CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Zoonotic Aspects of Edible Insects in the Czech Republic

Master's Thesis

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Declaration

I declare that my thesis had been completed independently; with usage of resources only added at the end of this work. I agree with sharing and saving this document in CULS Prague library for study purposes.

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Name of student

“If all insects on Earth disappeared, within 50 years all life on Earth would end. If all human beings disappeared from the Earth, within 50 years all forms of life would flourish.“

(Jonas Salk)

“Bug business as a natural complement to the existing food industry.“

(Guarino)

Acknowledgment

I would like to thank all the organisms, which are all together helping world with its sustainability of creating beautiful and health mother Earth! Thanks to higher principles-consciousness. Thank you, who were encouraged me a lot! Thank you, my closest people-family and friends. Thank you for love.

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Abstract

Entomophagy is a food practice followed by 2 billion people worldwide and can be helpful with improving and ensuring food safety and food security, which is related to the new world conception One Health. This is connected with food-borne diseases, so potential of insects can be well sustainably utilized, but only if they are farming properly, with the right Biosecurity plans, which means mainly keeping sufficient hygiene and correct storage. Deeper we go in exploring insects and its good side, perfect nutritional value; we also strike on potential hazards of its consumption. Diploma thesis was in first stage focused on microbiological agents (family Enterobacteriaceae, *Salmonella* spp*. and Escherichia coli*) of given specimen of insects (*Tenebrio molitor, Zophobas morio, Gryllus assimillis*). Aim of this thesis was to overview norms for microbiological examination in insect's species; related to microbiology of tree given specimens *(Gryllus assimillis, Tenebrio molitor, Zophobas morio)* from three different distributors with the focus on family Enterobacteriaceae (genes *Salmonella* spp*., E. coli*) and to outline, whether insects are no more dangerous than meat products as alternative source of protein. To fulfill the targets were, in stage two of this thesis, formed two hypotheses. We used values of CFU/g (colony-forming units), which vary with every species. Hypothesis one was, that in raw insect (concretely *Zophobas morio*) there will ocure higher number of CFU/g (concretely *E. coli*) than in boiled insect. Hypothesis two was that content of *E. coli* in insects (concretely *Zophobas morio*) will be comparable with content of *E. coli* in meat products when the same treatment will be used. As a main norm was used norm ČSN 56 96 09. First part of the thesis was done according to norms ČSN EN ISO 6579: for detection of *Salmonella* spp.; ČSN ISO 16649–2: for the detection of *Escherichia coli* and ČSN ISO 21528: the detection and of Enterobacteriaceae. Millions of bacteria (1 500 000 CFU/ g) had been found in all examined species, so it is unacceptable value (insect should be heat-treated following of recommendation in this study). In washed insects samples there was values lower (150 000 CFU). Value 10 CFU/g in third examination was also same in all three types of insects and finally meet the limits in norm ČSN 56 96 09 (insects compared with meat). The second part of the thesis showed very different results from the first one. In this part, a data set from meat from 2016 to which had been applied the same treatment as applied in insects, was used. Yet the values fell into created norms, which was in contrast to the results from part one. High numbers in part one can be caused by different

season of harvesting insects/examination, stress of animals, or longer time before examination. In second part was used STATISTICA 12 CZ. According to Pearson's chi-squared test, (elected significant level p was 0.05), between raw and boiled *Zophobas morio*, there does not exist statistically significant difference. In raw meat and in raw insects our result was also higher than p (0.05), so suitability for consummation is the same. The same result was for third examination, boiled *Zophobas morio*, where also our result was higher than p (0.05), which means that there is no statistically significant difference between boiled meat and *Zophobas morio*). As it is seen difference between first part and second part of this thesis, there is need of future interests according to low sources of examinations from Netherland and Belgium.

Key words: microbiology, food safety, entomophagy, *E.coli*

Contents

List of Tables and List of Figures

List of Abbreviations Used in the Thesis

Organizations

OIE: World Organization for Animal Health

FAO: Food and Agriculture Organization of the United Nations

WHO: World Health Organization

FBO: Food Business Operator (under HACCP)

FDA: The Food Defect Action Levels

FSA: Food Standards Agency

HACCP: Hazard Analysis and critical control points (under FAO)

CCP: Critical control points

OSN: United Nations, UN

EC: European Commission

TFEU: Treaty on the Functioning of the European Union

ECDC: European Centre for Disease Prevention and Control

CDC: Centers for Disease Control and Prevention

EFSA: European Food Safety Authority

EMA: European Medicines Agency

CAI: Czech Accreditation Institute

SVI: State Veterinary Institute Prague

MENDELU: Mendel University Brno

CULS: Czech University of Life Sciences Prague

FAPPZ: Faculty of Agrobiology, Food and Natural Resources

VRI: Veterinary Research Institute, Brno

NIPH: The National Institute of Public Health

Scientific names

PCR: Polymerase chain reaction

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

IgG; IgM: Immunoglobulin

ATB: Antibiotic

GWP: Global warming potential

EU: Fossil energy uses

LU: Land use

EDCs: Endocrine disrupting chemicals

HPP: High pressure production

Microbiology

CFU and others: in Annex of this thesis

Media

MAC: MacConkey agar

MYP: Mannitol Yolk Polymyxin

DTM: Dermatophyte Test medium

TBX: Tryptone Bile Glucuronic agar

XLD: Xylose Lysine deoxycholate agar

RVS: Rappaport-Vassiliadis Soya Peptone broth

GTK: Plate Count agar

WPA: Wheat Peptone agar

DRBC: Dichloran Rose Bengal Chloramphenicol agar

DC: Deoxycholate Citrate agar

MAL: Mannitol-arabinosa-lactose

PRECIS: Brilliance Salmonella agar

Others

PCE: Good Conversion Efficiency

SLT: Shigella like toxin

1. INTRODUCTION AND LITERATURE REVIEW

The Universal Declaration of Human Rights states that food is a primary right of all people (Belluco et al., 2013). In these days arise needs to find alternative source of food for future population. Insect consumption by humans has always been a worldwide practice. This food habit dates back to prehistory and even though there was big change in way of looking at insects throughout Neolithic revolution (Van Itterbeeck and van Huis 2012), according to Paoletti (2005) and Halloran (2015), entomophagy is still a common practice in many regions of the world. However, there are few examples of national regulations that directly govern insects for human consumption. Moreover, more and more people have just found that entomophagy can be good way for sustainability of ensuring food (Yen, 2009). The term "entomophagy" is from the Greek words ἔντομον éntomon, "insects", and φᾰ́γεῖν phage, "eat", which together means the use of insects as food (Kouřimská, 2015). This minilivestock-insects could be of the great interest as a possible solution to two world crisis (famine as well as obesity), due to their capability to satisfy two head requirements: Firstly, they are an important source of protein and other nutrients; Secondly, their use as food has ecological advantages outperform conventional meat and, in the long run, insects have huge economic benefits (FAO, 2010, Oonincx, 2010, Dicke, 2013, Conference Insects to feed the world, 2014). Important part is good management of insects; precise overview and summary of the previous works about nutritional perfect properties of insects with new technology how to breed it constantly and sustainably can be found on pages livinfarms.com.

As it is generally known-everything is connected and we are one-so health status of pets and consumers of insects are in correlation with humans state. Insects can be regarded as safe, but only properly managed and consumed. Insects farming has the same technical characteristics as other animal production systems, there is need of the access to water and feed (substrate) to supply energy and nutrients for growth and system for collection of intestinal content (frass). The production is impacted by the physical conditions (small scale/large scale, or level of technological management solutions etc.) and the level of biosecurity in place to prevent introduction of e.g. microorganisms from the surrounding environment (Conference Insects to feed the world, 2014; EFSA, 2015).

Potential hazards, in relation to the insect's consumption, are allergies, chemical, microbial and parasitical risks (Belluco et al., 2016), which in some cases should be determined and limited by law for ensuring public health with aim to create food sustainable for human consumption. From the hygienic point of view should be also pointed out that some insects produce strong pharmacological compounds which are known as toxins for vertebrates. They may also contain residues of pesticides and heavy metals from the ecosystem. Necessary is to consider also allergic reactions which have been documented e. g. for various kinds of Orthoptera FAO (2013); Kouřimská (2015).

According to Rietschel (2002); Borkovcová (2009), the main problem in the World is globalization, which nowadays adopts mainly west economic values and natural sources of food and traditional life are gone. Result is often consuming old, conserved and unhealthy foods. Thus, western interest in this topic is growing, as demonstrated by FAO, EU and research project "Sustainable production of Insects Proteins" funded by The Netherlands Ministry of Economic Affairs and implemented by Wageningen University (Belucco et al., 2013). Grabowski et al. (2016) mentioned that edible insects has been introduced officially as foodstuffs into the European Union market in Belgium and the Netherlands, presenting national regulations in order to ensure food safety in 2014, but he add that limits in Netherlands and Belgium are not so relevant (Opperhuizer, 2014; Néve, 2014).

Entomophagy can also be found, on a small scale, in other EU countries (e.g. Germany).There still miss norm (FAO, WTO, WHO, EFSA) for ensuring human health by setting of correct HACCP. Because of this have been tried to invent partial recommendation norm, which could be applicable to practice examination of microbiological agents of given specimen of Insects (because of course for another species it can be different) and may it be clue for this main organizations for ensuring public health–with connecting to One-Health conception.

In these days people know that class Insecta has big future in lives of human race throughout many ways (Coufalová, 2014), although for the moment, it is difficult to predict whether edible insects will become the "food of the future Sogari (2015). In case of Western population attitudes toward food are frequently characterized by the rejection of certain food sources for psychological rather than logical reasons (DeFoliart 1999; Paoletti and Dreon 2005).

The conference-Insects to feed the world, held in 2014, was a milestone in the recognition of the professional insect industry. Feed industry leaders, insect breeders, universities, NGO's and other stakeholders gathered for the first time. As a conclusion, Insects for feed and food are viable solution for the protein deficit problem. The higher perspective from class Insecta has mealworm, according to its modesty and thus practicality, which is condition in this economical world Oonincx (2014) i Bednářová (2013).

1.2. Nutritional and Enriching Edible Insects

Many of authors e.g. Kouřimská (2015) mentioned that from a nutritional point of view, the insects contain significant protein content (depending on the type and development stage of the insects are from 20 to 76 %), which is higher than in many plants e. g. 14% (Belluco et al., 2013). Most species of insects contain sufficient amounts of amino acids which meets the nutritional needs of humans. Digestibility of the insect's protein is about 89 %. The differences in fat content, are very substantial (2-50 %) and depends on many factors. Larvae and pupae contain the highest amount of fat, in adults fat content is generally lower. Smykal (2014) brings interesting study about removal of juvenile hormone in insects and thus prolonged stage of larvae. Fatty acid composition at each species varies; there is highest influence of the host plant, on which the insects feed. In comparison with animal fats the content of essential fatty acids is higher (Ramos-Elorduy 1998; Collavo at al., 2005; Dlouhý, 2007; according to Kouřimská (2015) the total content of polyunsaturated fatty acids may be up to 70 %. But these sums are decreasing in process of storage (Marinovová, 2015). In her researches Kouřimská (2015) stated that edible insects contain less carbohydrate than protein and fat. Their content is usually in the range of 1-10 %. Carbohydrates in insects are represented mainly by chitin, whose content is different for different types and ranges usually between 5-16 %. Edible insects contains large amounts of trace elements, e.g. K, Na, Ca, Cu, Fe, Zn, Mn, and P (Fialová, 2011; Oonincx, 2012); from vitamins there are vitamin A, carotene, B vitamins and vitamins D, E, K and C (Kouřimská, 2015).

There are signs of big future of Insects in medicine. Insect's cuticle produce enzymes, which destroy many of pathogenous bacteria and viruses (Kašparová, 2009). Borkovcová (2009) points out, that insect's products works as a easers with arthralgia, have anti-inflammation effects and favorably works in developing of children brain (concentration benefit).

Insects can be therefore used in medicine, improve quality of condition of sportsmen (Kudlová, 2009) and moreover it can be used for space travel for high quality and concentrate nutrients (Katayama et al., 2007; Bednářová, 2013).

According Oonincx (2012) are expected increasing of demand for animal protein, which between 2012 and 2050 may increase up to 70-80 %) (Conference Insects to feed the world, 2014). An advantage of insects is good conversion efficiency (PCE), which is the animal's ability to use nutrients for increasing of their own body mass. From an economic perspective is important how much feed in kg an animal needs to be consumed to gain 1 kg of live weight. For the production of one kilogram of high-quality animal protein is required many times larger amounts of vegetable protein, than is needed for insects. For beef is even up to 10 kg in compares with insects, where is for 1 kg of body mass need 1.7 kg. Moreover, insect's usability is up to 90 % after processing compare to beef, only 50 % according Nierostková (2015). Insects have an important fiber content making them a nutritionally balanced foodstuff (Collavo at al., 2005).

Energy use due to the production of 1 kg of edible protein is very low, but quality of conversion logically depends on quality of feed. When fed grain-based diets at a scale of economic relevance, populations of crickets, so any insects, showed little improvement in PCE compared to broiler chickens fed similar diets. When fed processed, organic side-streams of relatively high quality, cricket populations achieved a harvestable size. According to Lundy (2015) it means that the efficiency of any insects production system and, therefore, its protein contribution and ecological impact, will depend on the quality of the insects diet. Storage of insects is connected with everything, which have been said above. Quality of storage is the most important, critical point of manipulation with insects (Belluco et al., 2013).

1.3. Environmental and Ethical Impact

According to Oonincx (2012) Insect can play big role in global warming potential (GWP), fossil energy uses (EU), and land use (LU). Insect's farms emit a fraction of harmful gases compared to livestock (Oonincx, 2010; Van Huis, 2010, Baker, 2013). According to Belluco (et al., 2016) increasing yields through agricultural intensification reduces environmental sustainability and animal welfare. Overall, insects leaves a much smaller

ecological footprint (Raloff, 2008), which can have a positive impact on the state of the global climate (Nyman, 2012). Therefore, if there is some danger of whole world warming, insects can be very helpful (Premalatha, 2011).

Situnayake (2016) described new economic situation as a following several lines:

"We're looking at being able to generate an income that's above the average US household income, from a very small capital outlay. Currently we can't grow crickets cheaply enough to compete with other feed, such as fish meal. We're just not there yet, but we will be very soon.

The core part of who we are is a hub through which all information that farmers are generating and everything they're learning about what works and what doesn't, flows back through us. We can then capture this data and analyze it to come up with engineering solutions for future iterations and so on.

The amazing thing is that you can do it in places that you can't usually do livestock, and you can make genuinely significant money with a small space".

After the patenting process is complete, Tiny farms located in Mexico will license out the patent of technology to cricket farmers, who will produce the insects. Exo, Thinksect, Chapul, and Crik Nutrition are the four next brands in USA, which are interested in raising insects, but there are many more on the horizon (Situnayake, 2016).

Bednářová (2014) points out, that for Europeans is the worst to seeing of insect before consummation. Many people in "civilized world" lost basic natural instinct-kill what they want to eat. That is reason why is still more suitable for them to buy commercial feedstuffs, where many cases they cannot imagine procedure of food and these foods are in many cases expensive and les healthy.

1.4. Food Security and Food Safety

Food security should be always mentioned at the beginning, before food safety, because it is main problem of the mother Earth (even it is more the case of economy, than insufficiency). According to Shaw (2012) food safety is a term for relatively rich countries that have a luxury of having sufficient food to allow them to make rules about what is safe to eat. Food safety is in relation with the One Health conception, which is connected to One Health Triad (showed in Figure 1). The One Health Triad is completed by

Epidemiologic Triad, which can be seen in Figure 2. Veterinary epidemiology/ veterinary services has mission to control and prevent population and eliminate risks of diseases, mainly zoonoses. They cooperate with OIE (Paris), which is in connection with WTO. OIE check reports from SVI, which is called Health Report System (OIE, 2007).

Rumpold (2013) points out that entomophagy in the future will comprise commitments in terms of certain risks that must be considered and ensured by safety prevention system. This includes the possible content of allergenic and toxic substances, as well as antinutritive substances and the presence of pathogens. Further information and studies required for a thorough assessment of the nutritional potential of edible insects and the proper treatment and classification of decontamination methods, which must be developed in order to ensure food safety.

