agriculture

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Clostridia in Insect Processed Animal Proteins—Is an Epidemiological Problem Possible?

Tomasz Grenda [1,*], Krzysztof Kwiatek [1], Magdalena Goldsztejn [1], Magdalena Sapała [1], Nina Kozieł [1] and Piotr Domaradzki [2]

[1] Department of Hygiene of Animal Feeding Stuffs, National Veterinary Research Institute, Partyzantow 57, 24-100 Pulawy, Poland; kwiatekk@piwet.pulawy.pl (K.K.); magdalena.kozak@piwet.pulawy.pl (M.G.); magdalena.grabczak@piwet.pulawy.pl (M.S.); nina.koziel@piwet.pulawy.pl (N.K.)

[2] Department of Commodity Science and Animal Raw Materials Processing, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland; piotr.domaradzki@up.lublin.pl

* Correspondence: tomasz.grenda@piwet.pulawy.pl

Simple Summary: In the last few decades, insect feed has been considered as a potential future resource of sustainable material for animal feeding. Insect feed is recognized as contributing to dietary demands, such as the nutritional composition and valuable amino acids profile, and is rich in micronutrients. The aim of this study was the evaluation of insect processed animal protein (IPAP) contamination by *Clostridium* spp.—the spore-formers that are the most difficult to eliminate during the thermal processes of feed production. In particular, we screened for the occurrence of pathogenic species of Clostridia. The study was conducted on experimental poultry feeds with insect PAP (IPAP) added. The differences between the *Clostridium* spp. contamination levels in the control (without the addition of IPAP) and experimental (with the addition of IPAP) groups were monitored. The *Clostridium* spp. level in feed compounds with the addition of IPAP were found to be significantly higher in comparison to the control groups. Genes determining the botulinum toxin production in one sample of IPAP and the occurrence of phenotypically and genetically similar strains to *C. botulinum* species were noted. Considering the microbiological safety of IPAP and expanding the possibility of its use in livestock animal feeding, it seems to be reasonable to provide a complex risk assessment on the potential distribution of Clostridia, to assure the safety and sustainable development of the IPAP industry and to answer whether this is an epidemiologically significant problem.

Abstract: The aim of this study was the evaluation of the insect processed animal protein (IPAP) contamination level by *Clostridium* spp. Particularly, we screened for the occurrence of pathogenic species of Clostridia. The samples of IPAP were derived from yellow mealworm (*Tenebrio molitor)* and black soldier fly (*Hermetia illucens*) available in the Polish market. The IPAPs were added to experimental feeds for poultry. The differences between the contamination levels of the control (without the addition of IPAP) and experimental (with the addition of IPAP) groups were monitored. The samples were also examined by culture and PCR-based methods to detect 16S rDNA and genes determining botulinum toxin (BoNT) production. Statistical significance was noticed among the feed with the IPAP addition, as well as an increase of contamination by *Clostridium* spp. In one sample of IPAP, the occurrence of *ntnh* and *bont/D* genes determining the production of BoNT/D was noticed. However, a positive result was noticed only at the step of the liquid culture; the *Clostridium botulinum* type D strain was not isolated. Phenotypically, and according to the 16S rDNA analysis, genetically similar strains to *C. botulinum* species were isolated. Considering the microbiological safety of IPAP and expanding possibility of its use in livestock animal feed, it seems to be reasonable to provide complex risk assessment on the potential transfer of Clostridia into feed compounds, to assure the safety and sustainable development of insect PAP industry.

Keywords: *Clostridium* spp.; contamination level; BoNT-producing Clostridia; PCR; insect PAP

Citation: Grenda, T.; Kwiatek, K.; Goldsztejn, M.; Sapała, M.; Kozieł, N.; Domaradzki, P. Clostridia in Insect Processed Animal Proteins—Is an Epidemiological Problem Possible? *Agriculture* **2021**, *11*, 270. https://doi.org/10.3390/agriculture11030270

