MICRO-ORGANISMS IN A NEWLY DEVELOPED FISH AND REPTILE FEED BASED ON INSECTS.

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ABSTRACT

Insect species are promising for industrial animal feed production as an alternative protein source especially to soybean meal. These species include the black soldier fly (*Hermetia illucens*) and silkworms such as yellow mealworms (*Tenebrio molitor*). The goal of this study was to determine the presence and specification of microbial organisms in the new, insect-containing, reptile and fish feed developed by New Generation Nutrition (NGN), and whether these feeds met the requirements of the Implementing Regulation (EU) number 142/2011. The feeds produced by NGN contained *Hermetia illucens, Alphitobius diaperinus, Tenebrio molitor,* white fish and herbs. A literature search indicates that there could be multiple microbiological risks in these ingredients, regarding bacteria, fungi and molds. According to the literature, especially heat resistant spore producing bacteria (*Clostridium perfringens* and *Bacillus cereus*) need special attention. In this research, the samples from the products produced by NGN were tested for the presence of *Salmonella,* Enterobacteriaceae and *Clostridium perfringens.* The samples did not contain *Salmonella* or other Enterobacteriaceae, but did most likely contain *C. perfringens*. The presence of *C. perfringens* in the feed should be further specified by an officially certified laboratory. Our findings indicated that the NGN feed did not meet the Implementing Regulation (EU) nr 142/201 Therefore, the product is not yet ready for the market.

KEYWORDS

Insects as animal feed, *Hermetia illucens*, *Alphitobius diaperinus*, *Tenebrio molitor*, food safety, *Clostridium perfringens*, Enterobacteriaceae, *Salmonella*.

CONTENTS

INTRODUCTION

INSECTS AS ANIMAL FEED

The current production of animal feed has been identified as a major contributor to environmental issues because of land occupation, acidification, energy use and water dependence.[1] Soy and fishmeal are important useful sources of protein for animal feed, but the production of these sources has been linked to environmental issues.[1] To reduce these issues, a different source of protein is needed. As the nutritive needs of monogastric animals, fish in particular, include a high quality and quantity of protein in the diet, replacing soy and fishmeal is a challenge.

Insects are currently considered as a new protein source for feed with acceptable nutritive properties.[1] Insects are employed as feed in aquaculture and livestock and also used in the pet industry. For fishmeal there has been a high demand and consequent high prices, together with increasing production pressure on aquaculture. This has also led to research into the development of insect proteins for aquaculture and livestock, to eventually supplement fishmeal.[2] Insects have good quality amino acids and are rich in essential amino acids, whereas vegetable protein sources are usually deficient in limiting amino acids like lysine, methionine and leucine.[1]

The most promising species of insects for industrial feed production includes the black soldier fly (*Hermetia illucens*) and silkworms such as yellow mealworms (*Tenebrio molitor*). Mealworms are promising alternatives especially to soybean meal as a protein source.[2]

Insects are rich in nutrients and moisture, a disadvantage is that this provides a favorable environment for microbial growth and survival. Because of their biological makeup: microbial safety; toxicity; inorganic compounds; and the use of waste as insect feed, several issues related to food safety are distinct to insects. The critical factor in the commercialization of edible insect feed on a global scale will be the determination of optimal preservation methods. Live insects are usually transported in ice coolers shortly after collection and washing. They can be re-contaminated during the drying process through air or soil, which makes hygienic practices during processing of great importance. Additional heating or cooling steps are recommended before they are ready for consumption.[3]

NEW GENERATION NUTRITION

New Generation Nutrition (NGN) is a company based in ‘s-Hertogenbosch, The Netherlands, that develops and produces semi-moist feeds involving insect ingredients.[4] The insects used are *Hermetia illucens, Alphitobius diaperinus, Tenebrio molitor*. A mixture containing these insects is that basis of the animal feeds. The company produces reptile feed where herbs (marigold, curcuma, chamomile, garlic, aniseed, cardamom, cinnamon, dandelion, rooibos, peppermint, thyme and nettle) are also added to the mixture. The feed for fish was a mixture of the insects with an animal by-product of white fish (whiting and codfish). These two feeds are ready to go to the market but need research-based information about the presence of micro-organisms in their feed. This is required by the European law.