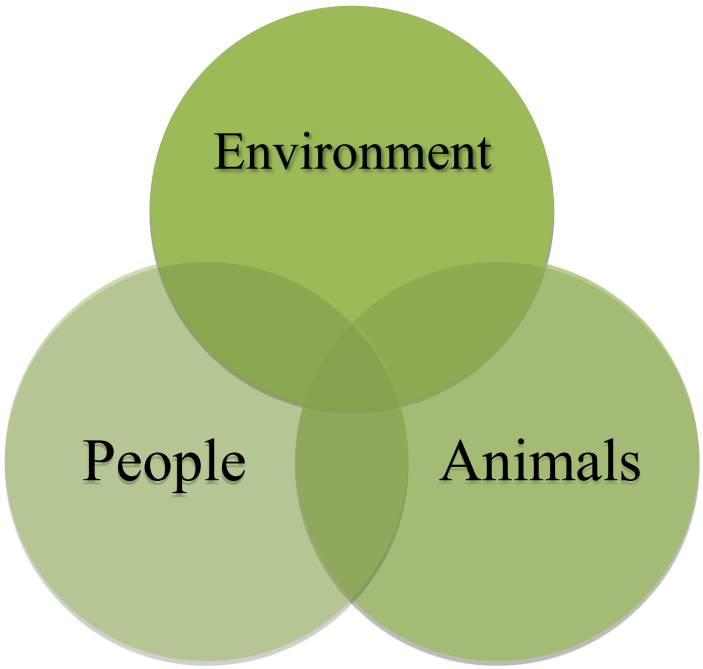

Figure 1: Healthy animals plus healthy environment plus healthy people = Healthy World; design by Coufalová (2016).

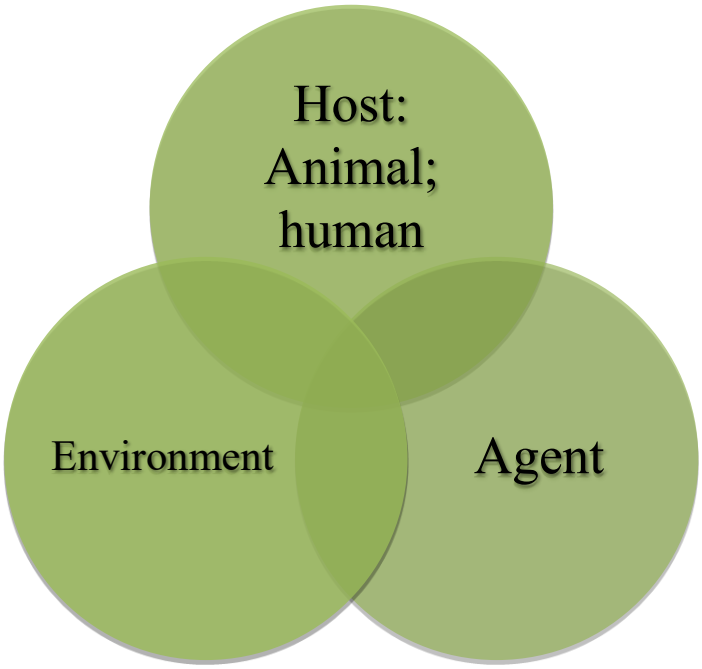

Figure 2: Epidemiologic Triad-host plus environment plus infectious agent (design by Coufalová, 2016), according Capinera (2010).

Although food safety is a major concern, it can undermine the importance of nature conservation, traditional food culture, food security, and potential economic development. Entomophagy should be viewed holistically and development of future legislation-must takes place into plan of sustainability of nature (Halloran, 2015).

1.4.1. Risks from Food

There are many hazards associated with food and eating (Shawn, 2012) e.g. chemicals, microorganisms (e. g. bacteria often producing toxins; or natural toxins-e.g. aflatoxins–fungus in nuts and grain–potential carcinogens) are one side of coin with physical hazards as a second one (Shawn, 2012). Same author also later formulated a base of Food Safety: Risk = hazard x exposure. If it is reduced exposure of particular hazard we eliminate risk. Basic of explanation of term risk can be seen in Figure 3. Interesting fact is that risk from eating insects is lower than e.g. being killed by car.

According to Shawn (2015) should be realized that also food preservatives against spoilage are dangerous. In case of insects it is good to mention that animals can absorb harbor of toxins from the plant they eat and via food chain we will be also exposed to them.

It is crucial to know that some of the toxins are not destroyed by cooking. Bacterial toxins can be by plasmids transferred from one species to another and some of bacteria can grow at refrigerator temperature (*Listeria monocytogenes* which can cause abortion) and not quickly cooled meat is always good environment for bacteria too (Shawn, 2015).

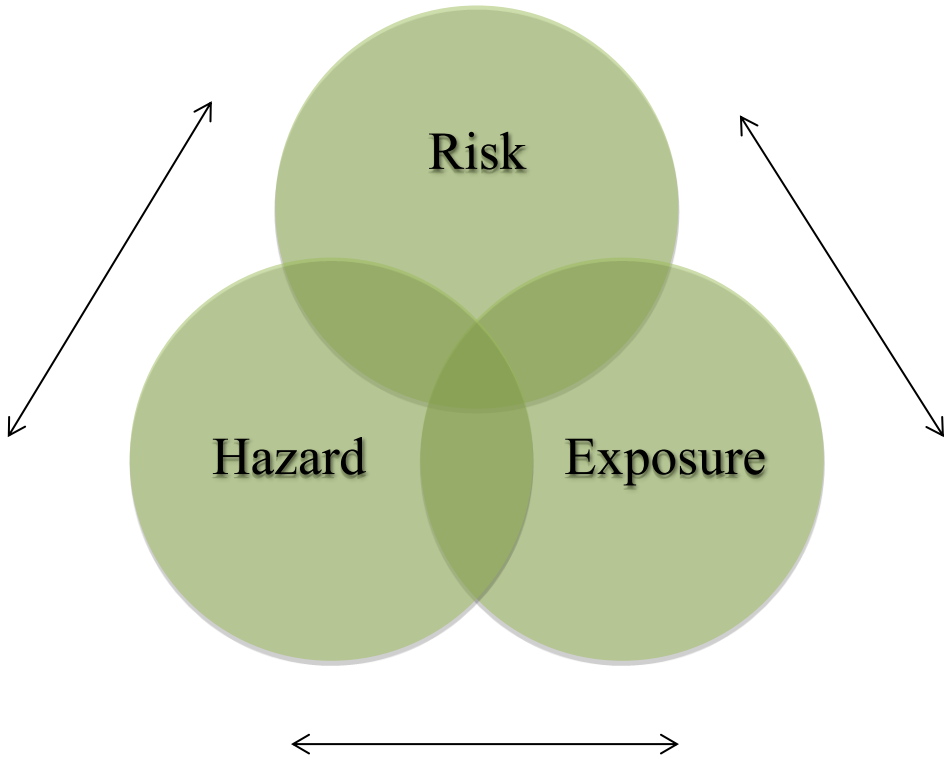

Figure 3: Epidemiologic Triad - host plus environment plus infectious agent (design by Coufalová, 2016), according Capinera (2010).

For determination of risk exist Regulatory committee's experts, necessary to advising government minister who make law decisions. It is assessing whether particular risk is acceptable or not which is very important, because it is continuously giving advice to consumers. Contaminants are set by governments and international bodies (*Codex Alimentarius*) to minimize exposure food contamination of hazards and ensure One Health sustainability (Shawn, 2015).

Food-borne diseases are main public health problem. They consider morbidity, disability, long term consequences and even mortality. There are four billion causes of diarrhea annually. Causes of Food-borne diseases are pathogenic bacteria, parasites, viruses and chemicals (FAO, 2007).

Shaw (2015) wisely says that food, as most other thing on earth, is richly inhabited by bacteria. Every meal has its own natural flora. It can be called **natural bacterial ecology of food**. The vast majority of bacteria are beneficial. Only very small of bacterial species or strains are harmful–cause illnesses. The bacteria, that cause disease is termed pathogenic. Bacteria are the major cause of Food-borne diseases worldwide (except single viral pathogen norovirus). Food-borne illness causes by bacteria starts from mild gastroenteritis to life-threatening diseases. As a following (and for this part of microbiological world, the most important bacterial pathogens) will be some of them pointed out, but it is good to know, that there are much more of bacterial pathogens. The biggest importance has *Esherichia coli, Esherichia coli O157:H7* as a typical food-borne illness example of genus *Salmonella* and *Staphylococcus*.

1.4.2. Bacteria

Domain Bacteria were for first seen by Antonie van Leeuwenhoek, thanks to his microscope. Bacteria are prokaryotes-without nuclei. They are evolutionary success-constantly evolving into strains and species which have been known for long time as a Charles Darwin's concept of 'survival of the fittest'. For bacteria is very suitable ecological niche inside humans and throughout food its simple way how to reach it. Because of these reasons there arises new and new food borne pathogens nowadays and constantly (Shawn, 2015). Classification of bacteria may be done from many points of views. Into most important division is counted inter alia classification according to their shape. There are

three main shapes: sphere shape in cocci bacteria, rod shape in bacillus bacteria and spiral shape in vibrio, spirillum and spirochete which is evident in Figure 4.

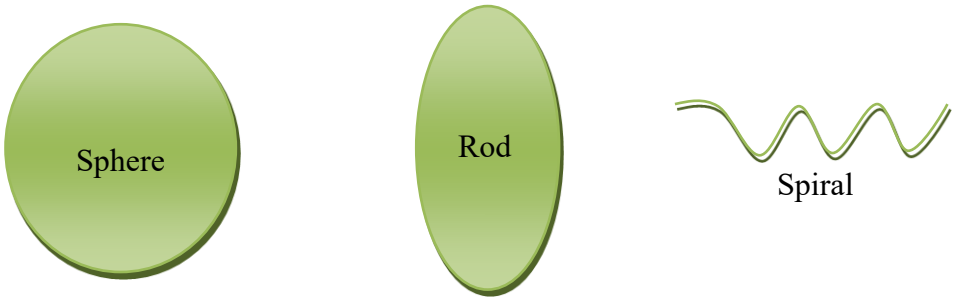

Figure 4: Basic shape classification of bacteria (design by Coufalová, 2016; according to Shaw 2015).

As it is important to divide bacteria there exist methods for basic analysis of bacterial morphology, thus staining of bacteria by Gram can be found in norm ČSN EN ISO 7218.

The difference between Gram positive and Gram negative bacteria is through cell membranes (cell walls). Gram positive can hold color of crystal violet and this ability is not present in Gram negative. The difference is shown in Figure 5.

Gram negative bacteria have two membranes and one cell wall; Enterobacteriaceae e.g. *Salmonella* spp. will stain pink/ red because fuchsine, a component of Gram's stain, is soluble in outer bacterial membrane of Gram negative bacteria.

Gram positive bacteria have one membrane (don't have outer membrane) and one cell wall; e.g. *Streptococcus mutans* will stain purple/ blue because the crystal violet in the Gram's stain binds to the outer polysaccharide cell wall of Gram positive bacteria. Everything is in around 10 μm (Shaw, 2015; Lamont et. al., 2013).

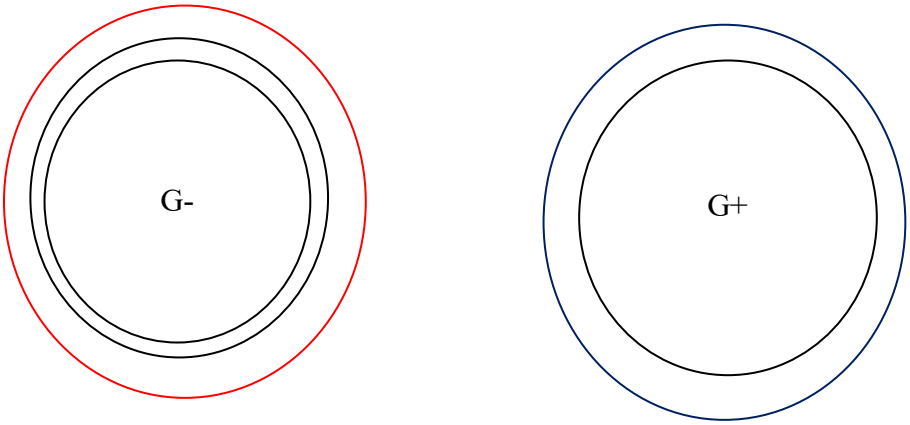

Figure 5: The differences in cell membrane/ cell wall organization of Gram positive and Gram negative bacteria (design by Coufalová, 2016; according to Shaw 2015 and Lamont et. al., 2013).

Viruses, like bacteria, are everywhere but they need to have the host cell for multiplying. Viruses infect only specific species cells and often specific cell types within the species. Specialty of viruses is determined by proteins protruding from capsule which bind with specific proteins on host cell (Shawn, 2015) mentions also history of viruses. Luis Pasteur and virus of rabies is well known historical event. Chamberlain-Pasteur filter had shown that here is even smaller organism than bacteria. But there are very few viruses associated with food-borne diseases. But norovirus, like with irony, cause most of the world Food-borne diseases. Its course is mild but has economic impact.

Viruses contained only nucleic acid proteins and sometimes lipids and only with it can alert host cell's biochemistry (synthetic apparatus) to replicate their own structures. Nowadays the most ringing problems are Ebola and AIDS viruses. At least viruses can be transmitted only by direct contact, because they cannot survive long without host cell (Shawn, 2015).

Parasites are animals or plants, which are taking and utilize nutrients from host and gives nothing in return. Importance has that part of animal kingdom, where are parasites, that have human host and are transmitted via food. Food-borne parasites are all zoonoses and common all over the world. All parasites are killed by sufficiently high temperature-cooking. Only problem is with raw and undercooked food. Parasites are more common in

developing countries, because of poor water source and non-sufficient food hygiene. Parasites range from protozoa (single celled) to tapeworms (complex creatures). They are ranked according to lifestyle, not phylogenetic point of view. In developed word is common parasite *Toxoplasma gondii* (Shawn, 2015).

Fungi grow as saprophytes, parasites, or both by using specific proteolytic, glycolytic, or lipolytic enzymes to extracellularly break down substrates and to absorb the products of digestion through the fungal cell envelope (Cole at al., 1996).

World Health Organization (WHO, 2015) prepares five keys for safer food for developing countries as well as for so called developed countries. Aim is maintain accurate hygiene. These keys are: Keep clean, Separate raw and cooked, Cook thoroughly, Keep food at safe temperatures and Use safe water and raw materials.

1.4.3. Enterobacteriaceae

Enterobacteriaceae is one of the most important families of microorganisms. Gram negative rods live predominately in the intestine. Enterobacteriaceae is family of many genera and species, which patogenity is widely scaled. They are facultative aerobic, fermenting glucose and others substrates. Card of Enterobacteriaceae is usually by stool sample as described Votava (2010).

Escherichia is big genus of bacteria and *Escherichia coli* is a species of Gram negative, non- spore forming, motile (flagellate), rod-shaped bacteria. It is naturally gut micro flora member of mammals (e.g. synthesis of vit K) and it is the most common bacteria species (Shawn, 2015). Thus native *E. coli* is not pathogenic, but *E. coli* conjugate with others bacteria, then it is able to acquire toxin, which is coding plasma and the new *E.coli* strain is able make further synthesis of toxins (*E. coli* plus *Shigella* conjugation–cause dysentery–*E. coli* 0157: H7) (Morgan, 2011). The types of *E. coli* that can cause diarrhea can be transmitted through contaminated water or food, or through contact with animals or persons. *E. coli* consists of a diverse group of bacteria. Pathogenic *E. coli* strains are categorized into pathotypes, but it can be said that pathotypes *E. coli* are only small group (CDC, 2015).

E. coli 0157: H7 lives in intestine of farm animals (e. g. cattle, lamb, venison, pork), thus is possible, that their meat will be contaminated by bacterium. Thoroughly cooked meat therefore kills bacteria, because they are only on surface (both-sides). But exception is minced meat, where is "pink middle of hamburger" and any other food that goes into contact with *E. coli* brings potential risk cause of *E. coli* 0157: H7 food-borne illness-unpasteurized milk, vegetables, manured-base fertilizers (Shawn, 2015).

Shawn, 2015 point out also symptoms of *E. coli* 0157: H7, which are infecting bloody diarrhea, abdominal pain, tenderness, but without fever. It begins one to ten days after ingestion of *E. coli* contaminated food (time depends on bacterial contamination level infected intestinal epithelium: speed, onset, toxin production). Usually, there is no lasting effect, but if more serious disease will develop, it can have cell-toxic effect, which can cause necrosis and infections and can be fatal. In case of Shigella like toxin (SLT) it can be involved to circulatory system. *E. coli* can be transmitted from person to person and by cross contamination in the kitchen.