Academic Editors: Vito Laudadio and Vincenzo Tufarelli

Received: 19 February 2021
Accepted: 18 March 2021
Published: 21 March 2021

1. Introduction

The incorporation of insect processed animal protein (IPAP) into livestock rations could be considered as a sustainable strategy to enhancing animal nutrition. A high nutritional value, environmental friendliness, economic benefits, and decreased vulnerability of insects to diseases are the most emphasised advantageous of insects use for feed production. IPAP is recognized as approaching dietary demands, such as the nutritional composition and valuable amino acid profile [1], and is rich in the micronutrients Ca, Mg, Zn, Fe, and Cu. The ecologically advantageous aspects of insect rearing have been raised, e.g., the environmental impacts on greenhouse gas production and water consumption by insects are comparable or lower to livestock animals [2]. The economic benefits of insect farming were also discussed, such as the small land area required to produce one kg of protein and the possibility to bioconvert organic waste material [3]. The high potential biomass increase is also profitable, as is the low feed cost, the ability to rear in high density, the short duration of the larval stage, and the low vulnerability to diseases. Among several insect species tested as animal feed, the most promising are the black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) [3–5].

Despite the remarkable advantages of incorporating IPAP as a component of animal feed or IPAP as feed ingredients for formulation of livestock diet, there are safety concerns. Some insects contain repellent or toxic chemicals as a defence mechanism; many species cause allergic reactions and may contribute to thiamine deficiency [6,7]. In the literature, serious bacterial and viral diseases, such as cases of botulism, bovine tuberculosis, influenza A, and parasitosis caused by entomophagy, were described [5,8,9]; however, these cases were observed in natural environment, not after feeding animals with IPAP derived from farming insects. Pathogenic species, e.g., *Clostridium botulinum*, *Staphylococcus aureus*, *Pseudomonas aureginosa*, and *Bacillus cereus*, were isolated from insect guts [10–15]. Potential safety risks associated with inclusion of PAP should be considered, and a risk assessment should be provided for future perspectives.

Spore-forming bacteria should be considered, in particular, for the safety aspects due to technological problems with their elimination. Many literature reports indicated insects as a potential source of pathogenic Clostridia and their toxins, especially causing botulism cases [14–19]. Botulism is a paralyzing disease of animals, birds, and humans with symptoms including a flaccid paralysis (weakness or slackness) in various muscles caused by the most potent, naturally occurring in the environment toxins produced by Clostridia—particularly, *C. botulinum,* which characterizes a high heterogeneity. Based on the physiological differences and 16S rRNA gene sequencing, the strains of this pathogen are classified into four metabolic groups: group I comprises all type A and proteolytic strains of types B and F, group II all type E and nonproteolytic strains of types B and F, group III type C and D strains, and group IV type G strains. In addition to *C. botulinum,* other microorganisms considered as nontoxigenic are also related to these groups (including some toxigenic strains of *Clostridium butyricum*, *Clostridium baratii,* and the recently described *Clostridium sporogenes*) [20–24].

The detection of *C. botulinum* using laboratory diagnostics for botulism poses a great challenge due to the diversity and occurrence of nontoxigenic, phenotypically similar species. According to their metabolic properties and 16SrRNA analysis, *C. sporogenes* and the recently described *Clostridium tepidum* [20] are considered to be related to group I, *Clostridium beijerinckii*, *C. butyricum,* and *Clostridium taeniosporium* to group II, *Clostridium novyi* to group III, and *Clostridium subterminale* and *Clostridium schirmacherense* to group IV [21–24]. Research conducted on the group I, II, and III genomes showed that *bont* genes are frequently plasmid-borne. Group III contains numerous plasmids carrying different toxin genes. These genes could be also found in other Clostridia, and some are able to move among different plasmids though the same physiological group [21,25].

Horizontal transferring was observed within and between species of *Clostridium*. Mobile element occurrence, particularly group III genomes (the most frequently cause of botulism cases in animals), is associated with plasticity of the genome and gene transfer

event mobility [26]. In addition to *C. botulinum*, unusual strains from related species have been described that were able to produce botulinum toxins (BoNTs), and toxins produced by them were causative agents of botulism cases. These include the strains of *C. baratii*, *C. butyricum*, *Clodtridium argentinense*, and *C. sporogenes*. Botulinum cluster genes were also detected in Gram-negative bacteria. Evidence was cited, taking the example of *Chryseobacterium piperi* isolated from freshwater sediments; however, the biological activity of *bont*-related genes was not noted [27].