REQUIREMENTS FROM THE LAW

The Implementing Regulation (EU) nr 142/2011 states seven processes of which at least one is required to be done in order to bring an animal feed product to the market. In the case of NGN the seventh processing method will be used.

To complete this process, two steps need to be done:

1: All relative dangers of the raw material used need to be identified with regard to the origin of the material and the animal safety status of the area in which the processing method will be used.

2: Daily sampling of the end product during a period of thirty days, which complies with the following:

i) Samples taken immediately after processing: *Clostridium perfringens*: none in 1 g

ii) Samples taken after storage: *Salmonella*: none in 25g: n=5, c=0, m=0, M=0

Enterobacteriaceae: n=5, c=2, m=10, M=300 in 1 g.

In which: n= amount of samples

m= threshold value of the amount of bacteria present

M= maximum amount of bacteria, the amount should always be smaller

c= amount of samples in which the bacteria count is acceptable if between m and M, as long as the other samples are not bigger than m.[5]

CLOSTRIDIUM PERFRINGENS

Clostridium perfringens is found in the intestinal tracts and faeces of animals and humans, as well as in soil. These bacteria may survive in soil as spores for several months.[6] There are 'heat resistant' and 'heat sensitive' spores, where the 'heat resistant' ones survive heating at 100 ºC for 60 min and the 'heat sensitive' do not.[7] There are five types of *C. perfringens*: type A to E. All of the *C. perfringens* types produce a number of potent, immunologically distinct exotoxins which cause the local and systemic effects seen in enterotoxaemias in animals and humans.[6] Type A is the main important one for food borne diseases in humans and causes mainly watery diarrhea.[8]

ENTEROBACTERIACEAE, *SALMONELLA*

The Enterobacteriaceae which are of veterinary importance include *Escherichia coli, Salmonella* serotypes, *Yersinia* species as the major enteric and systemic pathogens, and *Proteus* species, *Enterobacter aerogenes* and *Klebsiella pneumoniaea* as the opportunistic pathogens. *Salmonella* serotypes infect many mammals, birds and reptiles, mainly after consumption, and are excreted through faeces. They are present worldwide in water, soil, animal feeds, raw meat and offal, and vegetable material. The consequences of salmonellosis range from subclinical carrier status to acute fatal septicaemia.[6]

RESEARCH GOAL

The goal of this study was to determine the quantity of bacterial contents, which are present in the new products developed by NGN and whether this met the requirements of the Implementing Regulation (EU) number 142/2011.

HYPOTHESIS

H0 = The NGN feed does not meet the two steps of the seventh process of the Implementing Regulation (EU) nr 142/2011.

Ha = The NGN feed does meet the two steps of the seventh process of the Implementing Regulation (EU) nr 142/2011.

MATERIAL AND METHODS

For step one: a literature study was done to investigate which microbes are known to be present in the raw material used, in this case the insects (*Hermetia illucens*, *Alphitobius diaperinus* and *Tenebrio molitor*), the white fish (whiting and codfish) and the herbs (marigold, curcuma, chamomile, garlic, aniseed, cardamom, cinnamon, dandelion, rooibos, peppermint, thyme and nettle). The outcome of this study would indicate if NGN should invest in any further in vitro research in the future for the food safety status of their feeds. This first step of this research had no effect on which microbes were to be tested in step two.

For step two: samples of 25 grams from the end product by NGN were collected over five weeks. NGN took samples from fish and reptile feeds immediately after production. During production the feeds were exposed to a heating step of 90 ºC. The samples were produced and packaged under non-sterile, conditions. To make sure unbiased samples were used, NGN sent in total twenty-one samples of each feed from five different batches. The first batch consisted one sample. Further batches consisted of five samples. The samples were tested the day after production, after a storage of 17 hours at ambient temperature.

BACTERIOLOGICAL EXAMINATIONS

A 10^{-1} dilution was created by diluting 25 grams of feed in 225 ml of Buffered Peptone Water (BPW). Afterwards tenfold dilutions were made in 9 ml peptone physiological saline solution tubes until 10^{-5}.

To indicate the level of micro-organisms in the samples from the new products by NGN, a total aerobic plate count was performed. The total aerobic plate count (TAC) was performed by incubation for 72±3 hours at 30±1 ºC on Petrifilm Aerobic Count Plates (AC), as validated by the Association Française de Normalisation (AFNOR). Counting was performed on plates containing 15 to 300 colonies.[9]

For the detection of *Clostridium perfringens* the ISO guidelines were used[10,11] after an accumulation with Rapid Perfringens medium (RPM)[9]. This detection method was first performed on an identified strain of *C. perfringens* from the laboratory as a positive control. Detection was performed for living bacteria as well as spores. Spores were activated by heating ten separate tubes, with five milliliters of the -1 dilution each, in an 80 ºC water bath for 10 minutes.[12,13] To detect the *C. perfringens*, one milliliter of the -1 dilution was added to an RPM tube and cultivated under anaerobe conditions for 48±2 hours at 46 ºC. If a positive reaction, a stormy fermentation reaction, was observed in the tubes after 18-24 hours[14], the tubes were immediately processed. Inoculation was performed on Tryptose Sulfite Cycloserine (TSC)-agar and cultivated under anaerobe conditions for 20±2 hours at 37 ºC. Five specific colonies

(characteristics of the colonies were compared with the positive control) were taken from the TSC plates and inoculated on a new TSC plate under the same conditions. Confirmation of these colonies was performed by transferring a small amount of the colony material with an inoculation loop into 10 ml Thioglycollate (TG) medium tubes and cultivating these for 24±2 hours under anaerobe conditions at 37 ºC. Afterwards five drops from the TG tubes, that showed bacterial growth (turbulence), were transferred to tubes containing a Durham tube and 9 ml of Lactose Sulfite (LS) broth. These LS tubes were cultivated for 24±2 hours in a 46 ºC water bath. The LS tubes that showed black coloring and formation of gas in the Durham tube indicated the presence of *C. perfringens*.

ISO guideline 6579 was used for the detection of Salmonella. [15]

Enterobacteriaceae enumeration was performed by incubation for 24±2 hours at 37 ºC on Petrifilm Enterobacteriaceae Count Plates (EB), as validated by the AFNOR.

STATISTICAL ANALYSIS

For the total aerobic plate count an independent samples t-test was performed to compare the mean value of all samples for the fish and reptile feed. An ANOVA test with a Tukey's range test was done to compare the mean values of each batch per individual feed. A probability value (p-value) lower than 0.05 was considered significant. Statistics were performed in IBM SPSS Statistics 25.

RESULTS

STEP 1: THE LITERATURE RESEARCH

INSECTS

The literature search demonstrated that information on microbial presence on edible insects and insect-based foods is limited. The articles found showed that the microbial quality of the insects is still not fully revealed.[16] In general, the taxonomy of insect pathogens is separate from vertebrate pathogens and can be regarded as harmless to humans. Insects also have a high diversity of associated micro-organisms, probably not pathogen for humans, in their gut flora. This microbiological composition can be affected by degutting or fasting the insects for one or two days. Spores of various micro-organism may be present, including micro-organisms that grow on edible insect products. These can contribute to the degradation of edible products.[3]

In a laboratory experiment by Klunder et al. (2012) bacterial spores were found to survive when boiling *Tenebrio molitor* larvae in water. Even after boiling, the spores have the potential to germinate, and the bacteria to grow in favorable conditions, like temperatures around 30 ºC and a moist environment. This might cause food spoilage. The spore-forming bacteria found in this experiment were present in the insect gut and on the skin and were likely to have been soil-borne. The most frequent fungi found were species of *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* and *Phycomycetes* of which *Aspergillus*, *Penicillium* and *Fusarium* are associated with mycotoxin production. [3]

The research by C. Garofalo et al. (2017) showed microbial counts (log cfu g^{-1}) of <2.00 for *Enterobacteriacea,* total mesophilic aerobes, lactic acid bacteria, *Clostridium perfringens* spores and yeasts in dried *Tenebrio molitor*. Futhermore microbial counts of 2.21 ±0.20 and 2.30±0.20 were reported for molds. *Salmonella* spp. and *Listeria monocytogenes* were absent in 25 g.[12]

D. Vanderweyer et al. (2017) described the microbial quality of fresh edible mealworms was tested by comparing the bacterial community composition of *Tenebrio molitor* from different production cycles and rearing companies in The Netherlands and Belgium. Mealworm communities were mainly dominated by *Spiroplasma* and *Erwinia* species. *Spiroplasma* and *Erwinia* are generally not considered as foodborne pathogens.[16] The research by J. Jung et al. (2018) also found *T. molitor* specific *Spiroplasma* species.[17] The *T. molitor* of one company in the D. Vanderweyer et al. (2017) study was found to harbor some Enterobacteriaceae species: *Cronobacter,* a human pathogen. A spore-forming class of *Clostridia* was also found*,* which might be pathogenic as well. There was also a noticeable differences in the bacterial community composition found between different mealworm rearing companies and *T. molitor* production cycles from the same company. [16]

Another article by D. Vanderweyer et al (2017) described that a considerable log reduction can be obtained after blanching *Tenebrio molitor.* This log reduction counts for the total viable count, Enterobacteriaceae, lactic acid bacteria, yeasts and molds and psychrotrophs, except for aerobic endospores. The total viable counts for all samples used were maximally 3.4 ± 0.8 log cfu/g.[18] In a different experiment by Vanderweyer et al. (2017) neither *Salmonella* nor *Listeria monocytogenes* were identified in *Tenebrio molitor.*[19]

J. Stoops et al. (2016) showed that *T. molitor*, sold for human consumption, contained *Propionibacterium* sp.*, Lactobacillus* sp.*, Streptococcus* sp.*, Haemophilus* sp.*,* Enterobacteriaceae*, Staphylococcus* sp., *Pseudomonas* sp. and *Clostridium* species.[20]

H. Jeon et al. (2011) did research on the bacterial communities in the gut of *Hermetia illucens* larvae, that had been fed three different diets, was analyzed. Some Enterobacteriaceae (*E. coli*, *Klebsiella* sp., *Enterobacter* sp. and others) were found. Also, *Enterococcus caccae*, *Clostridium* sp., *Bacillus* sp., *Streptococcus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. were detected. The results showed that the type of food directly influenced bacterial diversity in the larvae.[21,22]

N. Grabowski et al. (2017) studied the microbiology of processed edible insect products. These products contained dried *Alphitobius diaperinus* and *Tenebrio molitor*, and powdered *Hermetia illucens* and *T. molitor*. They were tested for Enterobacteriaceae, *Staphylococcos sp.*, *Bacillus sp.*, *Salmonella, Listeria monocytogenes, Escherichia coli* and yeasts and molds. *A. diaperinus* contained *Penicillium* spp.. *T. molitor* contained *Listeria ivanovii, Penicillium* spp.*, Mucor* spp.. *H. illucens* contained *Bacillus cereus, Aspergillus* spp.*, Cryptococcus neoformans*. All samples were negative for *Salmonella*, *L. monocytogenes*, *E. coli* and *S. aureus.* [23]

FISH

In the research by M.B. Hofda et al. the microflora of commercially farmed, harvested, and processed prerigor cod was characterized. The fish was obtained from Aukra, Norway and rinsed under water after filleting and kept on ice. A wide variety of bacteria, including *Photobacterium phosphoreum, Pseudomonas* spp., *Flavobacterium* sp., and *Bacillus cereus*, were obtained directly from the fish muscle at the beginning of the storage.[24]

HERBS AND SPICES

Spices and herbs could be exposed to microbial contamination during pre- and post-harvest. The traditional method of drying spices and herbs post-harvest is to spread them out on the ground to dry under the sun. This could potentially expose them to contamination. [25]

To gain insight on the prevalence of *Salmonella*, *Bacillus cereus, Clostridium perfringens* and *E. coli* in spices and herbs collected from retail and production premises in the United Kingdom, S.K. Sagoo et al. (2009) performed a detailed analysis. *Salmonella* spp. have been found in a

wide variety of spices and herbs. The rates of contamination range between 0.6% and 14%. In a few samples *E. coli* was found at levels of $>10^2$ in 25 gr.[25]

According to the International Commission on Microbiological Specifications for Foods (ICMF) (2005) spore forming bacteria (*B. cereus, C. perfringens*) are frequently found in spices and herbs, but usually at low levels.[26] *B. cereus* has also been reported in a variety of herbs and spices sampled in The Netherlands, at values $\geq 10^4$cfu g^{-1}.[25] *Bacillus subtilis* is commonly found in many spices. [26] Of the *Bacillus* spp. found at high levels in the study by S.K. Sagoo et al. (2009), 58% were identified as *B. subtilis*, which has only occasionally been the cause of food poisoning linked to spices.[25]

C. perfringens has also been found in several spices. Values of <500 cfu g^{-1} were usually found, rarely $\geq 10^3$cfu g^{-1}.[26] The prevalence of *C. perfringens* at $\geq 10^3$cfu g^{-1} in the UK in 2004 was 0.4% and in other studies it ranged from 0% to 7.6%.[25]

The Dutch Food and Consumer Product Safety Authority (NVWA) investigated from what types of food commodities *C. perfringens* could be isolated. This included also a group of spices and herbs. The results demonstrated that 410 (14%) out of the 2890 spices and herbs samples were positive for *C. perfringens.* In 98% of samples, from all the different groups, which were tested positive for *C. perfringens*, the concentrations did not exceed 1000 cfu g^{-1}. However, it was stated that the results did not derive from a random survey. The investigated samples were not taken randomly but within the framework of special projects and therefore fell into a small number of food commodity groups.[8]

STEP 2: NGN FEED SAMPLES

The results and outcome of the statistical analysis for the total aerobic plate count are shown below in Table 1.

Total aerobic plate count (TAC)		
Production batch	**F mean log(cfu/gr)**	**R mean log(cfu/gr)**
1	5.00*	6.12*
2	5.25±0.03	6.49±0.02
3	5.47±0.03	6.37±0.03
4	6.08±0.03	6.67±0.05
5	6.07±0.03	6.78±0.04
Mean	5.68±0.09	6.56±0.04

Table 1. Total aerobic plate count for every production batch of the fish and reptile feed produced by NGN. F=fish feed, R=reptile feed, cfu=colony forming units.

Table 1 shows that the mean value of the total aerobic plate count (TAC) for the reptile feed is significantly higher than the value for the fish feed ($p<0.001$).

For the fish feed there is a significant difference between the mean values of the production batches ($p<0.001$). There is no significant difference between batch four and five ($p=1.00$). **

For the reptile feed there is a significant difference between the mean values of the production batches ($p<0.001$). There is no significant difference between batch two and three ($p=0.14$), and batch four and five ($p=0.21$). **

*there is no mean ± standard error available for batch one, since there was only one value available.

**batch one wasn't included in the post hoc ANOVA test, since there was only one value available.

Table 2 shows whether the Enterobacteriaceae enumeration results of the samples followed the Implementing Regulation (EU) nr 142/2011. In almost all samples no Enterobacteriaceae were detected. In two samples an amount of Enterobacteriaceae colonies was found between zero and ten, therefore all the samples complied with the Implementing Regulation (EU) nr 142/2011. Because there were almost no colonies detected, no statistical analysis was performed.

Enterobacteriaceae		
Production batch	**F (n/N)**	**R (n/N)**
1	0/1	0/1
2	0/5	0/5
3	0/5	0/5
4	0/5	0/5
5	0/5	0/5
Total	0/21	0/21

Table 2. Compliance with The Implementing Regulation (EU) nr 142/2011 for Enterobacteriaceae for every production batch from the fish and reptile feed produces by NGN. N=number of total samples, n=number of samples which did not comply with The Implementing Regulation (EU) nr 142/2011, F=fish feed, R=reptile feed.

The results of the detection of *Salmonella* in the samples and whether they followed the Implementing Regulation (EU) nr 142/2011 are shown in Table 3. No *Salmonella* were found in any of the samples, therefore no statistical analysis was performed. For the detection of *Salmonella*, all the samples complied with the Implementing Regulation (EU) nr 142/2011.

Salmonella		
Production batch	**F (n/N)**	**R (n/N)**
1	0/1	0/1
2	0/5	0/5
3	0/5	0/5
4	0/5	0/5
5	0/5	0/5
Total	0/21	0/21

Table 3. Compliance with The Implementing Regulation (EU) nr 142/2011 for *Salmonella* for every production batch from the fish and reptile feed produces by NGN. N=number of total samples, n=number of samples which did not comply with The Implementing Regulation (EU) nr 142/2011, F=fish feed, R=reptile feed.

Table 4 shows whether the *C. perfringens* detection results of the samples comply with the Implementing Regulation (EU) nr 142/2011.

Clostridium perfringens				
Production batch	**F L (n/N)**	**F S (n/N)**	**R L (n/N)**	**R S (n/N)**
1	0/1	0/1	0/1	1/1
2	1/5	0/5	1/5	1/5
3	3/5	0/5	2/5	1/5
4	2/5	0/5	2/5	0/5
5	0/5	1/5	4/5	1/5
Total	6/21	1/21	9/21	4/21

Table 4. Compliance with The Implementing Regulation (EU) nr 142/2011 for *Clostridium perfringens* for every production batch from the fish and reptile feed produces by NGN. N=number of total samples, n=number of samples which did not comply with The Implementing Regulation (EU) nr 142/2011, F= fish feed, R= reptile feed, L= living, S= spores.

C. perfringens was more observed in the living (L) samples than in the spore (S) samples. In all batches *C. perfringens* was detected either in the living samples or the spore samples, or in both. Except for the first batch of the fish feed. No statistical analysis was performed, for the results only showed the presence (yes or no) of *C. perfringens*.

DISCUSSION

Information from literature is scarce when it comes to the base products used for the NGN feeds, as the microbial research on edible insects and insect-based foods is very limited. This also applies to the other ingredients for the feed. The Implementing Regulation (EU) nr 142/2011 states that "all relative dangers of the raw material used need to be identified regarding the origin of the material and the animal safety status of the area in which the processing method will be used". It is unknown where the insects, fish and the herbs used for the products of NGN originate from, how they are produced, what the husbandry conditions are and how they are processed for the product. This makes it difficult to find conclusive literature regarding the risk of the use of these products for the specific feed. Especially for fresh whiting no literature was found to indicate the possible risks.

According to the literature some *Bacillus* sp.[21,22,23,24,25,26], mainly *Bacillus cereus*[23,24,25,26], could be present in the base products of the feed and serve as a potential risk. Since these are heat resistant endospore forming bacteria, it would be wise to have the NGN feed researched for the presence of this bacteria. D. Vanderweyer et al. (2017) also states that bacterial spores and their survival need special attention, whatever way edible insects are processed and whatever insect species is considered.[18]

The literature states that *Streptococcus* sp.[20,21,22], *Pseudomonas* sp.[20,21,22,24], *Staphylococcus* sp.[20,21,22,23], *Flavobacterium* sp.[24] may also be a potential risk for the feed and should be looked into. Fungi and molds could be found in the base products according to the literature.[3,12,18,23] The fungi and molds, which are associated with mycotoxin production were mainly *Aspergillus*, *Penicillium* and *Fusarium*.[3,23] This could be a potential risk for the feed, especially since it is a semi-moist feed[4]. More research is vital on this subject, especially regarding the shelf life of the product and the impact (the amount of) bacteria, fungi and molds would have on this matter.

With regard to the bacteriological examinations, the following results are of interest. For the total aerobic plate counts, some statistically significant differences were found between the mean values of the production batches for the fish and the reptile feed. It was assumed that no changes to the production process by NGN were made during the sampling for this research, that may have caused the differences in these results, yet this was not confirmed and should be investigated. The mean value of the total aerobic plate counts for the reptile feed were significantly higher than for the fish feed. If the two feeds were produced separately, the different ingredients of the feeds might account for the difference. The two feeds contained the same insects but a different by-product. This makes it likely that this difference in total aerobic plate count could be attributed to the various by-products. The literature also states the potential for contamination of spices and herbs[25]. Therefore, the herbs and spices might be a bigger contamination source than the white fish for the NGN feeds. The packaging of the feed samples was done under non-sterile conditions. Therefore, the feed could also have been contaminated by the packaging. To exclude this source of contamination, it is important that in future microbial examinations, the feed should be collected and packaged in a sterile manner.

It is recommended to add a heating or cooling step to the production of insect-containing products, for it is likely to have obtained contaminated insects.[3] The absence of *Salmonella* or other Enterobacteriaceae in the sampled feed, makes it likely that the heating step of 90 ºC, done by NGN, is enough to exterminate these bacteria. Especially since Enterobacteriaceae could be present in the insects, based on the literature[12,16,20,21,22]. According to the available literature *Salmonella* was not detected in the insects[12,19,23], but could be present in the spices and herbs[25].

Clostridium perfringens was detected in all batches, except for the first batch of the fish feed. They were found directly from the product or through activation of spores present in the product. It is advisable to have the *C. perfringens*, cultivated from the samples, tested for the sequence of the DNA to confirm that it is *C. perfringens* and which type. The presence of *C. perfringens* in the feed does not comply with The Implementing Regulation (EU) nr 142/2011. In the literature, *C. perfringens* has been described in *T. molitor*[12] and in spices and herbs[8,25,26]. *Clostridium* sp. were detected in *T. molitor*[16,20] and *H. illucens*[21]. These *Clostridium* sp. could contain *C. perfringens.* The presence of *C. perfringens* in the samples by NGN could therefore be assigned to the possibility of this bacteria being present in the base products used. Furthermore, it is important to eliminate the possibility of the feed being contaminated by the process of producing the feed. This could be a motivation for NGN to check their hygiene protocol.

It is important to notice that the bacteriological examinations done during this research were different from the Implementing Regulation (EU) nr 142/2011. First, the Implementing Regulation (EU) nr 142/2011 states that the samples should be tested immediately after processing or storage. It is not entirely clear what that means and therefore it is uncertain if the immediate testing after processing or storage was achieved during this research. In this research it was also not possible to test samples for 30 days, due to the limited time available for a master research study. Instead it was decided to have five test days with samples from five production weeks. Having more sample testing days gives a higher probability of detecting (more) bacteria than in the five days during this research. Therefore, to officially comply with the Implementing Regulation (EU) nr 142/2011, all the 30 sample testing days should be performed.

During the research 25 grams of sampled feed were diluted in 225 ml, which gives 0.1 gr product/ml. The detection of *C. perfringens* was done by using one milliliter of the diluted product, which gives an indication of bacteria present in 0.1 gram of product, according to the ISO guidelines.[10,11] This was done five times for every batch which makes a total of 0.5 gram of product tested for the presence of *C. perfringens*. Not in every sample *C. perfringens* was

detected. The Implementing Regulation (EU) nr 142/2011 states that in one gram of product, no *C. perfringens* should be present. In theory, there is still a chance that in the samples that were negative for *C. perfringens* in this research, *C. perfringens* would have been present if the entire one gram of product was tested.

This research was performed in an unofficially certified laboratory by an unofficially certified laboratory technician. Therefore, the results from this research can merely be used as an indication instead of official evidence for the Implementation Regulation (EU) nr 142/2011.

CONCLUSION

Literature on food safety is scarce when it comes to the base products (*T. molitor, H. illucens, A. diaperinus,* cod, whiting, spices and herbs) used for the new fish and reptile feeds produced by NGN. In the available literature, it became clear that these base products might contain a wide range of micro-organisms with a potential risk for animal or human health. Especially heat resistant spore producing bacteria (*C. perfringens* and *B. cereus*) need special attention. The samples from the products produced by NGN did not contain *Salmonella* or other Enterobacteriaceae but did most likely contain *C. perfringens*. NGN is therefore advised to investigate where this contamination with *C. perfringens* came from and to determine a strategy on how to eliminate these bacteria from the feed. The presence of *C. perfringens* and other microbiological risks regarding the bacteria, fungi and molds found in this study should be researched by an officially certified laboratory. Therefore, the NGN feed does not meet the two steps of the seventh process of the Implementing Regulation (EU) nr 142/2011 (H0) and therefore the products are not yet ready for the market.

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