Salmonella spp. are rod-shaped, Gram negative, flagellate bacteria that do not form spores. *S. enterica* is normally present in the intestines of cattle and poultry, but if it is present in egg, or meat is the cause of *Salmonella* spp. food-borne illness. The highest risk of outbreak of salmonellosis can be found in poultry meat. *S. enterica* can infect egg whites and later egg yolk, which is important, because egg yolk is commonly used in a raw in some foods (mayonnaise), or it is consumed as a "runny" (i.e. uncooked) yolks. *S. enterica* is not killed by freezing, but it is destroyed by cooking: $x \geq 60$ °C for 2-6 minutes (Shawn, 2015).

Shawn (2015) presents also basic symptoms of salmonellosis, which are *diarrhoea*, vomiting and fever. They begin 8 hours to 3 days after infection. The time from infection to symptoms depends on the number of bacteria contaminating the food. The illness usually lasts for around 5 days and most people recover completely. *Salmonella* spp. can be dangerous in the case of immune-compromised people (e.g. AIDS sufferers). In other rare cases *Salmonella* spp. can be cause of inflammations connected with arthritis.

1.4.4. Food Surveillance

Food surveillance is the most precise and continuous way to determine food residue intake. Food surveillance is policing correct processing of food in many ways. It is long-term rolling program of analysis of particular residues. Food laws are accompanied with food policy to check complying of producers with laws. Food surveillance is the most common tool of food policing (Shawn, 2015). If there exist unacceptable residues or microbiological counts there are many ways how food laws can be policed and sanctioned (Kirchsteiger-Meier, 2014).

It can be found many ways of infection causes by microorganisms. Microbial surveillance is science which operates with microbial causes of infection. Infections may be caused by bacteria (including mycobacteria, chlamydiae, mycoplasmas and rickettsiae), viruses, fungi, or parasites. Infection may be divided to endogenous or exogenous. In endogenous infections, the microorganism (usually a bacterium) is a component of the patient and his indigenous flora. Endogenous infections can occur when the microorganism is aspirated from the upper to the lower respiratory tract or when it penetrates the skin or mucosal barrier and as a result of trauma or surgery. In contrast, in exogenous infections, the microorganism is acquired from the environment (e.g., from medium, water, animal and other person) or from another person or an animal (Cole at al., 1996).

It is very important to know behavior of microorganisms, because nearly all acute food-related diseases are caused by bacteria or viruses. Microbial surveillance involved sampling and cultivating of pathogens (Shawn, 2015).

Specimens for examination are selected on the basis of signs and symptoms, should be representative of the disease process, and should be collected before administration of antimicrobial agents. The specimen amount and the rapidity of transport to the laboratory influence the test results.

For examination are created so called microbial criteria which are there for stipulation of type of microorganism, group of microorganism, or toxin production by microorganism and microorganism must either be not present at all, be present only a limited number of samples, or be present as less than specified number or amount in given quantity of a food or food ingredient (Cole at al., 1996). This is included in norm ČSN 56 9609 with parasites, toxins or metabolites per units of weight, volume, flat or a production batch.

For insects safety, from negative point of view, is in many literatures, used term alien substance for insects in the food, which can be organic or inorganic. Alien substances have many ways how to entrance food chains, it can come from many different infectious agents, mainly contaminated water, fallout from polluted air, from medium, like consequence of used agrochemicals, during technology processing of raw resources for production of food, their package and transportation (Kudlová, 2009).

According to FAO (2013) allergies are an increasing problem in Western populations, contrary to developing countries where their prevalence is far lower. There is logically higher risk of contamination of pesticides in areas of intensive production (Paoletti, 2005). The hygiene hypothesis states that the high prevalence of allergies in Western populations is induced by a lack of exposure to pathogens, including intestinal parasites, and to increased vaccination practices during childhood.

The consequences for the pathogenesis of asthma and allergies following increased consumption of chitin through the promotion of insects as food are unpredictable, noted by FAO (2013). Hypersensitivity on insects with general symptoms is seen more often, but cause can be latent (Fialová 2011). However, if allergies are catalyzed by a lack of exposure to chitinous substances in childhood, as suggested, increasing the consumption of insects in early childhood could, by extension, support better protection against allergies later in life (FAO, 2013).

1.4.5. Food Preserve

The author Shaw (2012) warns that protection against undesirable microbes is crucial. Food risk has to be managed because it is simply not acceptable for people to die as a result of eating. There exist food standards determined by *Codex Alimenarius* Comission (in cooperation with FAO/ WHO/ OIE).

Louis Pasteur and Joseph Lister are two men good to be mentioned according to improvements of food safety throughout history. First man showed that high temperature for short time can kill undesirable microorganism in food and second followed him with antiseptic postoperative–infection and disinfectants which are still, in different structures, used as a killers of many food-borne diseases. In these days are for microbiological pre-surveillance/ prevention frequently used refrigerators and chemical preservatives (Shaw, 2012).

Properly managed insect farm could remain free from pathogens by avoiding contact with wild insects (Templeton, 2006; Hazeleger, 2008) and other sources of contamination. Sugar has been traditional preserve for long time. Nowadays is also used food irradiation, which discovered Wilhelm Rontgen. These X-rays are form of electromagnetic radiation like gamma rays, which kills also parasites. Later, Henri Becquerel discovered radioactivity, which is connected with husbands Curie's (Shaw, 2012). Klundera (2012) highlights drying or acidification of insects, Fialová (20011) inclines to lyofilization, which is way of 100 % preserve of nutrients. Bednářová (2013) mentioned modern form of capsules. Researchers from the University of Khon Kaen in Thailand reported that microbiological tests showed that the shelf life of crickets boiled for 10 minutes and then packed with air and kept at 0 °C was 30 days. The shelf life was extended beyond 30 days when the crickets were packed under vacuum at 0 °C (EFSA, 2015).

1.5. Legislation

In European Union, there are four countries, which performed risks assessment related to Insects as a food or a feed. These countries are Belgium, France, Iceland and Netherlands (EFSA, 2015). The goal of the European Food Safety Authority (EFSA) is to provide the EU institutions as well as Member States with independent scientific advice and support in questions of food safety. The EFSA is occupied with any topics having direct or indirect effect on the safety of food, including question with connection with animal health and animal protection (Kirchsteiger-Meier, 2014). Previously there were logically no limitations in legislation, which defend selling bugs as a food. Nowadays The European Commission (EC) asked the European Food Safety Authority (EFSA) to assess the microbiological, chemical and environmental risks arising from the production and consumption of insects as food and feed with aim to cover the main steps from the production chain up to consumption by pets, food producing animals and humans. EFSA was requested to provide an overall conclusion based on the above assessment, on the potential risks posed by the use of insects in food and feed (EFSA, 2015).

Thus nowadays new scientific opinion on insects arises in EFSA Journal (EFSA, 2015). Insects with comparison to the non-processed sources of protein of animal origin and its occurrence of hazards are comparable; because of currently allowed feed materials which are used as substrate to feed insects and other non-processed sources of protein of animal origin as well. The possibility of occurrence of microbiological hazards is expected.

Substrates like kitchen waste, human and animal manure are also considered as hazards. For insects fed on these substrates is thus need of these hazards to be specifically assessed. The substrates that will be included in the production will depend on the legislative framework-including availability, the applicability in the specific farming system and the cost. Due to the different requirements, the substrate preference will differ also among the different insect's species. The specific production methods, the substrate used, the stage of harvest, the insect's species and developmental stage, as well as the methods for further processing will all have an impact on the occurrence and levels of biological and chemical contaminants in food and feed products derived from insects. Hazards according to EFSA (2015) are related to the environment are expected to be comparable to other animal production systems. The opinion also identifies the uncertainties (lack of knowledge) related to possible hazards when insects are used as food and feed and notes that there are no enough systematically collected data on animal and human consumption of insects (EFSA, 2015).

Even if the intestine is emptied before harvesting, frass will remain in the substrate and can contaminate the insects. Some of the microbiota may become pathogenic to the insects under stress circumstances. Also, like other animals, insects will have a microbiota on their surface and some of these are pathogenic to insects. The questions here are whether any of these microbes, including viruses, are pathogenic to other than insects or to humans and animals and, if so, whether they could be transferred through food and feed containing insects or products thereof (EFSA, 2015). FAO (2013) explained terms microbial flora of insects which is composed of bacteria of different genera: *Staphylococcus, Streptococcus, Bacillus, Proteus, Pseudomonas, Escherichia, Micrococcus, Lactobacillus and Acinetobacter.* Pathogenic bacteria of insects (entomopathogenic) are regarded as a harmless to animals and humans, because the hosts are so phylogenetically different (FAO, 2013). Opperhuizer (2014) add, that only 15% of the coliform bacterias (mainy *E. coli*) in insects are comparable to E. coli in mamals and birds. The same organization shows, that specific studies on the microbiological safety of insects specifically reared or harvested for food or feed production are rare in the scientific literature so further research for better assessment of microbiological and chemical risks from insects as food and feed including studies on the occurrence of hazards when using particular substrates, like food waste and manure is recommended (FAO, 2013).

Antimicrobial resistance is a natural and widespread phenomenon, which is amplified by the use of antibiotics in all the sectors involved: human medicine, agriculture, animal husbandry. All organisms that can cause disease in humans and/or animals (bacteria, viruses, fungi, parasites) reveal a remarkable ability to adapt, to evolve and to survive by developing resistance to each and every therapeutic compound administered (to all classes of drugs). A recent and comprehensive report by ECDC, EFSA and EMA (2015) has been set. In insects farming, the use of antimicrobials is reported in the scientific literature for emergency treatment in case of diseases caused by bacteria, fungi or microsporidia which can seriously damage farmed insects. Finally, some authors report the use of antimicrobials in order to accelerate nymphal development, increase survival and adult longevity in insects, concluding that streptomycin has potential in rearing *N. viridula*, especially in improving quality of field-collected adults, by mitigating the introduction of pathogenic bacteria, and improving the quality of the population. As a concept of HACCP exist Food Surveillance schemes, where random samples of food and products are examined at the point of sale or from the farm (CCP), which is in connection with ČSN 56 9609. HACCP analyzed for residues of chemicals, physical, allergic (proteins caused) or biological (microbiological) content. Followed process is set to maintain Food Safety. In first step hazards are analyzed, then are determined critical control points; after which follows establishing of limits for critical control points. Establish monitoring procedures for critical control points is next step and establish corrective actions go after. At the end is established verification procedures and record system.

If levels exceed the limits, court action might be taken. Thus first method is Random sample and second method is Total diet surveys (TDSs) samples, where duplicate of meal consumers eat are send to laboratory. This examination last for longer time. And third, Enforcement sampling, is taken when there is concern that food safety problem already exists and is immediate process. For example, if *Listeria monocytogenes* is found, the regulatory authority might close the restaurant and withdraw food from shops. National surveillance schemes (NSSs), within country policing schemes, involved samplings usually random and secret, which are the most suitable way for public health. Compliance with the legislation is policed by surveillance schemes, in which food samples are taken and analyzed for residues, microbiological contaminants and additives (Shawn, 2015). Direct examination of microorganisms is basic, but for disproving of possibility of epidemiology agent is necessary, also by law, to detect exact species (Votava, 2010).

This concept of HACCP is connected with GMP (good manufacture practice), where is included food safety plus clean water, good products and quality. GMP was established by WHO.

Shawn (2015) alert that safe to eat means farm in such a way that contamination with harmful bacteria, viruses, parasites, prions and toxic chemicals is as low as possible. Minimum risks means to produce as safe as to cause no harm for consumer. For maintenance contaminants hazards under border of harm are set limits by governments and international bodies (*Codex Alimentarius*). Laws set levels and all producers and manufacturers, retailers must comply with them. All developed countries have food legislation, but some developing countries have no effective or poorly effective food legislation. In developing countries it is necessary to solve the food security problem first, than food safety.

There are also international food laws, which ensure import and export food safety. Food laws are accompanied with food policy to check complying of producers with laws. Food surveillance is the most common of food policing. When food is imported from different country it should be assumed, if the country has sufficient food laws and efficient surveillance schemes (Shawn, 2015).

There are similarities throughout the world in case of safe food legislation. General principles are international. The USA has federal food agency (FDA, 2014)-*US Codes* - *"Levels of natural or unavoidable defects in foods that present no health hazards for humans."*

Nowadays FDA's response to inquiry to be pushed to create at least some basic regulation and thus was created FDA Guidance on edible insects as Foods (FDA, 2016).

For closer detail there is e.g. United Kingdom parliament, which has first discussion and its results and proposal are dent to Congress, which set rules. Second discussion is out of parliament-Committee deliberation, which can last for months. Then there is voting and Act is send to monarch. When once an Act is on the statutes, e.g. Food Standards Agency in the UK, and can prosecute people who infringe what is written down. Courts determine whether is guilty or not, or how to be penalted (fines, or prison) under *Food Act*. New Zealand Food Act is based on similar principles. If production or store is not done properly, consequences may be great (Shawn, 2015).

Legislation in EU can be best explained by Tim Corrigan from WTO (2016) and Karpíšková (2016):

"This topic has yet to be addressed within the work of the Committee on Sanitary and Phytosanitary (SPS) Measures but I would not be surprised if it would be in the not-so-distant future. The issue of novel foods and accessing certain markets has come up in the Committee before and is a recurring discussion, but has never explicitly referred to entomophagy".

The EU classifies the presence of visible insects larvae in food as "foreign bodies," as reported in the RASFF (Rapid Alert System for Food and Feed) database. According to EFSA (2015) safety aspects of insects that are bred for feed or the human consumption (fractions as a protein preparations or other extracts obtained from insects) are included in regulatory literature of Belgium, but insects harvested in the wild are not considered. Other Countries, which have at least some legislation about treatment of insects for human consumption, are France, Iceland and Netherlands. Other countries replied that they have not performed a risk assessment on insects: Austria, Bulgaria, Croatia, Cyprus, Czech Republic, Estonia, Finland, Greece, Latvia, Lithuania, Luxembourg, Norway, Poland, Portugal, Hungary, Slovak Republic, Slovenia, Switzerland and United Kingdom.

In the Czech Republic and in the others countries, there exist three very important norms, which should be mentioned and remembered. First is Act on Food and Tobacco Products number 110/1999 Sb. Second is number 274/2003 amending law about Public Health. And last is regulation number 287/1999 Sb., about veterinarian demands on product of animals (Kudlová, 2009).

Neither in EU, nor in the Czech Republic still does not exist legislative rules for using insects as a food (Bednářová, 2013). However, there is expectation of change. Nowadays is insects perceived as big advantage and fact and there is big discussion in EU for arise of legislation Czech republic was initiative country for eating insects but in paradox, is not first land which allows us to do entomophagy as a daily ad normal habit (Festival „Do Zobáku", 2014). Maximum of the residuals from insects, which can cause illness are discussing in London, where is also placed question: Should be insects termed as a food in law? Next question was trade with insects, which is also not allowed yet (Festival „Do Zobáku", 2014). In 2008 was in Mendel University, Brno discussed question of entomophagy and thanks to this event were also initiated and suggested, with help of prof.

MVDr. Jiří Ruprich Ph.D., species of bugs suitable for consummation for human in EU (Bednářová, 2013). There is expectation for insects to be allowed to be manipulated and eat soon and in all ways in the Czech Republic. There was held conference „Insects to Feed the World" at Wageningen University, which cooperated with Ede city (Germany), with help of FAO, which discussed further about entomophagy (Festival „Do Zobáku", 2014).

For correct run of industrial system, there exist European Parliament's and council's regulations. In regulation (ES) number 853/2004 of April 2004, there are sets specifics hygiene rules for food of animal's origin, which was later completed by regulation (ES) number 219/2009. European Parliament and council mandate norm (ES) 852/2004 about hygiene of food and prevention of contamination caused by animals and pests, that regulation of these animals is one of the most important and basic duty for operator of food company (Bednářová, 2013). QH91146 Comprihesive method of protection of stored grain and milled cereals products against rodenst and insects with the employment of authomatic processes (NIPH). In the EU, insects hasn't been accepted yet. Reports can be found in the RASFF (Rapid Alert System for Food and Feed 2012) database, where insects is classified mainly as unauthorized novel food ingredient in food supplement (Belluco et al., 2013). In extension, it is determined basic hygienic demands in all aspects food industry from outside areas and equipment to personal hygiene of employees. In addition from grocery point of view as such–preparation, cooking, storage and transporting must be done in correct hygienic conditions and in right temperature. Food Business Operator (FBO) must maintain processes to be sustainable in bases with Hazard Analysis and Critical Control Points (HACCP) rules. Food companies should also follow regulation (ES) number 178/2002 (2014), where are sets main points of correct producing.