The BoNT-related toxin (BoNT/En) produced by *Enterococcus faecium* was described by Zhang et al. [28]. The obtained results indicated a 29–38% identity with other BoNTs. However, the new toxin described was not toxic to mice. The most frequently seen botulism symptoms in animals are caused by toxins produced by *C. botulinum* group III, which are BoNT/C and D and their mosaic variants [18]. The lethality of botulinum toxins depends on the animal species' individual sensitivity, e.g., for mice, the median toxic dose is estimated at 0.1 ng/kg, whilst the mouse lethal dose (MLD) of BoNT/C for cattle is 3.88 MLD/kg [29]. Birds are sensitive to BoNT/C and resistant to BoNT/D, e.g., the MLD50 of BoNT/C reported for fowl is 2000/kg, whilst BoNT/D is as high as 100.000/kg. Cattle are sensitive to both BoNT/C and BoNT/D. Among avian species, chickens, pheasants, and turkeys are more sensitive to BoNT/C when compared with ducks [30,31].

Legislation on the use of insects as a feed differs widely between countries worldwide. In Europe, legislation regarding the use of IPAP in animal feeding seems to be affected by Bovine Spongiform Encephalopathy (BSE), which causes a real threat to consumer safety. New challenges in ensuring the safety of feed and food force dynamic legislative changes [32,33]. According to Regulation (EU) 2017/893 [34], feeding with nonruminant PAP is authorized for aquaculture animals only. Fat from insects is permitted for feeding every animal species, and PAPs are only permitted for aquaculture. The extent of authorization for use is currently under discussion and is one of the main problems in the sustainability of feed production with the addition of IPAP. The processing standards and microbiological requirements are described in the 142/2011 EU Regulation [35].

According to the aforementioned regulation, insects could be submitted to the processing methods (one–five and seven). Samples of the final products taken during or on withdrawal from storage at the processing plant must comply with the standards limited only to Enterobacteriaceae number in 1 g and *Salmonella* absence in 25 g. According to the same regulation, there are requirements regarding processing method number seven. In this method, the sampling of the final product is conducted on a daily basis over a period of 30 production days in compliance with the microbiological standards, including requirements limited to the Enterobacteriaceae number in 1 g (n = 5; c = 2; m = 10 colony-forming units (cfu)/g; M = 300 cfu/g, where: n is the number of samples to be tested, m the threshold value for the number of bacteria in 1g; M the maximum value for the number of bacteria in 1g, and c = the number of samples in the bacterial count that are between m and M, the samples still being acceptable if the bacterial count of the other samples is m or less), the *Salmonella* absence in 25 g (n = 5), and *C. perfringens* absence in 1 g (described in detail in Annex IV, p. "G" of the 142/2011 EU Regulation) [36].

The influence of *Clostridium* spp. on the PAP quality is limited only to one processing method and to the *C. perfringens* species. The *Clostridium* spp. influence on microbiological safety is discussed; however, this is not framed in the legislative requirements. In comparison to other pathogenic Clostridia, e.g., *C. botulinum, C. perfringens* possess characteristic phenotypic features and are easy to detect and control [36]. However, the *C. perfringens* occurrence does not exclude contamination by BoNT-producing Clostridia, which are harmful for animal and human health.

The aim of this study was the evaluation of the contamination level of *Clostridium* spp. due to the addition of IPAP, derived from mealworms (*T. molitor*) and black soldier flies (*H. illucens*), to experimental poultry compounds. Particularly, the occurrence and distribution of pathogenic species of Clostridia were screened, as these are phenotypically and genetically similar to *C. botulinum* species.

2. Materials and Methods

2.1. Sample Collections

The samples for this study's purposes were collected in the frame of the project "Development of a strategy for the use of alternative sources of insect protein in animal nutrition enabling the development of its production in the territory of the Republic of Poland" financially supported by the National Centre for Research and Development in the years 2018–2020. The aim of the project was to develop a strategy for the use of alternative protein sources for entities operating in this field in the territory of the Republic of Poland. This project involved the assessment of environmental and nutritional requirements, as well as the impact of potential threats to poultry (broiler chickens) health when using feed with insect protein.

This communication focused only on the influence of the IPAP addition to feed compounds on the uprising of the Clostridia level and potential distribution of pathogenic species from this genus in feed compounds (without an analysis of the particular percentage of the addition, which was considered only in the nutritional experiment).

The analyses were carried out on 52 samples, which included 10 samples of IPAPs derived from yellow mealworms (*T. molitor*)—5 samples—and black soldier flies (*H. illucens*)—5 samples—and 42 samples of compound feed with different levels of insect PAP additions (Table 1). The samples of the compound feed with a "0%" IPAP addition was classified as the control groups, whilst the others with different contents of insect IPAP addition were the experimental groups.

Table 1. Insect processed animal protein (IPAP) and feed samples analyzed during the experiment.

Number of Samples	Mealworm IPAP	Black Soldier IPAP	Compound Feed Samples with and without Addition of Mealworm IPAP		Compound Feed Samples with and without Addition of Black Soldier Fly IPAP	
			Control Group	Experimental Group	Control Group	Experimental Group
1	MM1	BSFM1	MCS1-0% *	MCS-6.5% *	BSFCS1-0% *	BSFCS-20% *
2	MM2	BSFM2	MCS2-0%	MCS-13%	BSFCS2-0%	BSFCS-30%
3	MM3	BSFM3	MCS3-0%	MCS-16%	BSFCS3-0%	BSFCS-40%
4	MM4	BSFM4	MCG1-0%	MCS-19%	BSFCG1-0%	BSFCG-17%
5	MM5	BSFM5	MCG2-0%	MCS-25%	BSFCG2-0%	BSFCG-25%
6			MCG3-0%	MCG-5.5%	BSFCG3-0%	BSFCG-34%
7			MCF1-0%	MCG-11%	BSFCF1-0%	BSFCF-13%
8			MCF2-0%	MCG-13.5%	BSFCF2-0%	BSFCF-20%
9			MCF3-0%	MCG-16%	BSFCF3-0%	BSFCF-27%
10				MCG-22%		
11				MCF-4.5%		
12				MCF-9%		
13				MCF-11%		
14				MCF-13%		
15				MCF-17%		
Total number of samples	52					

*—Percentage of IPAP; in particular, feed compounds, e.g., 0% means feed compound without the addition of IPAP, substituted with soybean meal, and 6.5% means feed compound with the addition of 6.5% of insect IPAP. MM 1, 2, 3, etc.—mealworm IPAP of different batches. BSFM 1, 2, 3, etc.—black soldier IPAP of different batches. MCS, MCG, and MCF—different types of feed compounds (S: starter, G: Grower, and F: Finisher) with an adequate percent of soy meal substitution by mealworm IPAP. BSFCS, BSFCG, and BSFCF—different types of feed compounds (S: starter, G: Grower, and F: Finisher) with an adequate percent of soy meal substitution by black soldier IPAP.

The mentioned IPAP samples were derived from different registered European producers proceeding in accordance with the 142/2011 EU Regulation [35] (Table 2), and the mixtures of IPAP (each mixture was composed of five IPAP samples derived from mealworms or black soldier flies) were added to particular feed compounds for poultry (broiler chickens) according to the recipes included in Tables 3 and 4, with the nutritional

composition corresponding to the needs at each stage of chicken growth described in Tables 5 and 6. All analyzed PAP and compound feed samples possessed a moisture content below 15%.

Table 2. Origin of IPAP used in the experiment.

IPAP Samples	Producer Location (Country)
MM1	Poland
MM2	Poland
MM3	Denmark
MM4	France
MM5	Spain
BSFM1	Poland
BSFM2	Germany
BSFM3	Ireland
BSFM4	France
BSFM5	France

MM1, MM2 etc. means IPAP derived from mealworm (*Tenebrio molitor*); BSFM1, BSFM2 etc. means IPAP derived from black soldier fly (*Hermetia Illucens*).

All feed compounds were prepared by the Experimental Station of the Department of Animal Nutrition of Poznań University of Life Sciences (Miedzychód, Poland). The IPAP samples were collected between November 2018 and April 2019. All the IPAP samples were stored according to the producers demands, and the expiration date was not exceeded. Each feed compound batch (with all components described in Tables 3 and 4) was cold-pelleted (without steam and at a maximum of 65 °C) using a Scorpion pellet press (BMG Pelleting Experts, