation in locusts (order Orthoptera).

Evaluation of the Gram stains suggests an alteration of the microbial communities between 24 and 48h of starvation. For practical reasons, no Gram stains were analyzed at 0 hours. There is a predominance of Gram-positive cocci at 24h (68%) and at 48h, Gram-positive cocci values equal Gram-negative cocci (48%). Bacilli were observed in crickets starved for 24h (4% Gram-positive and 4% Gram-negative) but not for 48h.

Another study where the same rearing conditions of the present were used (including the feed composition), showed that the predominant phyla of crickets were Proteobacteria, Bacteroidetes (Gram negative) and Firmicutes (Gram positive) However, no fasting period was applied to the crickets (Fernandez-Cassi et al. 2020).

Starvation did not significantly reduce the total fat content of both males (p= 0.13 for 24h and p= 0.57 for 48h) and females (p= 0.13 for 24h and p= 0.98 for 48h). However, significant sex differences were observed overall, regarding the fat content of crickets (p=0.01), showing a higher fat content in males. These results are conflicting with the ones of Kulma et al. (2019), where females were found to have higher total fat content. The sex dependent variation of the fat content could eventually be explained by the variation in body composition that occurs with age, showed by Lipsitz and McFarlane (1971), since the exact age of the crickets used in this study was not thoroughly monitored. However, crickets were collected for analysis within 2-7 days after the last molt at adult stage but presented with different ages since the nymphalid stage differed between groups. Moreover, the small number of samples allowed a greater variation of the results, thus failing to detect smaller differences. Additionally, grinding after freeze-drying acted as a source of error since the weights of the samples were relatively small and some fat was lost during this step (Prost and Wrebiakowsi 1972).

VI Conclusion

This research aimed to find a correlation between starvation, microbial load, and fat content of edible crickets. The conclusions of the study are:

- *Enterobacteriaceae* counts were reduced significantly with a starvation period of 24h, while starvation for 24h or 48h did not reduce TAC nor fat.
- The microbial load of crickets did not vary with sex.

Hence, starving edible crickets for 24h prior to slaughter will reduce the *Enterobacteriaceae* counts without significant fat loss, although longer starvation periods do not appear to be beneficial.

For future research, the age of the animals used should be more homogeneous, as in this study crickets with different ages were used in the trials. Considering these new findings, insect farmers should consider whether or not to apply a starvation period, seeing as the outcome is limited. Ultimately, further research on the impact of facility materials, rearing methods, hygiene practices and feed on the microbiome of insects is needed in order to produce safe foods in the insect industry.

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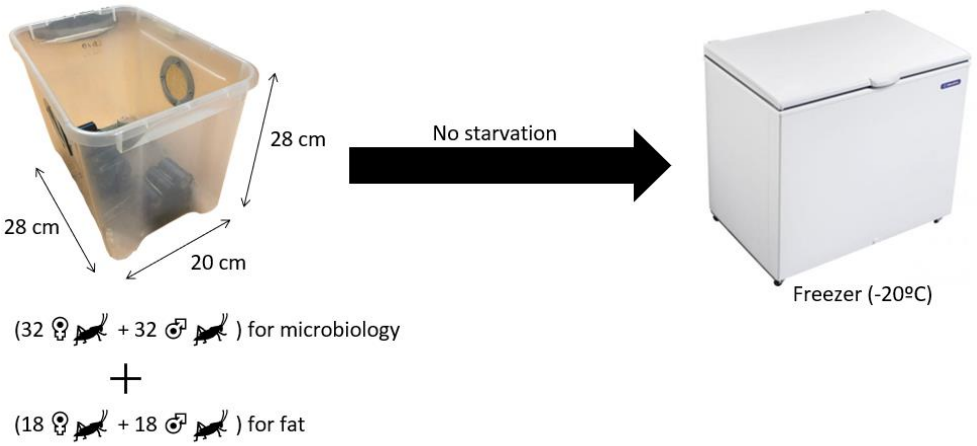
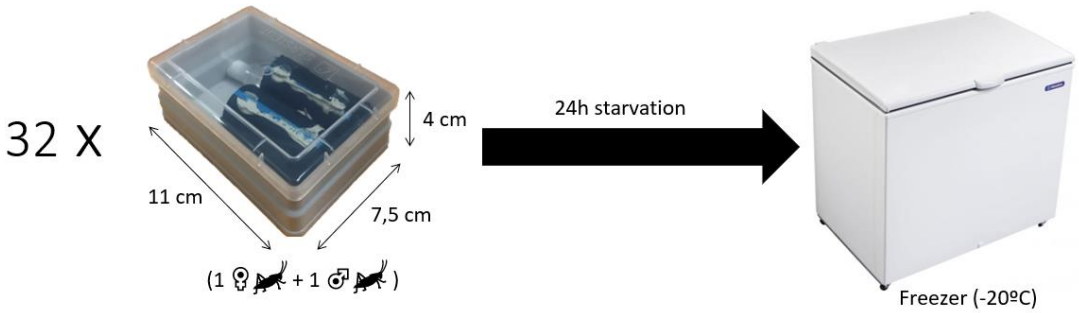
VIII Annexes

Annex I- Sample collection

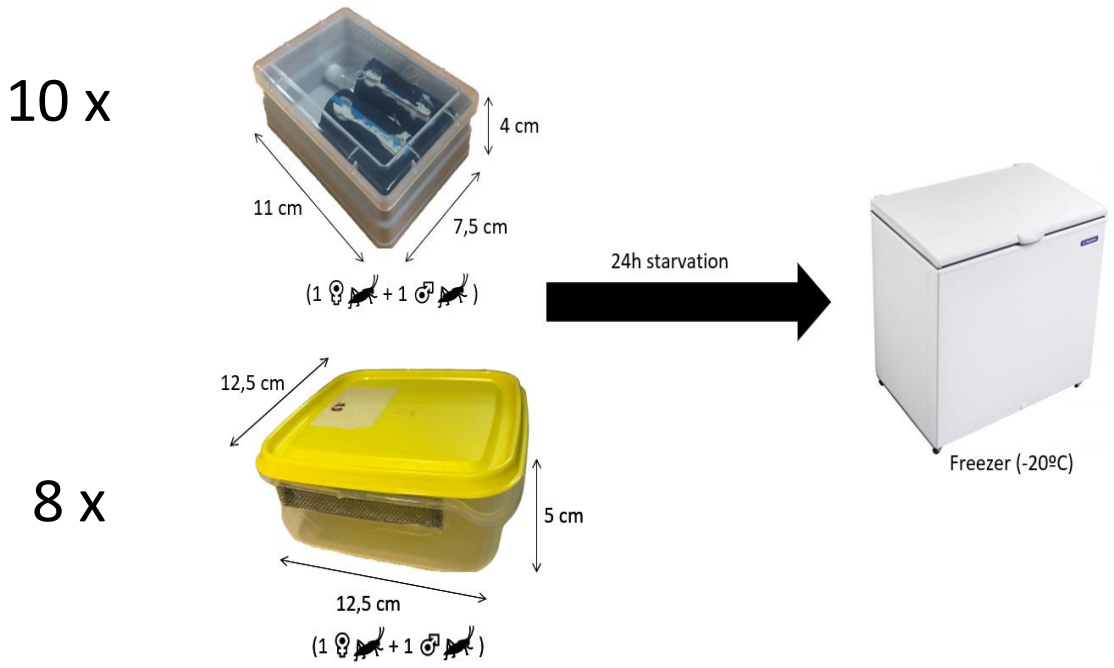
Sample collection for microbiological analysis and fat quantification for T0



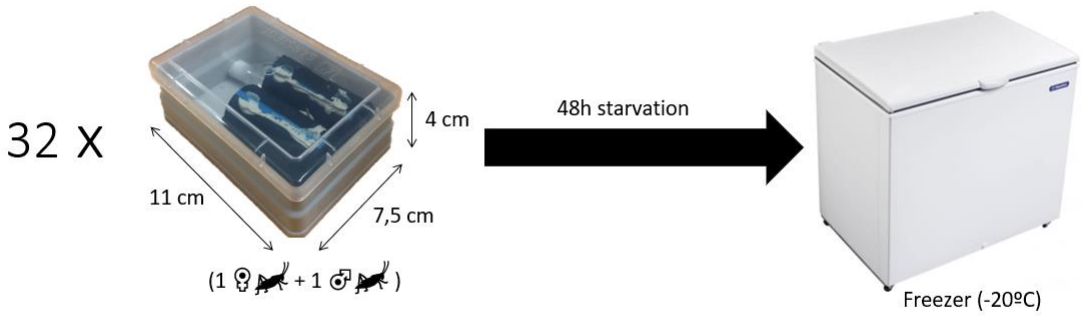
Sample collection for microbiological analysis for T24