Detailed regulations regarding the establishment of personal insects farm or company is recommends in work of Janíček (2013). It's necessary to know that delivery or sale of dangerous food is an offense–EU–General Food Regulations (EU, 2014).

As it was said on the very beginning there arises important law Commission Regulation (EC) number 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs with appendix: Commission Regulation (EC) number 1441/2007 of 5 December 2007 amending Regulation (EC) number 2073/2005 on microbiological criteria for foodstuffs (Kouřimská, 2015). In regulation ES n. 1881/2006 are listed maxima of limits of the contaminants for the most risky alien substances in some food.

Under United Nations (OSN) was issued tract, where are set limits of natural or inevitable deficiencies in food for human consummation. It was done by department for pharmaceuticals and food „Food and Drug Administration“. Limits for insects are set in brochure „Levels of food disorders”. Further we can find here also rodents, hair or fungi (FDA, 2014). For example in peanut butter (per 100 g), it is normal state to be of 30 fragments of insects, in the same volume it is count like normal 2 500 aphids for 10 g hop cones and in canned tomatoes for 500 g up to 10 eggs of flies or 5 eggs and one worm.

Insects in the Czech Republic are still seen like pests more than food. There is regulation (CS) number 356/2008 sb. In this document can be found in annex number 3 storehouse pests with figures.

In case of insects according to Bednářová (2013) will occur a need of security especially in case of insect’s usage in a logical economical clever circle as a inverter of energy of biomass or waste to be further used like a food.

Ministry of Agriculture of the Czech Republic has already prepared Regulation of the European Parliament and the Council 2015/2283 about new food, which will come into force after 1. 1. 2018 (Ministry of Agriculture of the Czech Republic).

2. AIM OF THE THESIS

Diploma thesis was focused on microbiological agents (family Enterobacteriaceae, *Salmonella* spp. and *Escherichia coli*) of given specimen of insects (*Tenebrio molitor, Zophobas morio, Gryllus assimillis*).

The aim of this thesis was:

1) to give an overview on norms for microbiological examination in insects species

2) to examine microbiology of tree given specimens *(Gryllus assimillis, Tenebrio molitor, Zophobas morio)* from three different distributors with the focus on family Enterobacteriaceae (genes *Salmonella* spp*., E. coli*)

3) to find out whether insects are no more dangerous than meat products as alternative source of protein

To fulfill the targets were formed two hypotheses:

H1 In raw insect (concretely *Zophobas morio*) there will be higher number of CFU *(E. coli)* than in boiled insect.

H2 Content of *E. coli* in in insects (concretely *Zophobas morio)* will be comparable to content of *E. coli* in other meat products when the same treatment will be used (insects are no more dangerous from biosecurity point of view).

3. MATERIAL AND METHODS

3.1. Information Sources

There were collected information from primary sources as a Web of Science, Thomson Reuters, database Scopus and Ebrary. In bachelor study were worked with 67 citations and in diploma thesis with 54 citations plus norms and others. Next were addressed groups of people, who are specialists in this study problematics. The main organizations questioned were FAO, WHO, and Ministry of Agriculture of the Czech Republic. All of which have sent me EFSA and other recent documents (see chapter References).

The study was following bachelor thesis (Coufalová, 2014), where can be found questionnaires, with most important question about willingness to eat insects in the future throughout the generations. The main result from bachelor study was confirmation that children are much more willing to eat insects in the future than older people. It means that in the future we can expect eating of insects on daily bases. Next result was that respondents prefer rather salty taste of insects, than sweet. In case of nutritional value, people were not informed (about 70% of respondents selected not correct values of insect qualities). Partial results were besides, that respondent's the most preferred insects from pictures of questionnaire was from order Orthoptera–crickets, which was one of the reasons, why was cricket included in master thesis (Coufalová, 2014). Mealworms were selected in case of simple preparation and easy breeding. This species of insects from order Coleoptera, were the best valued by respondents in case of study of Bednářová (2013). Studies about chemistry and nutrients in insets had been examined, thus it had been chosen to work with other important criterion of eating insects–microbiological point of view.

Logically it was followed through collection of information about microbiology risks which can occur by consummation of insects. The next information were collected during spring (2015) in order to evaluate how insects could be safely used as a food and to discuss nutritional data to justify, why insects food sources can no longer be neglected, even there is a potential risk of transfer of diseases.

3.2. Research Study

The inherent research study was realized in field conditions by collecting of insects. The diploma thesis was done in Prague and also in Brno, because these localities are the most frequented in case of distribution of insects to zoos. The samples of insects were taken during 2015-2016 from each distributor, with subsequent laboratory examination at State Veterinary Institute, Prague (SVI Prague in the part of laboratories of Food Hygiene (Hygiena Potravin, in Czech) at the Department of microbiology, with help of professionals-trained specialists.

3.2.1. Usage of Invertebrates

In the experiment were used three species of insect: *Gryllus assimillis "*crickets"*, Tenebrio molitor* "smaller mealworm" and *Zophobas morio* "bigger mealworm", which were chosen from the representative distributors in Prague (Pet Center, Happy Zoo and Super Zoo), where is offered as a feed stuff. These species (see Figure 6) were evaluated as a very popular for consumers, which can be found in previous studies (Coufalová, 2014).

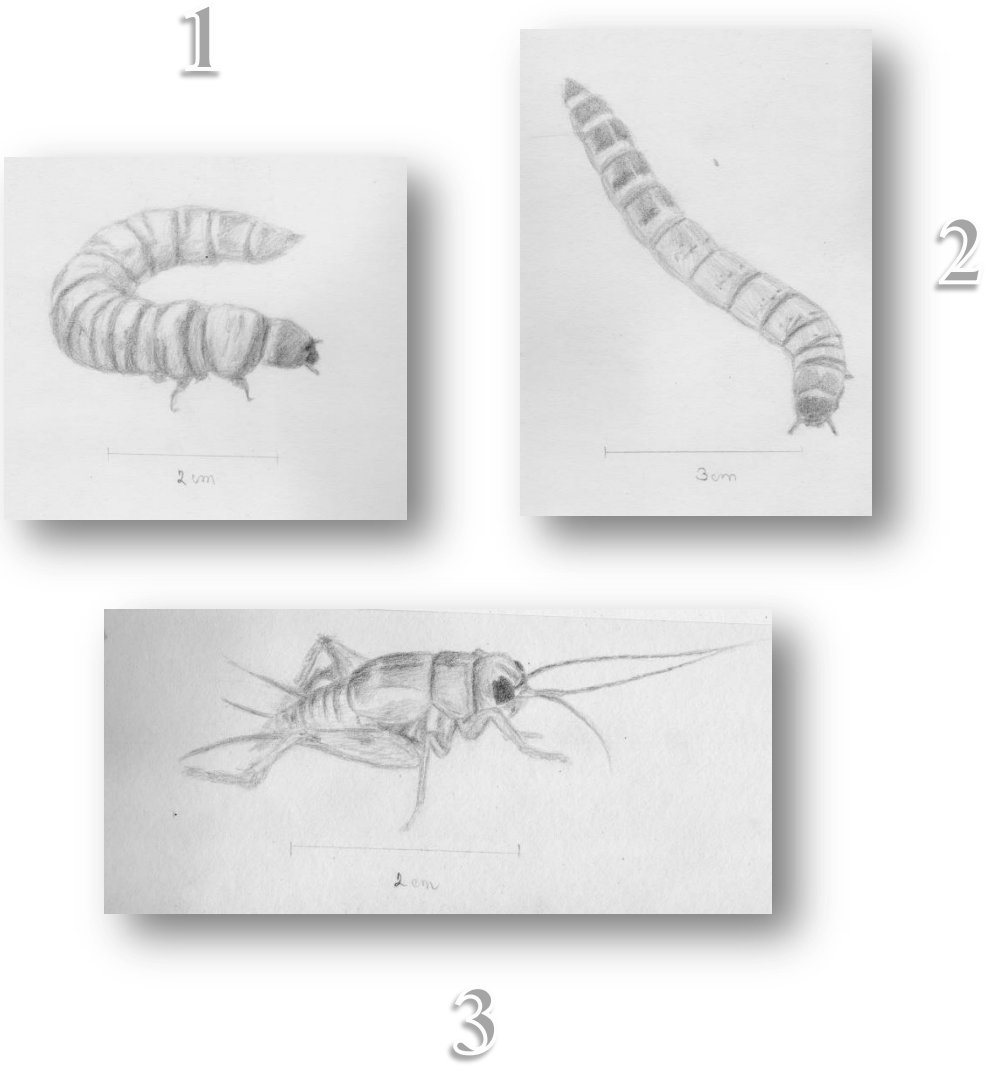

Figure 6: Three chosen species of class Insecta. 1: *Tenebrio molitor* "smaller mealworm". 2: *Zophobas morio* "bigger mealworm". 3: *Gryllus assimillis "*cricket" (design: Coufalová, 2014)

3.2.2. Sampling and Transport

By consumers best-rated species was mealworm (*Tenebrio molitor*), that is reason why it was chosen for our examination. According Oonincx (2014) and Bednářová (2013), mealworm is the most promising of the class Insecta, due its modesty (nowadays, economic it is based on modesty, together with practicality). Environmental impact of mealworms compared to other animal products offer the lowest risk (Coufalová, 2014). Mealworm should be considered as a more sustainable alternative to chicken, pork and beef (Oonincx, 2012; Joots van Itterbeeck 2012). Crickets were chosen according to their similar properties. They are a bit harder for managing but their taste and ratio from in scale of preferences of consumers are also high.

In the Czech Republic, there are commercially available edible insects larvae mainly of *Tenebrio molitor*, also known as so-called. "flour beetles" larvae, than *Zophobas morio*, crickets and cockroaches (Kašparová, 2009). Mealworms both species can be found in Figure 7.

Figure 7: *Tenebrio molitor* and *Zophobas morio* (bigger one); (Coufalová, 2014).

Insects were collected into sterilized jars and were immediately transported to the microbiological testing at SVI Prague. It was used three different species of insects (nine mixed samples, each by 100 g) to determine their potential hazard there. Samples of collected insects were carefully labeled (owner, number of sample, delivery date) and entered into the laboratory report and then were placed in a refrigerator (+3-5 °C). After cooling, the samples were weighed according to the type of required analysis–in our insects it was 25 g.

As mentioned above insects samples were processed at the State Veterinary Institute Prague (SVI Prague) in laboratories of Food Hygiene. Each procedure was carried out according to mandatory standard: ČSN EN ISO 7218 (2008). This standard involves examination of qualitative and quantitative, laboratory equipment and hygienic practices.

3.2.3. Microbiological Examination of Insects and Legislative Framework

For my research work at SVI Prague it was necessary to complete the basic course in microbiology and I received a certificate from the course for laboratory workers at Mendel University Brno. It was organized by the Agentura Slavíčková s.r.o. (links to annex, where can be found also basic equipment used of laboratory).

For my work in laboratory of SVI Prague, there raised a necessity, to be familiar with the Czech norms, and methodological procedures in order to proceed in the evaluation of edible insects as a food.

State Veterinary Institute (SVI) is an organization established by the Ministry of Agriculture of the Czech Republic and deals with laboratory diagnostics in the field of safety of raw materials and foods of animal and plant origin, feed and water. SVI Prague is accredited laboratory by CAI CR. Microbiological studies were done according the three Czech norms for food hygiene:

1. ČSN ISO 21528: Microbiology of food and animal feeding stuffs – Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 2: Colony count method.

2. ČSN EN ISO 6579: Microbiology of food and animal feeding stuffs – Horizontal method for detection of *Salmonella* spp.

3. ČSN ISO 16649–2: Microbiology of food and animal feeding stuffs–Horizontal methods for the detection and enumeration of ß-glucuronidase-positive *Escherichia coli* - Part 2: Colony count technique at 44 °C using 5-bromo-4-chloro-3-indoyl ß-D-glucuronide.

Further consultations relating primarily to the legislative procedures for determining the microbiological content in edible insects were consulted in cooperation with workers of Faculty of Veterinary Research Institute (VRI, Brno) and Faculty of Agrobiology, Food and Natural Resources (CULS, Prague). According to Karpíšková (2016) it is first of all necessary to mention basic European Union regulations, which are superior Czech norms. According to these three standards is possible to look at insects as it was food.

The main regulation is (EC) number 178/2002 of the European Parliament and of Council: laying down the general principles and requirements of food law, establishing the European Food of 28 January 2002. Next basic norm is norm for work with foodstuff is regulation (EC) number 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. The samples of edible insects in laboratory practice were treated as other foods that are normally investigated by the Czech technical standards. It was norm ČSN EN ISO 7218 (560103): Microbiology of food and animal feeding stuffs: General requirements and guidance for microbiological examinations of March 2008. This norm is available at workplace SVI Prague.

3.2.4. Main Devices Used in this Study

There were used two basic machines at SVI Prague: microprocessor dilutive instrument Dilumat that performs primary (ten-fold) sample dilution (calculates how much of the solution must be added to make it ten-fold dilution). In the other words, it is able to weight sample volume and make appropriate amount of peptone water (BPW), or other diluents, to form solution. Next used instrument is a homogenizer with type blades Stomacher (big mixer), where the sample should be processed for at least 1 min. Both Dilumat and Stomacher can be found in Figure 8.

Figure 8: Dilumat (right side) and Stomacher (left side); (Coufalová, 2016).

3.2.5. Culture Media Types Used in this Study

Violet red bile glucose agar (VČŽG, in Czech); medium used for family Enterobacteriaceae is seen in Figure 9. Shelf life of this agar is (with storage in cold and dry conditions) four hours.

Agar contains yeast extract, peptone, sodium chloride, bile salts, glucose, neutral red, crystal blue, and agar.

Figure 9: Agar VČŽG for identification of Enterobacteriaceae (Coufalová, 2016).

Rappaport vasiliadis (RVS, in Czech): was like medium used for detection *Salmonella spp*. in first step of examination. RVS medium is visible in Figure 10.

This medium contains peptone, magnesium chloride, sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate. In this agar is presented inhibitor with malachite green. Shelf life of this agar is (with storage in cold and dry conditions) two weeks.

XLD solid medium: was used for *Salmonella* spp. in a second step and it can be seen in Figure 11. Medium is composed of xylose, lysine, sodium deoxycholate, lactose, sucrose, sodium thiosulfate, ammonium ferric citrate, phenol red and brilliant green agar.

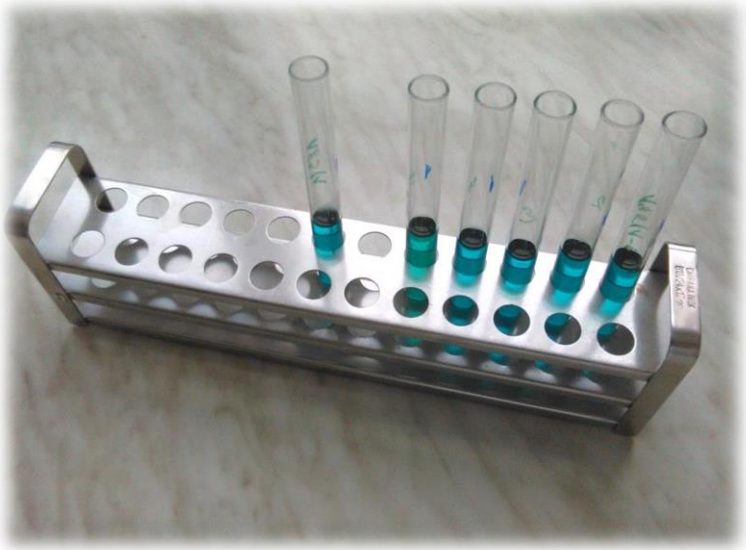

Figure 10: RVS liquid medium for detection of *Salmonella* spp. (Coufalová, 2016).

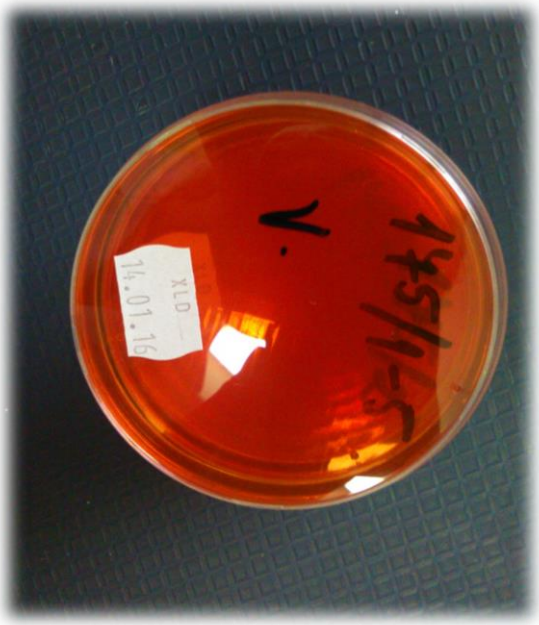

Figure 11: XLD agar for detection of *Salmonella* spp. (Coufalová, 2016).

TBX medium: was used for the detection of *Escherichia coli* and it is presented in Figure 12.

This medium contains tryptone, bile salts and glucuronide. Shelf life of this agar is (with storage in cold and dry conditions) two weeks.

Reaction is oxidatively negative, there is no cleaving of oxidase.

From this agar arise *E. coli* with characteristic green colorization.

Figure 12: TBX: medium for the detection of *Escherichia coli* (Coufalová, 2016).

3.2.6. Treatment of Insects Used in Study

There was necessity to find out potential microbiological pathogens in insects, thus examination was divided to three parts: insects with no treatment, washed insects (+10 °C) and boiled insects (+100 °C) in water bath for one minit. Boiling is better for reducing microorgainsms than roasting (Opperhuizer, 2014), so we had chosen boiling.

From every mixed sample, which had volume 100 g was weight 25 g of sample for each examination: Enterobacteriaceae (see protocol in chapter 3.2.5.), *Salmonella* spp. (see protocol in chapter 3.2.6.) and *E. coli* (see protocol in chapter 3.2.7.).

Monitoring was carried out repeatedly, in three samplings, always with above mentioned methodological procedures (3.2.5.-3.2.7.).

Altogether, in first part, were processed nine mixed samples of insects from different distributors with three methods of treatments and then were used three laboratory methods for evaluation of presence of microbiological agents. Detailed sampling is described in Figure 13.

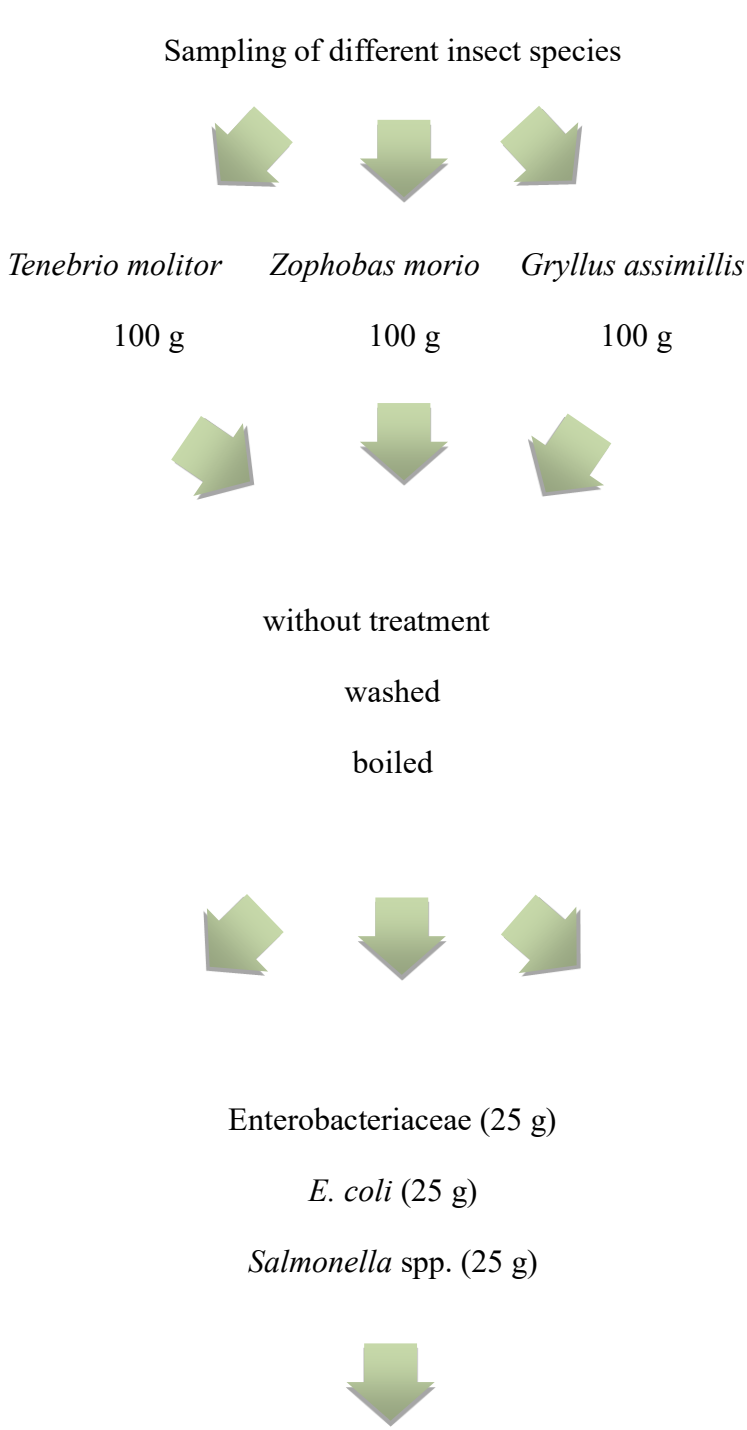

Figure 13: Sampling of three insects species

3.2.7. Detection of Colonies of Bacteria Enterobacteriaceae in Insects

For detection of microorganisms are used many methods. All of the process of counting colonies used in this study can be found in standards.

For the proof of family Enterobacteriaceae was followed ISO 21528-1; CSN EN ISO 21528-Microbiology of food and animal feeding stuffs - Horizontal methods for the detection and enumeration of Enterobacteriaceae-Part 2: Technique of the colony count (cultivation-number 334).

For the detection of bacteria of the family Enterobacteriaceae was used agar VŠŽG-violet red, bile and glucose (see Figure 10). There should not be multiplication; this test serves to certificate Enterobacteriaceae–by suffusion of medium for this family created.

Principle: For identification of the bacteria Enterobacteriaceae is known reaction called fermentation of glucose with generation of a gas.

Procedure: Detection of Enterobacteriaceae is simply described in Figure 14 under which is example of Petri dish with sample (Figure 15). The test consists of the making of initial suspension and decimal dilutions (tenfold dilution).

To the two sterile Petri dishes with a sterile pipette is transferred by 1 ml of the test sample if liquid, or in the case of other products 1 ml of the initial suspension. The *inoculum* in each Petri dish is covered with about 10 ml of medium VČŽG, cooled to +44 °C and put to +47 °C water bath. The time that elapses between inoculating Petri dishes and pouring the *inoculum* medium on it should not exceed 15 minutes. The inoculum is mixed thoroughly with making "number eight" petri dish in a horizontal position, with allowing solidifying leaving Petri dishes to a cold horizontal surface. After complete solidification of the creation of the agar complex, the medium is overlaid with about 15 ml VČŽG, cooled and then is again allowed solidify. The inoculated Petri dishes are inverted (bottom up) and incubated in a thermostat at 37 °C for 24h +/- 2h.

Characteristic of the colonies are circularity, purple- pink color, size 1-2 mm in diameter, and bacteria are surrounded by a purple halo zone or zones without precipitation.

Family: ***Enterobacteriaceae – enumeration***

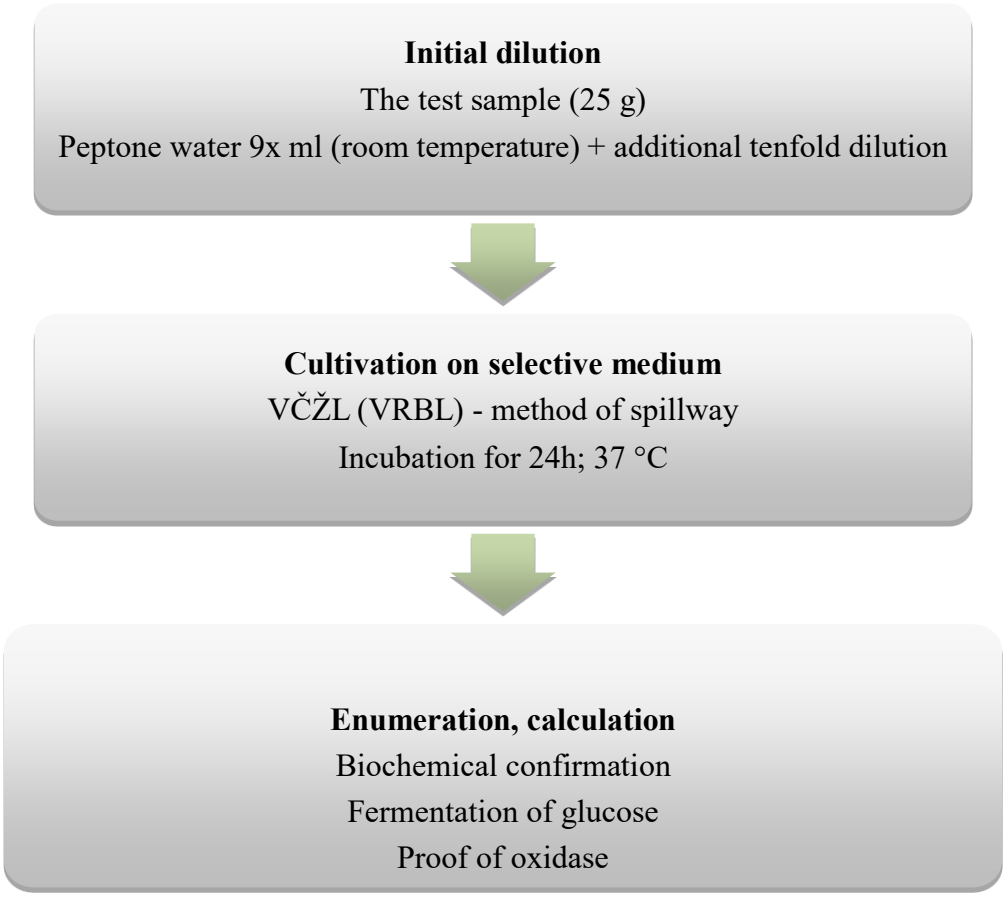

Figure 14: Detection of Enterobacteriaceae – flow chart (Coufalová, 2016)

Figure 15: Petri dish with sample: ENT = species; 1 = quantity of ml; 276 = number of sample; 2 = sequence of Petri dishes.

3.2.8. Detection of Colonies of Bacteria *Salmonella* spp. in Insects

Correct examination is ensured by standard ČSN EN ISO 6579 Microbiology of food and animal feeding stuffs-Horizontal method for the detection of *Salmonella* (cultivation-number 307).

For avian samples is used selective medium PRECIS (there is huge potential to find *Salmonella*-e.g.: melange, bombaze milk, selected dairy products). If on the agar PRECIS will grow salmonella colonies, the sample goes to bacteriology = *Salmonella*-confirmation (typization). On the other samples, with lower risk of *Salmonella* appearance is used medium XLD (see Figure 12). Process is concentrated only on multiplication if microorganisms (tenfold dilution) and there is no counts, because *Salmonella* shouldn't be present in 25 g of sample.

Principle: Reaction is fermentation of xylose, lysine decarboxylation, reduction of sulfate to hydrogen sulfide. The reaction with ammonium ferric citrate to form iron sulfide is responsible for black coloration of colonies.

Procedure: Detection of salmonella is simply described in flow chart Figure 16. Detection of *Salmonella* requires four successive stages. It starts with backfill in the homogenization bag in Dilumat-25 g for the automatic addition of peptone water. Then the bag is put into a Stomacher for homogenization and incubated in a thermostat 18 hours (+/-2 hours) under standard (around 24 hours-depending on the type of food), at +37 °C.

The second day is 0.1 ml of sample put into resuscitation media Rappaport (Rappaport Vassiliadis medium with soya-RVS-liquid blue for medium enrichment; see Figure 11). Then is transported in to thermostat at + 41.5 °C (+/- 44 °C); for 24 hours (+/- 3 hours).

The next day is done revaccination on PRECIS or XLD-24 hours (+/-3 hours), +37 °C (+/- 1 °C). Then enumeration is done.

Suspect colonies of *Salmonella* obtained by inoculation are sub cultivated and confirmed with usage of appropriate biochemical and serological tests.

Typical colonies of *Salmonella* growing on XLD agar have a black center, are almost transparent and have a pinkish color due to the color change. Details of the calculation can be found in standards.

Genus: ***Salmonella* spp. - *method of detection***

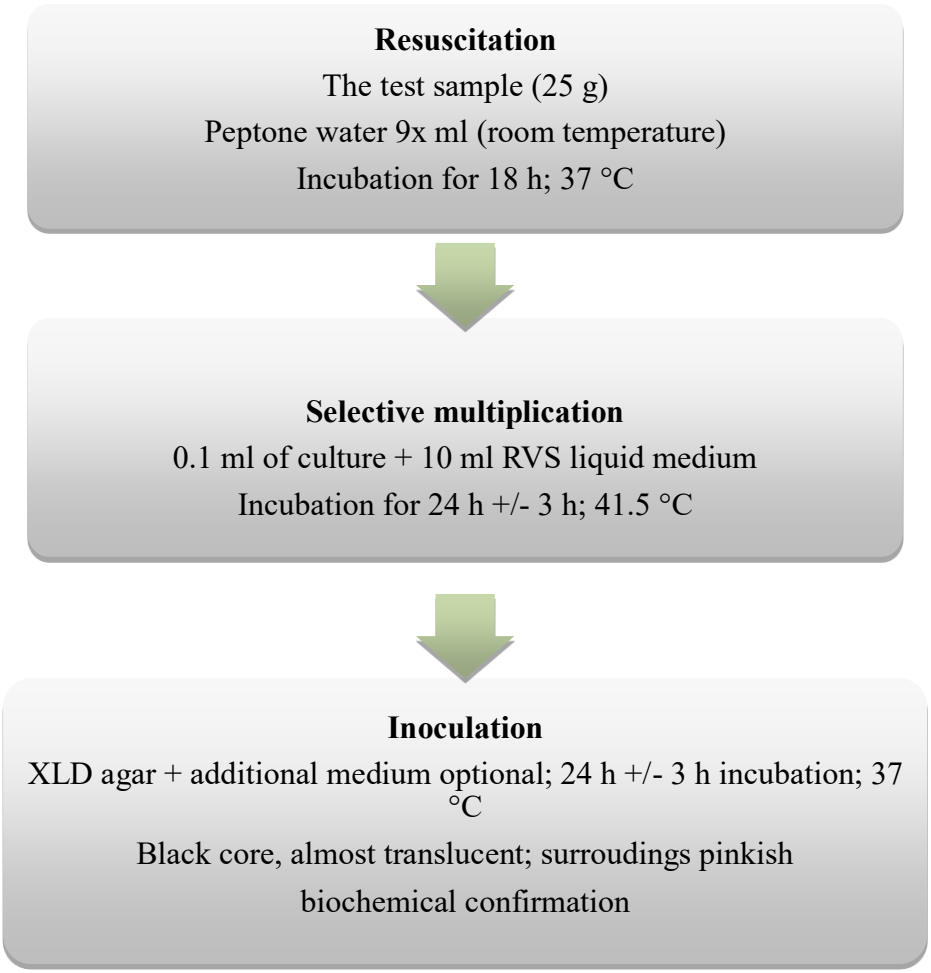

Figure 16: Detection of *Salmonella* spp. (Coufalová, 2016)

3.2.9. Detection of Colonies of Bacteria *Escherichia coli* in Insects

For the proof of *E. coli* is worked according to ISO 16649-Microbiology of food and animal feeding stuffs-Horizontal methods for the enumeration of ß-glucuronidase-positive *Escherichia coli*-Part 2: Colony count technique at +44 °C using 5-bromo-4-chloro-3-indoyl ß-D-glucuronide (no. 327).

Medium used is called TBX agar (tryptone, bile salts and glucuronide) and is chromogenous. *E. coli* is not subject of microbiological confirmation–does not continue to biochemistry.

Principle: Used is technique of counts of colonies cultivated at +44 °C in solid agar, which contains chromogenic substrate for detection of enzyme ß-glucuronidase.

Procedure: For easier view on detection of *E. coli* is applied flow chart Figure 17. Always used are simultaneously two of Petri dishes, which are inoculated with the specified volume of test solution (usually 1ml), or the designated volume of the initial suspension. The inoculum is suffused with 15 ml agar with tryptone, bile salts and glucuronide (TBX agar; see Figure 13). The time between introducing seed and potting medium should not exceed 15 minutes. Inoculated Petri dishes oriented "cap down" are placed in an incubator with the temperature maintained at +44 °C and incubated for 18h to 24h. The total incubation time should not exceed 24h. The calculation is specified in the standards.

Genus: ***Escherichia coli – enumeration***

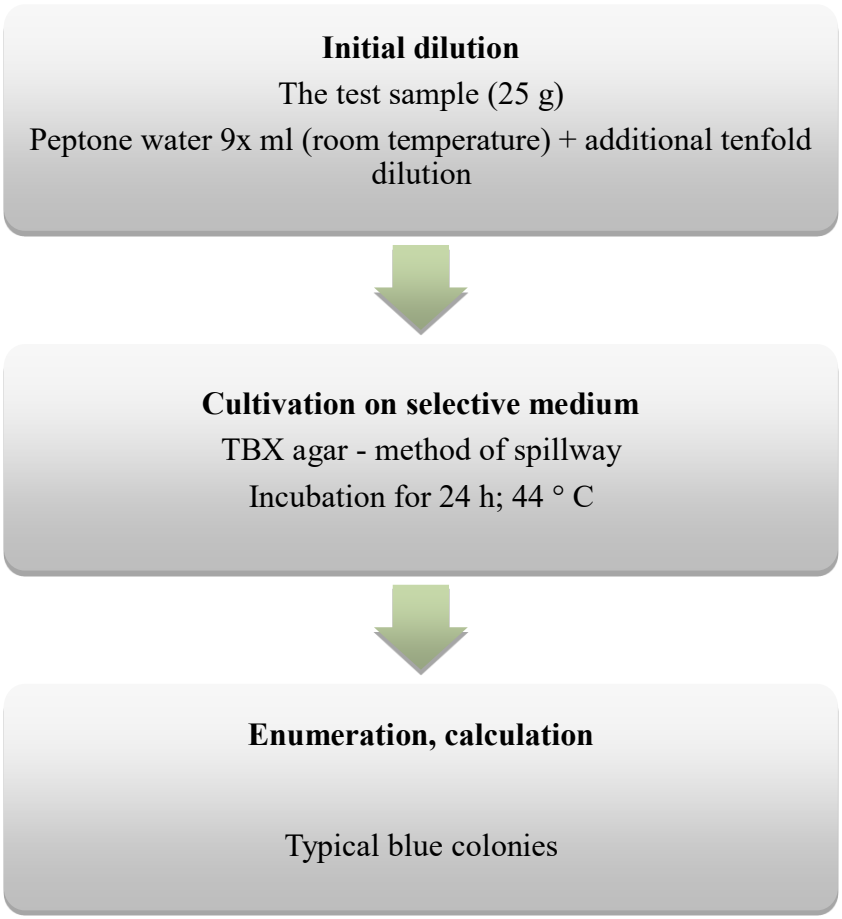

Figure 17: Detection of *Escherichia coli* – flow chart (Coufalová, 2016)

3.2.10. Comparison of *Zophobas morio* with Meat Samples (2016; SVI)

There was also interest to make comparison between raw and boiled edible insect with raw and boiled meat samples. Chosen was only *Zophobas morio* and *E. coli* in case of the most importance (Grabowski et. al. (2016). For this analysis were used data from SVI, Prague (with agreement of institute). Procedure followed norm ČSN 56 96 09: B.5.2. Tolerated values of food stuffs; individual species, groups, or subgroups, were categorized meat-or divided to in limit or out of limit following Commission Regulation (EC) No 2073/2005 of 15 November 2005. According this norm, were categorized meat samples (divided) to in limit or out of limit. **First** chosen category was raw-cooked meat products (with temperature min 70 °C, 10 minutes cooked and storage at 5 ° C. As a **second** samples were chosen raw meat products (so called semis).

3.2.11. Statistics

After the laboratory work, there were used statistical analyses in program STATISTICA 12 CZ available at http://www.statsoft.cz/podpora/ke-stazeni/trial-verze-statistica/.

In program STATISTICA 12 CZ, were examined three tests. First was concerned on comparison of number of CFU/g throughout raw and boiled insects-*Zophobas morio*. Second were task to find out whether there is significant difference in CFU/g between raw meat and chosen edible insects. The last one was between boiled meat and insects. All of these examinations, were done through Pearson's chi-squared test with elected significant level **p** was 0.05. All the results can be found in part RESULTS bellow.

First chosen category was Raw-cooked meat products (with temperature +70 °C, 10 minutes cooked and storage at +5 °C), where was, during 2016, examined 25 samples through *E. coli* and throughout these samples **no** *E. coli* was found. As a second samples were chosen raw meat products (so called semis), where were examined 1091 samples and from it were **32** out of limit.

4. RESULTS

Our results from SVI, Prague; can be found in appendices (according to Czech language used).

Aim of thesis was, in the **first part**, to examine the **CFU/g (Enterobacteriaceae, *E. coli* and *Salmonella* spp.)** in edible insects of following tree chosen specimens: ***Gryllus assimillis, Tenebrio molitor* and *Zophobas morio***; from three different distributors from Prague; with three different types of examination: **raw, boiled and washed** (see Table 1 to 3).

From tables can be seen, that the required microbiological limits (CFU/g); from norm ČSN 56 96 09: B.5.2. Tolerated values of food stuffs; individual species, groups, or subgroups, were categorized meat-or divided to in limit or out of limit following Commission Regulation (EC) No 2073/2005 of 15 November 2005; for **Enterobacteriaceae** and ***E. coli*** species **were met limits only if thermal processed-boiled** (see Table 1 and 2).

Different situation was in the case of presence of Salmonella, where all the examined samples were negative (meet the required hygienic limits according to norm mentioned above) (see tab. 3).

In the **second part**, there were chosen only **raw** and **boiled** samples and only ***Zophobas morio* and *E. coli*** (see Table 4) as a pathogen (to be statistically analyzed). In both cases (in contrast with first part), raw and boiled samples **all follow limits in norm ČSN 56 96 09**.

Table 1: The difference throughout family Enterobacteriaceae (CFU/g), between three species of insects and, in column category/evaluation, it is seen also difference between types of treatments.

Examination	**Species**	**Pathogen**	**Treatment**	**Result**	**Category**
23000	*Zophobas morio*	Enterobacteriaceae	Raw	$>1.5 * 10^6$	out of limit
23001	*Tenebrio molitor*	Enterobacteriaceae	Raw	$>1.5 * 10^6$	out of limit
23002	*Gryllus assimilis*	Enterobacteriaceae	Raw	$>1.5 * 10^6$	out of limit
801	*Zophobas morio*	Enterobacteriaceae	Washed	$>1.5 * 10^5$	out of limit
802	*Tenebrio molitor*	Enterobacteriaceae	Washed	$>1.5 * 10^5$	out of limit
803	*Gryllus assimilis*	Enterobacteriaceae	Washed	$>1.5 * 10^5$	out of limit
798	*Zophobas morio*	Enterobacteriaceae	Boiled	<10	in limit
799	*Tenebrio molitor*	Enterobacteriaceae	Boiled	<10	in limit
800	*Gryllus assimilis*	Enterobacteriaceae	Boiled	<10	in limit

Table 2: The difference throughout genus *E. coli* (CFU/g), between three species of insects and, in column category, it is seen difference between types of treatments.

Examination	**Species**	**Pathogen**	**Treatment**	**Result**	**Category**
23000	*Zophobas morio*	*E. coli*	No	$>1.5 * 10^6$	out of limit
23001	*Tenebrio molitor*	*E. coli*	No	$>1.5 * 10^6$	out of limit
23002	*Gryllus assimilis*	*E. coli*	No	$>1.5 * 10^6$	out of limit
801	*Zophobas morio*	*E. coli*	Washed	$>1.5 * 10^5$	out of limit
802	*Tenebrio molitor*	*E. coli*	Washed	$>1.5 * 10^5$	out of limit
803	*Gryllus assimilis*	*E. coli*	Washed	$>1.5 * 10^5$	out of limit
798	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
799	*Tenebrio molitor*	*E. coli*	Boiled	<10	in limit
800	*Gryllus assimilis*	*E. coli*	Boiled	<10	in limit

Table 3: The difference throughout *Salmonella* spp. (CFU/g), between three species of insects and, in column category, it is no out of limit sample.

Examination	**Species**	**Pathogen**	**Treatment**	**Result**	**Category**
23000	*Zophobas morio*	*Salmonella*	No	negative	in limit
23001	*Tenebrio molitor*	*Salmonella*	No	negative	in limit
23002	*Gryllus assimilis*	*Salmonella*	No	negative	in limit
801	*Zophobas morio*	*Salmonella*	Washed	negative	in limit
802	*Tenebrio molitor*	*Salmonella*	Washed	negative	in limit
803	*Gryllus assimilis*	*Salmonella*	Washed	negative	in limit
798	*Zophobas morio*	*Salmonella*	Boiled	negative	in limit
799	*Tenebrio molitor*	*Salmonella*	Boiled	negative	in limit
800	*Gryllus assimilis*	*Salmonella*	Boiled	negative	in limit

Table 4: The difference throughout genus *E. coli* (CFU/g) in *Zophobas morio*; in column category, it is seen no difference between types of treatments.

Examination	**Species**	**Pathogen**	**Treatment**	**Result**	**Category**
12086	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
12087	*Zophobas morio*	*E. coli*	Raw	<10	in limit
12565	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
12566	*Zophobas morio*	*E. coli*	Raw	<10	in limit
12567	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
12568	*Zophobas morio*	*E. coli*	Raw	<10	in limit
12999	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
13000	*Zophobas morio*	*E. coli*	Raw	<10	in limit
13001	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
13002	*Zophobas morio*	*E. coli*	Raw	<10	in limit
13003	*Zophobas morio*	*E. coli*	Boiled	<10	in limit
13004	*Zophobas morio*	*E. coli*	Raw	<10	in limit

There has been done comparison between raw and boiled insect with raw and boiled meat. For this analysis were used data from SVI, Prague (year 2015). Procedure followed norm ČSN 56 96 09: B.5.2. Tolerated values of food stuffs; individual species, groups, or subgroups. Meat was divided to two categories: 1) in limit or: 2) out of limit following

Commission Regulation (EC) No 2073/2005 of 15 November 2005. The limits for first chosen category **raw meat products** was for 5n (5 units of the same type) m= 500 CFU/g (M= 5000 CFU/g). For **in limit** evaluation are all **5n equal or lower than m** (For in limit evaluation, from these five samples can two (**c** value) overreach m, but no one should exceed M). As a second samples were chosen **raw-cooked meat products** (so called semis), where is assumption of 0 CFU/g seen as a best result. In case, that *E. coli* exceed 10 000 CFU/g it is potential risk of arise of disease. Recommended values for meat products in general laying in norm ČSN 56 9609; B. 5.2.1.2 Meat Products are m= 50 CFU/g; M= 500 CFU/g, when c= 1 (for in limits evaluation can only one sample exceed m, but no one should exceed M).

Limits in Netherlands and Belgium are 10^5 for minced meat used also for insects (Opperhuizer, 2014; Néve, 2014), but these tables are not so relevant (Grabowski et. al.; 2016).

1) Between raw and boiled *Zophobas morio*, there does not exist statistically significant difference in amount of CFU/g (Pearson's chi-squared test, $p > 0.05$).

2) According next examination, suitability for consummation of meat and insects was the same. There was found no statistical difference between meat and insects (Pearson's chi-squared test, $p > 0.05$).

3) The same result was for third examination, boiled *Zophobas morio*; there is no statistically significant difference in CFU/g between boiled meat and *Zophobas morio* (Pearson's chi-squared test, $p > 0.05$).

5. DISCUSSION

Entomophagy can be helpful with improving and ensuring of food safety and food security, with relation to the new world conception One Health. Connection with food-borne diseases it is seen, so potential of insects can be well sustainably utilized, but only if they are farming properly, with the right biosecurity plans, which means mainly keeping sufficient hygiene and correct storage.

Diploma thesis was focused on microbiological agents (family Enterobacteriaceae, *Salmonella spp. and Escherichia coli*) of given specimen of insects (*Tenebrio molitor, Zophobas morio, Gryllus assimillis*).

The aim of this thesis was:

1) to give an overview on exist norms for microbiological examination in insects species

2) to examine microbiology of tree given specimens *(Gryllus assimillis, Tenebrio molitor, Zophobas morio)* from three different distributors with the focus on family Enterobacteriaceae (genes *Salmonella* spp*., E. coli*)

3) to find out whether insects are no more dangerous than meat products as alternative source of protein

Ad 1) In the Czech Republic, there is still no norm (FAO, WTO, WHO, EFSA, Ministry of Agriculture of the Czech Republic, communication with the prominent experts) for ensuring health and set correct HACCP. Because of it will be tried, at the end of this chapter, to invent partial norm, which could be applicable to practice examination of microorganisms in this three species (because of course for another species it can be different) and may it be clue for this organizations for ensuring public health.

In Belgium and Netherlands, there exits some norm, but if we go back to *Salmonella* spp. and Enterobacteriaceae, Grabowski et. al. (2016) from Germany, who work on similar microbiological examination of insects for food point out: “So far, we have not found any *Salmonellae* in any of the insect samples we worked with. Few of them yielded *E. coli*, but Enterobacteriaceae counts were very high, especially if you analyze raw insects. The process hygiene criteria as recommended by the Dutch and the Belgian simply do not work in this sense!“

Ad 2) In a first part of examination; according to norm ČSN 56 9609; tab. B.6–Bacterial agents of diseases from food stuffs; is content of *Escherichia coli* satisfactory/ **in limits** in insects, **only in boiled form** (and in the form of direct consumption). In the **raw** state and **washed** were numbers higher than $10^{4,}$ which is **out of limit**. *Salmonella* spp. is unacceptable in 25 g and thus in this way insects are also suitable/ **in limit**.

Ad 3) In the second part–statistical analysis with *E. coli* and *Zophobas* morio only, was formed two hypotheses to fulfill the targets:

H1 In raw insect (concretely *Zophobas morio sp.*) there will be higher number of CFU *(E. coli)* than in boiled insect.

H2 Content of *E. coli* in in insects (concretely *Zophobas morio sp.*) will be comparable to content of *E. coli* in other meat products when the same treatment will be used (insects are no more dangerous from biosecurity point of view).

H1 was that **in raw insect** (concretely *Zophobas morio sp.*) **there will be higher number of CFU *(E. coli)* than in boiled insect**. This hypothesis was **not** confirmed (even when first examinations seem stay for it), because there was not found statistically significant difference between boiled and raw insects. It may be caused by stress of animals in winter period and transport, little amount of water (in first series of examinations), longer time from bring samples to examine them by SVI, different primary amount of insects, or different welfare conditions in primary distributors.

H2 was that content of *E. coli* in in insects (concretely *Zophobas morio sp.*) will be comparable to content of *E. coli* in meat and so insects are no more dangerous from biosecurity point of view than other meat products (when the same treatment was used). Comparison between **raw and boiled insect** with **raw and boiled meat** been done. Procedure followed norm ČSN 56 96 09: B.5.2. Tolerated values of food stuffs; individual species, groups, or subgroups. Meats were divided to two categories: 1) in limit or: 2) out of limit. Hypothesis two was confirmed, there is big sight of **comparability in meat and insects**.

As an addition only 15% of the coliform bacterias (mainy *E. coli*) in insects are comparable to E. coli in mamals and birds (Opperhuizen, 2014). Hoever E. coli is indicator of Noroirus, wich must be obsered in insects. Recently EFSA (2015) shows that both in Belgium and in the Netherlands, risk assessments have been developed for farming of insects for food production.

The report from the Scientific Committee of the Belgian Federal Agency for the Safety of the Food Chain provides some data about the microbiological status of insects specifically farmed for food production. High values of 107 CFU/g were measured for the total aerobic bacterial count, but also for the total anaerobic bacterial count and Enterobacteriaceae (facultative anaerobes) were found in a preliminary **Belgian** study on mealworms (*T. molitor*) and morio worms (*Zophobas atratus*). In another exploratory Belgian study on raw and frozen mealworms and locusts, were found similar high values (107–109 CFU/g).

In the risk assessment from the **Netherlands**, the results of a small-scale survey on the microbiological status of 55 insects products (locusts, lesser mealworms, mealworms and a mealworm snack) that had undergone **no treatment** apart from freeze-drying, are reported. In this study it was found that 59 % of the insects products tested exceeded the process hygiene criteria for aerobic bacteria in raw materials used in meat preparation (106 CFU/g), while of course cause is in direct contatt with frass excreata --- but boiled ok. The concentration of Enterobacteriaceae in 65 % of the samples exceeded the criteria for raw materials used in meat preparations (103 CFU/g). The study investigated the presence of *Clostridium perfringens, Salmonella* spp. and *Vibrio* and none of these were detected, which is for *Salmonella* spp. same as in our study.

In Germany, they been following Dutch and Belgian criteria, and it turned out that **unprocessed** insects usually do **not** meet the process hygiene criteria, while the food hygiene criteria are usually fulfilled-exactly like in our work (Grabowski et. al.; 2016).

In first part of our examination, were found $1{,}5x10^6$ = 1 500 000 CFU/g (for Enterobacteriaceae and also for *E. coli*) for raw insects (in all examined species). Values from our work are evaluated as too high in case of raw insects (millions!–unacceptable; e. g. normal volume of CFU is: 500–5000 for *E. coli*; in minced meat is limit 500 CFU/g (SVI, Prague; Opperhuizen, 2014; Néve, 2014). In washed insects samples there was found lower value: $1{,}5x10^5$ = 150 000 CFU. Value $1x10^{1\ =}$ 10 CFU-third examination-was also same in all three types of insects. This value finally falls into recommendation for norms. As it was compared, in our work was quite higher number for Enterobacteriaceae than in studies in Belgium and Netherlands. It was probably because of our longer time between reception and evaluation of samples and also because of many others factors e.g. quality of feeding. Also it can be caused by many others factors. In future there should be more frequented and detail studies, which will also confirm that insect are no more dangerous than other food sources.

From these three countries, can be seen, that there is need of heat treatment of insects with following of future values, which may be in the Czech Republic laying in norm ČSN 56 96 09, although in second part of my work there were found the fit values in this norm also in raw insects. According to Belluco et al. (2016) *Zophobas morio* states potential hazards in a high bacterial count.

The Codes (FDA, 2014)-Food Acts-must be followed by individual states, but how they do this is with respect to their own Sate Legislation–hence the state to state variability (Shawn, 2015). My opinion is, that in US Codes is a missing part-there is still no mention of entomophagy.

Also is scarcity in a HACCP, especially in CR, because if we logically look at it and ask companies, they will say: "*we do HACCAP for individual customer and its production"* (manager of Quality Safety Legality Company, 2016). So, there arises question how we ensure correct way with this analytic system? Random and secret policing may be the most effective way for examination of insects and other products. Direct examination of microorganisms is basic, but for disproving of possibility of epidemiology agent is necessary, also by law, to detect exact species (Votava, 2010).

For consultation of creation of first norm for microbiological criteria's of insects there were help from SVI Prague. Insects are novel food and they content chitin and other body composition, which are not in meat. Indeed, there occurred interesting thing about homogenization of all insects' body (chitin crustae left)-after passing Stomacher machine chitin layer was still present without homogenization (see picture 20). In other samples (e.g. meat) were always recorded accurately homogenized samples.

Proposal of insect microbiological examination was done. It have been worked with three types of insects treatment for creating norms was used only of these two following (insects washed by water will be maybe good medium for multiplication of the microorganisms and fungi; in this case, norm for washed insects may be unreasonable): from ČSN 56 96 09 I would recommend to add my proposals:

B.5.2.21.1. Insects in fresh quality; for home cooking or frying

B.5.2.21.2. Insects prepared by cooking or frying

Legend used in ČSN 56 96 09:

n is the sample size-the number of samples for testing, with aim to determine whether a product batch or part of the product will be evaluated as satisfactory or unsatisfactory according to the requirements of given microorganism set at the beginning

m is the amount of microorganisms, which is allowed in all samples of selecting n

M is the mass of microorganisms, which is still allowed for a number of sample that is less than or equal to c

c is a number, which means the number of samples from a selection n where is allowed the value of M

Table 5: Proposal and recommendation for microbiological examination of insects

B.5.2.21.1. Insects in fresh state; for home cooking or frying					
		n	C	**m**	M
Enterobacteriaceae	food stuffs for direct consumption	5	2	$5 . 10^5$	$5 . 10^6$
Escherichia coli	food stuffs for direct consumption	5	2	10^2 - 10^3	10^4
Salmonella spp.	food stuffs for direct consumption	5	0	0/10	-

Bases of this Table 5: recommendation was created according to norm's amount of CFU in raw meat B.5.2.1.2., was created limit for Enterobacteriaceae in three species of insects.

According to *E. coli* Tab.1 the B.6 was created limit for *Escherichia coli* in insects.

According to *Salmonella* spp. Tab.1 the B.6 was created limits for *Salmonella* spp. in insects.

As there were set maximum limits, very similar part of norms can be used for examination of microorganisms in insects-boiled form, because there is of course less of microorganisms.

In the future will be interested also create other parts of norms for frozen microorganisms (may be used criterion for frozen vegetable, or meat one, or better criterion B.5.2.2.3. Frozen mollusks and crustaceans–which are from same phylum like insects and contain also chitin substance, so following of these norms make sense,-dried and e.g. for flour forms of insects).

Our table can be used in norm ČSN 56 96 09: B.5.2. Tolerated values of food stuffs; individual species, groups, or subgroups.

Regarding the above conclusions the following recommendation are stated:

There should be standard **farms, or households, that** produce high quality, healthy and wholesome edible insects.

Methods of (**organic) waste management** offer big potential and thus should be deeply examined from this point of view in the future

The same food hygiene **criteria** as for **meat** can be used for Insects.

There should be **created HACCP; norms and standards for microbiological edible insects** examination (these norms should be enlarged for many species of class Insecta and for many genes and families of microorganisms; e.g. for *Campylobacter*, *Clostridiums perfringers*, *Bacilus cereus*, which is according to EFSA also very crucial form insect point of view). There should be recommendation for people who are **allergic** to sea food; insect classification belongs to the same phylum Arthropoda as a e.g. Crustaceans.

In the other hand, insects can **cure** some **allergic** problems, if eaten from childhood.

Insects can be helpful with famine in developing counties and also insects can reduce obesity in “developed” countries, where it can offer solution for **lack of nutrients for vegetarians and vegans.**

Insect should be **processed by heat treatment,** before humans eat them (After heat treatment Enterobacteriaceae felt into hygiene criteria (Opperhuizen, 2014).

In order to avoid the **toxic** effects of some species of insects, due to the synthesis of poisons, an accurate selection of species, should be thoroughly selected before farming.

Packaging of insects is very important (and correct **storage**), there should be avoiding of molds and others microorganisms. Different designs of packaging and forms (liquid, powder or paste), should be concluded to market.

Boiling is better for reducing microorganisms than roasting.

The same norms as form **meat,** can be used for instects (Opperhuizen, 2014)

Since there is shortage of information about edible insects further **researches** should be done (public relation).

In the future, can be worked with others **ways of preparation**, e.g. following Crustaceans, which are from same phylum like insects.

Welfare of Insects breeding should be also included.

Insects should be **periodically tested** on others microbiota as *Staphylococcus, Streptococcus, Bacillus, Proteus, Pseudomonas, Escherichia, Micrococcus, Lactobacillus and Acinetobacter*

Enumeration of pathogens or potential pathogens differs also according to way of processing so in the future is necessary to work with other **species of insects**.

Technology of the mechanisms of the new era should be learning and copying Insects principles of living, as a form of life, which is higher in biomass and in many other points.

6. CONCLUSIONS

Edible insects could be of the great interest as a possible solution to two world crisis (famine as well as obesity), due to their capability to satisfy two head requirements: they are an important source of nutrients and their use as food has ecological advantages outperform conventional meat and, in the long run, insects have huge economic benefits.

In my research was aim to determine, whether insects can be comparable to other food sources-from microbiological point of view. There was found that microbiology of insects is important part of all parts of the examinations of insects and that insect are no more dangerous than other food stuffs, but only if they are correctly processed. Also trivia are necessary, because such a things as amount of water or seasonality may cause potential harm. Simple hygienic measures (as appropriate cooking and/or freezing) should be applied during food processing, as suggested for poultry, pork, and fish. The legislative framework for insects microbiological examination in the Czech Republic still does not exist, thus there arise necessity of create norms.

For sustainable development of the World, there arises necessity, to look at every issue from global point of view. The more will be public informed about huge plusses of insects; the more will be acceptable for them thought of its consummation. I agree with opinion of Itterbeeck (2008); Kudlová (2009); Škrabalová (2009); van Huis A. (2012); Borkovcová (2014); Bednářová, 2014 and many others, that potential of Entomophagy is very widely significant.

Insects can follow One-health conception only if correctly storage, which seems to be the most valuable critical control point and, than entomophagy seems to be sustainable source of food; after all insects represents more than half of all known living organisms on the Mother Earth!

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APPENDICES

- Results; SVI, Prague
- Laboratory procedures-glossary
- MENDELU course
- Confirmations of practices (MENDELU; SVI)

Results, SVI, Prague

Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku	Teplota°C
23000	Zopholas morio	3,0
23001	Tenebrio molitor	3,0
23002	Gnyllus assimilis	3,0

Mikrobiologické vyšetření	23000	23001	23002
Escherichia coli	$>1,5x10^6$	$>1,5x10^6$	$>1,5x10^6$
Salmonella sp.	negativní	negativní	negativní
Enterobacteriaceae	$>1,5x10^6$	$>1,5x10^6$	$>1,5x10^6$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Použité metody stanovení:

Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.
Horizontální metody pro průkaz a stanovení počtu bakterií čeledi Enterobacteriaceae kultivací: ČSN ISO 21528 - 1, ČSN ISO 21528 - 2.
Horizontální metoda průkazu bakterií rodu Salmonella kultivací: ČSN EN ISO 6579.

* Takto označené metody nejsou předmětem akreditace.

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Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku
801	Zopholas morio
802	Tenebrio molito
803	Gnyllus assimilis

Mikrobiologické vyšetření	801	802	803
Escherichia coli	$>1,5x10^5$	$>1,5x10^5$	$>1,5x10^5$
Salmonella sp.	negativní	negativní	negativní
Enterobacteriaceae	$>1,5x10^5$	$>1,5x10^5$	$>1,5x10^5$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Poznámka:
Vzorky 801-803: živý hmyz, ošetřený oplachem vodou

Použité metody stanovení:
Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.
Horizontální metody pro průkaz a stanovení počtu bakterií čeledi Enterobacteriaceae kultivací: ČSN ISO 21528 - 1, ČSN ISO 21528 - 2.
Horizontální metoda průkazu bakterií rodu Salmonella kultivací: ČSN EN ISO 6579.

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Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku
798	Zopholas morio
799	Tenebrio molitor
800	Gnyllus assimilis

Mikrobiologické vyšetření	798	799	800
Escherichia coli	$<1x10^1$	$<1x10^1$	$<1x10^1$
Salmonella sp.	negativní	negativní	negativní
Enterobacteriaceae	$<1x10^1$	$<1x10^1$	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Poznámka:
Vzorky 798 -800: po tepelné úpravě smažením

Použité metody stanovení:
Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.
Horizontální metody pro průkaz a stanovení počtu bakterií čeledi Enterobacteriaceae kultivací: ČSN ISO 21528 - 1, ČSN ISO 21528 - 2.
Horizontální metoda průkazu bakterií rodu Salmonella kultivací: ČSN EN ISO 6579.

* Takto označené metody nejsou předmětem akreditace.
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Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku
12086	Zophobas morio, syrový

Mikrobiologické vyšetření	12086
Escherichia coli	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Použité metody stanovení:
Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.

* Takto označené metody nejsou předmětem akreditace.
Tento protokol může být reprodukován jedině celý, jeho části pouze se souhlasem SVÚ Praha. Výsledky laboratorního vyšetření se týkají pouze vzorků, uvedených v protokolu. Pokud se budete odkazovat na naše služby, použijte tuto citaci: "Zkoušeno Státním veterinárním ústavem Praha - Zkušební laboratoří hygieny potravin a krmiv, která je akreditována Českým institutem pro akreditaci, o.p.s. Protokol o zkoušce neznamená schválení zkoušeného předmětu orgánem udělujícím akreditaci.

Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku
12087	Zophobas morio, vařený

Mikrobiologické vyšetření	12087
Escherichia coli	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Použité metody stanovení:
Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.

* Takto označené metody nejsou předmětem akreditace.
Tento protokol může být reprodukován jedině celý, jeho části pouze se souhlasem SVÚ Praha. Výsledky laboratorního vyšetření se týkají pouze vzorků, uvedených v protokolu. Pokud se budete odkazovat na naše služby, použijte tuto citaci: "Zkoušeno Státním veterinárním ústavem Praha - Zkušební laboratoří hygieny potravin a krmiv, která je akreditována Českým institutem pro akreditaci, o.p.s. Protokol o zkoušce neznamená schválení zkoušeného předmětu orgánem udělujícím akreditaci.

Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku
12565	Zophobas morio, syrový
12566	Zophobas morio, syrový
12567	Zophobas morio, vařený
12568	Zophobas morio, vařený

Mikrobiologické vyšetření	12565	12566	12567	12568
Escherichia coli	$<1x10^1$	$<1x10^1$	$<1x10^1$	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Použité metody stanovení:

Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.

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Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku	Teplota°C
12999	Hmyz 100 g, vařený	3,0
13000	Hmyz 100 g, syrový	3,0

Mikrobiologické vyšetření	12999	13000
Escherichia coli	$<1x10^1$	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

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Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.

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Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku	Teplota°C
13001	Hmyz vařený	3,0
13002	Hmyz syrový	3,0

Mikrobiologické vyšetření	13001	13002
Escherichia coli	$<1x10^1$	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Použité metody stanovení:

Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.

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Výsledky laboratorního vyšetření

Č.vzorku	Popis vzorku	Teplota°C
13003	Hmyz vařený	3,0
13004	Hmyz syrový	3,0

Mikrobiologické vyšetření	13003	13004
Escherichia coli	$<1x10^1$	$<1x10^1$

Kvantitativní vyšetření vyjádřeno počtem KTJ/g (ml) vzorku. (KTJ - kolonie tvořící jednotky).
Průkaz patogenních mikroorganismů v navážce 25 g (pokud není u názvu parametru uvedeno jinak).

Použité metody stanovení:
Stanovení počtu Escherichia coli - technika počítání kolonií: ČSN ISO 16649 - 2.

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Laboratory procedures

Specimens selected for microbiological examination should reflect the disease process and be collected in sufficient quantity. Samples should allow complete microbiologic examination. The number of microorganisms per milliliter of a body fluid or per gram of tissue is variable, ranging from less than 1 to 10^8 or 10^{10} colony-forming units (CFU) (Cole at al., 1996).

Liquid and solid media are used in many methods, the cause of an infection is confirmed by isolating and culturing microorganism either in artificial media or in a living host. Bacteria and fungi are cultured in either liquid (broth) or on solid (agar) artificial media as was written by Cole at al. (1996). The same author also mentions that liquid media provide greater sensitivity for the isolation of small numbers of microorganisms. Growth in liquid media also cannot be most of the time quantitated. Solid media, although somewhat less sensitive than liquid media, provide isolated colonies that can be quantified if necessary and identified. Some genera and species can be recognized on the basis of their colony morphologies identification of bacteria (including mycobacteria) is based on growth characteristics (time required for growth to appear or the atmosphere in which growth occurs), colony and microscopic morphology, biochemical, physiologic, and, in some cases, antigenic or nucleotide sequence characteristics. The selection and number of tests

for bacterial identification depend upon the category of bacteria present (aerobic or anaerobic, Gram-positive or Gram-negative, cocci or bacilli) and the expertise of the microbiologist examining the culture Cole at al. (1996).

MEDELU Course

Basic behavior at specialized workplace

Personal hygiene belongs to the most important measures. With personal hygiene is meant to have washed hands, short nails, movements on workplace without mobile phone, be aware of drinking, eating or smoking at workplace.

In basic requirement of laboratory belongs absence of air conditioning against cross contamination of the bacteria.

Area should fulfill following sequences of location against cross contamination: first, there is reception of samples, then place for sterilization, analyzation space and place for cleaning stuffs.

In ideal case, there should be entrance through hygienic airlock, i. e. shower between so called “clean and dirty space”.

Control of the contamination is done by fallouts – in every sample deployment (That is reason why it is highly recommended to not open cap, but only lift of (Petri dishes) and through smears. Controls are done once months or abruptly (after general cleaning or after rebuilds).

Equipment of laboratory

Equipment and instruments are specified in norm ISO 7218.

The condition of the accreditation is, that instruments are correctly calibrated, which is controlled by superior laboratory (ČSN EN ISO 8655) and through this is possible to guarantee functionality. Every instrument should also have its own diary of service.

Basic equipment of laboratory are scales; homogenizator; pH meters, autoclave (sterilizer – with principle of moist heat steam cleaning: p + t = 121°C, 23 min; 101,5 kPa), hot air (dry heat) which can be seen in Figure 18.; incubators = thermostats (max six Petri dishes at

each other – for air flowing) which is demonstrated in Figure 19.; thermometers (with automatic sensors); fridges; freezers; water bath with adjustable temperature; microwave (gel for electrophoresis – PCR; or agar); optical microscope; germicide lamp (UV light); sterile loops; sterile pipettes; tubes (nowadays usually plastic); flasks; beakers for suffusion; sterile bags; sterile tweezers, scissors; “sticks” for rubbing off sample on a petri dish – for quantitative examination; Petri dishes; anaerostates.

Figure 13: Sterilizer; (Coufalová, 2016)

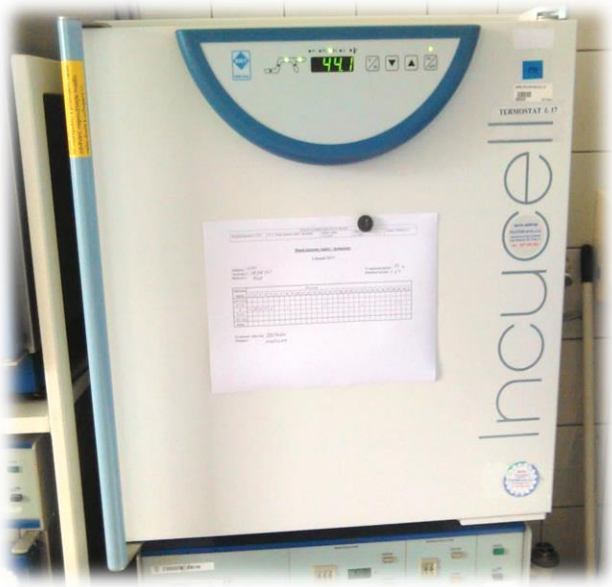


Figure 14: Thermostat (with calibrated thermometer); (Coufalová, 2016)

Microbiological Glossary

Inoculum: culture of microorganisms, put on fertile medium (MENDELU, 2015).

Culture: isolation of infectious agents frequently requires specialized media. Nonselective (noninhibitory) media permit the growth of many microorganisms. Selective media contain inhibitory substances that permit the isolation of specific types of microorganisms (Cole at al., 1996).

Culture medium: a simple or complex nutrient composition in liquid or solid form, used to maintain or increase the development of a microbial species under appropriate biological conditions.

Inoculation: with the aim of to have one cell, which brings clean colonies (used is cross smear) for dilution and for the next step of the whole process-bacteriological examination, (MENDELU, 2015).

Enumeration of microorganisms: counting of microorganisms from sample (MENDELU, 2015).

Serovar: group of bacteria (one species), which has the same antigenic properties (against different serotypes of the same species microorganisms they create different types of antibodies; antiserum is created by proper state institution (MENDELU, 2015).

Cultivation: gaining of microorganisms form the sample (MENDELU, 2015). For the cultivation are very important indicators temperature and time (mainly is used 24 hours of cultivation for ordinal bacteria, for *Staphylococcus* cultivation mean days and in fungi case even weeks). The temperature depends on the microorganism, which sets (SVI, 2016).

Cultivation quantitative: CPM/ CFU: total bacterial contamination on solid media – agars. Arise of colony which is divided according to specific signs (MENDELU, 2015).

Colony (one): population of microorganism, which arises by division of one mother cell (usually 10^6), which has same morphological properties/ signs: profile, form, edge. For examination of CFU it's used mainly GTK agar (MENDELU, 2015); colony = one cell on medium, while biochemistry confirmation is done only sometimes (SVI, 2016).

CFU: In microbiology, a colony-forming unit (CFU/ in Czech language: CPM – celkový počet mikroorganismů) is a unit used to estimate the number of viable bacteria or fungal cells in a sample. The visual appearance of a colony in a cell culture requires significant growth, and when counting colonies it is uncertain if the colony arose from one cell or a group of cells. Expressing results as colony-forming units reflects this uncertainty (MENDELU, 2015).

Cultivation qualitative: there is used dilution of the sample, with formation of the countable bacteria (MENDELU, 2015).

Ten folds dilution: 1 ml of sample plus 9 ml of diluent; * dilution 10^{-1} (plus 1 ml of this sample to the next diluent; * dilution 10^{-2}) (MENDELU, 2015). Detail often folds dilution can be found at the end.

Spillway of medium: in the empty Petri dish is put solution. Medium is heated to 45 °C, then cooled on the temperature of room and spilled on solution (MENDELU, 2015).

The rules of disposal of biological material

Waste pathogens free are disposed of as municipal waste.

The waste, where is risk of contamination belongs to a specific container, and then is burned with dry heat in an autoclave (121 ° C, 30 min).

In the event of sharp objects, the samples are placed in special containers for decontamination.

Decontamination of the table is very important and is carried out in two phases. The first phase is the chemical decontamination (AJATIN, or 70 % ethanol), in the second stage is utilized physical decontamination using UV - germicidal lamps) (MENDELU, 2015).

Basic methods

First of all is important to use sterile items with correct usage (MENDELU, 2015).

Microbial identification

For the first microbiological view, *micro-pathology*, for the sample is used one of the classical microbiological methods. Direct microscopy is done. If there are hesitations about genus or species, or it's important to be sure, which species it is, sample goes on bacteriological examination. If it's still not clear it is send to PCR – genetic probe, where genetic probes identify genus- or species-specific of DNA or RNA to confirmation (MENDELU, 2015). Thus preliminary identification is possible to ensure by arise of colony and cellular morphology. Growth characteristics depend on various conditions, utilization of carbohydrates and other substrates, enzymatic activity, immunoassays (immunofluorescence, immuno-peroxidase staining). Serodiagnosis is a high or rising titer of specific IgG antibodies or the presence of specific IgM antibodies may suggest or confirm a diagnosis (Cole at al., 1996).

In general, there is life time for usage of produced media, often it is around one day for liquid. Usually is applied 1 ml (or 0.1) of liquid agar and the medium is cooled to 45 ° C in a water bath or thermostat. For solid agars is life time longer, around one month (SVI, 2015).

The basic are universal media, e. g. meat peptone agar, Müller Hinton agar, blood agar GTK, WPA medium, or DRBC (MENDELU, 2015).

Selective media supports growth of wanted group of microorganisms, with effect of suppressing accompanying microorganisms. They can also contain inhibitor substance (e. g. ATB); (XLD agar, RVS) (MENDELU, 2015).

Next types of media are diagnostic media, which contain color like indicator (DTM agar) (MENDELU, 2015).

Chromogenous media are used in the food industry. Principe is chromogen, which is accepted by bacteria to its cell; bacteria must split substrate from chromogen through coenzyme, which is cleaved and colored (MENDELU, 2015) (TBX agar).

There is also mixed types - selectively diagnostic media - e. g. Endo agar, MAC or MYP agar (MENDELU, 2015).

Media for Enterobacteriaceae

For Enterobacteriaceae detection is used quantitatively Endo agar, which has many selective advantages, MacConkey agar, XLD or others (DC or MAL) (Votava, 2010).

Qualitative examination

Detection method (qualitative method) determines the presence or absence of a particular microorganisms in a defined quantity of product. Qualitative examination is used for the detection of specific micro-organism, where is aim to counts numbers, so the result should be readable. The minimum weight is of 10 g, but it depends on the microorganisms species examined - for *Salmonella* spp. is 10-25 g; for the others from 10 to 12 g.

After adding of peptone water in to sample by machine, it takes one day in the incubator. This step is called multiplication, and for certain procedures is followed by resuscitation phase, where if there is a pathogen present even in an inactive state, the resuscitation liquid can prepare environment so that we can see searched microorganisms.

Second day is used selective medium (solid media, agar), which are important for the propagation of a certain type of bacteria and other microorganisms are suppressed. On this media inoculation are used and this agars are mainly chromogenic for easier assessment of the results. Usually, inoculation takes approximately 1-5 days.

Quantitative analysis–CPM/ CFU

Quantitative analysis is sequentially identical to a qualitative, but using a different dilution. In the case of the test for counting of CFU (total number of microorganisms), the sample is inoculated, but not go to the thermostat.

In quantitative testing microorganisms are obtained after multiplication, regardless to the number of microorganisms and according to morphological characters it is possible to isolate specific microorganisms. This examination is not accurate, because even after homogenization remains extended portion of microorganisms in the sample differently than homogeneously. The greater the number of the dilution, the greater the number of error may occur.

Section of quantitative analysis includes quantitative examination of Enterobacteriaceae family (from which it is important genus *E. coli* and *Salmonella* spp.). Batch size should be at least 5 or 10 g for Dilumat machine with subsequently added diluents medium (with arise of suspension). Then is homogenized and ten folds dilution to a Petri dish is used (usually is pipetted 1 cm^3 = 1 ml of suspension). It is necessary to use sterile equipment; important is heat treatment of the neck of the flasks of the liquid media in glass. With gyratory agitation is medium allowed to achieve solidify. A sample is usually stored in a thermostat for 24 hours. Suitable medium is produced according to microorganism, which is examined and there are differences between families, genes and species – total number of microorganisms rise in 30 °C, Enterobacteriaceae in 37 °C and for other is temperature around 20 °C. Then, after the formation of the typical colonies the subtracted result is enumerated.

A tenfold dilution-in detail

Ten- fold dilutions of samples are examined in broth dilution device-medium–Dilumat, which automatically count, how much of the solution should be added to achieve ten- fold dilutions - 1:10 suspension. First dilution represents colonies and it is equal the number 10 microorganisms times 10; i.e. it is pipetted one milliliter of examined solution to nine ml of diluent - physiological solution = * 10^{-1}. Throughout expectation is done as much of the dilutions, as necessary – this background is given by skill, practice and professionalism of the workers. For example, if we expect the sample will be 300 000 microorganisms it is necessary to dilute 5x.

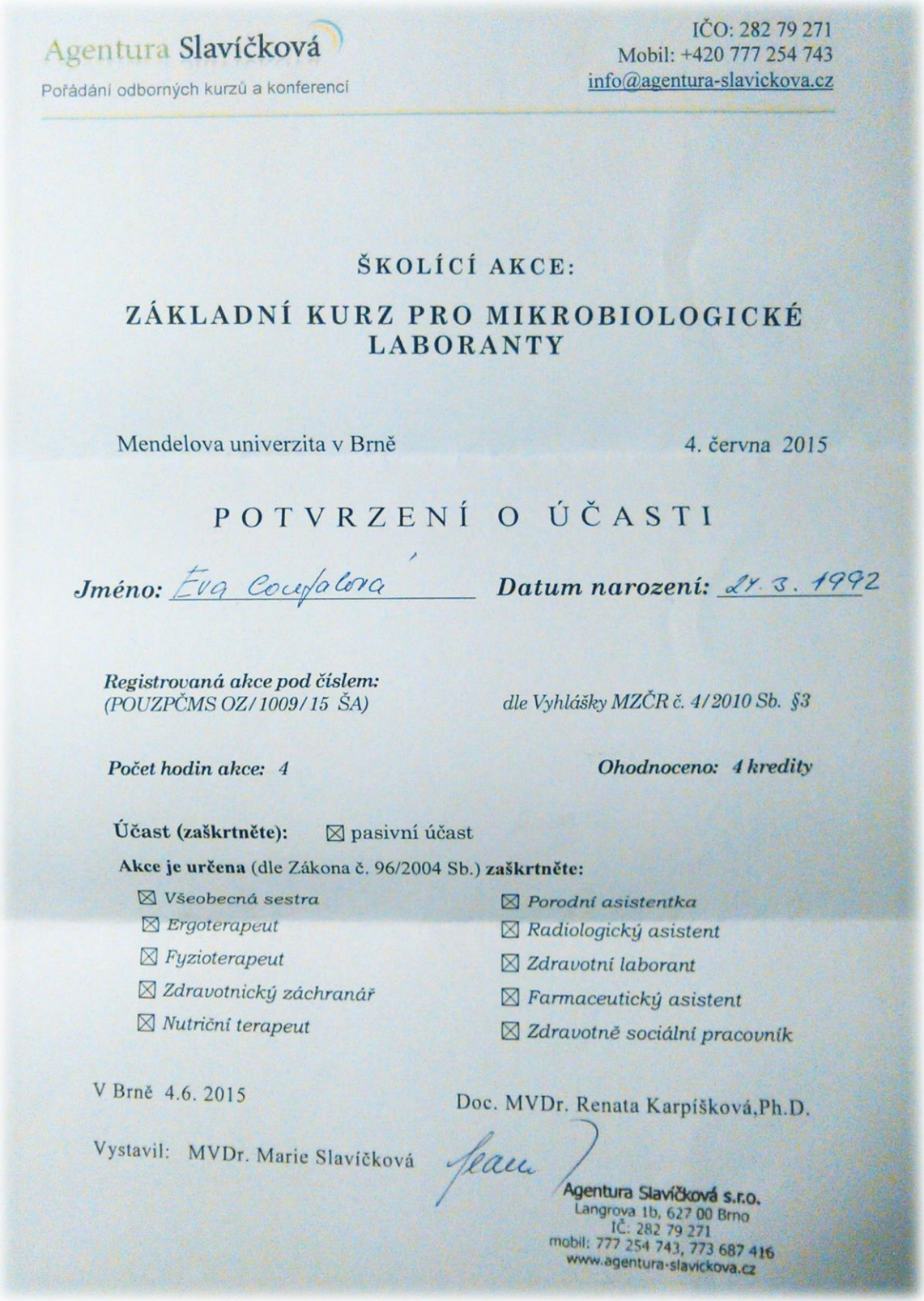
(Note: For liquid samples is done directly 10^{-1} without the "zero" dilution, because it is already in a homogenizer bag, Erlenmeyer flask).

Practical calculation example

Calculation of the final density of microorganisms is multiplied by the number of dilutions (100 times, if was used of two dilutions and a million times when was used of ten dilutions e.g.: 6x 1 000 000; one worker count of 35, the second 38= differential 3 000 000).

Figure 20: Laboratory work at SVI Prague (Coufalová, 2016)

Confirmations of practices (MENDELU; SVI)



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ŠKOLÍCÍ AKCE:

ZÁKLADNÍ KURZ PRO MIKROBIOLOGICKÉ LABORANTY

Mendelova univerzita v Brně 4. června 2015

POTVRZENÍ O ÚČASTI

Jméno: Eva Coufalova ***Datum narození:*** 27. 3. 1992

Registrovaná akce pod číslem:
(POUZPČMS OZ/1009/15 ŠA)

dle Vyhlášky MZČR č. 4/2010 Sb. §3

Počet hodin akce: *4*

Ohodnoceno: *4 kredity*

Účast (zaškrtněte): ☒ pasivní účast

Akce je určena (dle Zákona č. 96/2004 Sb.) **zaškrtněte:**

- ☒ *Všeobecná sestra*
- ☒ *Ergoterapeut*
- ☒ *Fyzioterapeut*
- ☒ *Zdravotnický záchranář*
- ☒ *Nutriční terapeut*
- ☒ *Porodní asistentka*
- ☒ *Radiologický asistent*
- ☒ *Zdravotní laborant*
- ☒ *Farmaceutický asistent*
- ☒ *Zdravotně sociální pracovník*

V Brně 4.6. 2015

Doc. MVDr. Renata Karpíšková,Ph.D.

Vystavil: MVDr. Marie Slavíčková

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PRAHA

SVÚ

Potvrzení o vykonání praxe

Praktická část k diplomové práci byla provedena v prostorech Státního veterinárního ústavu Praha, přičemž si praktikant osvojil základní mikrobiologické postupy.

Praha,

20. ledna 2016

Praktikant:

Bc. Eva Cavalová

MVDr. JAN KUČERA

Státní veterinární ústav Praha
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