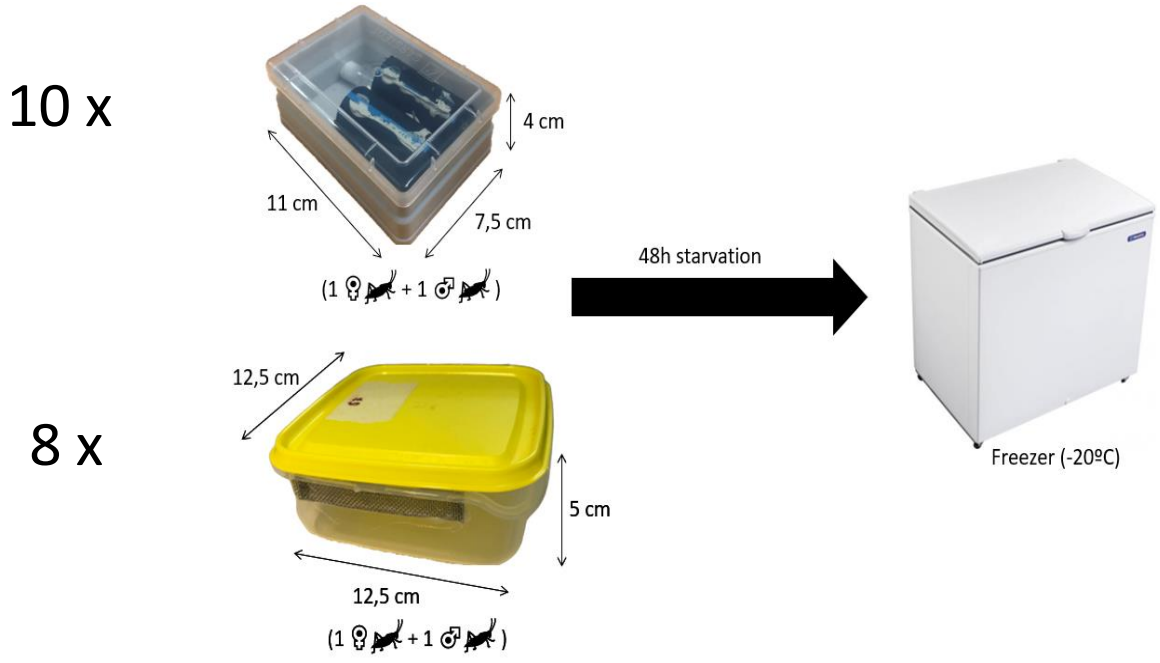
Sample collection for fat quantification for T24



Sample separation for microbiological analysis for T48



Sample separation for fat quantification for T48



Annex II- Materials used

Figure 1. Plastic boxes where crickets were reared (16.5W x 14D x 14H cm).

Figure 2. Plastic boxes where crickets were reared (28W x 20D x 28H cm)

Figure 3. Empty (A) and complete (B) yellow lid box (12.5W x 12.5D x 5H cm)

Figure 4. Empty (A) and complete (B) transparent lid boxes (11W x 7.5D x 4H cm)

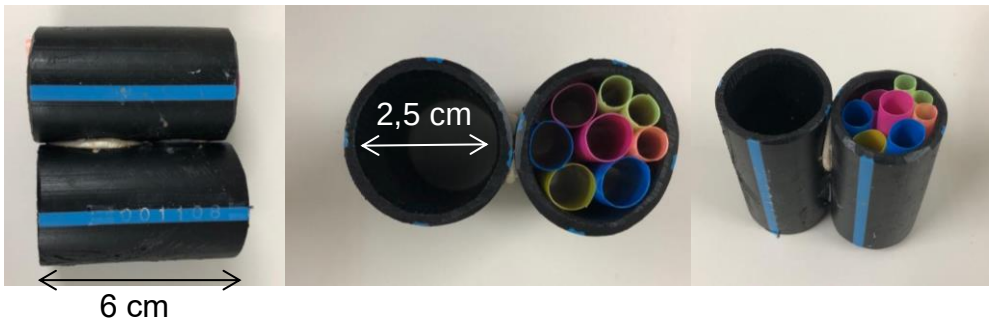


Figure 5. Glued black water piping tubes (L6 x Ø 2.5 cm)

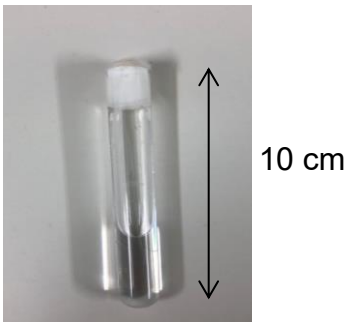


Figure 6. Water tube (L10 cm)

Figure 7. Feed provided to the crickets

Figure 8. Sand cups for oviposition

Figure 9. Material used to catch crickets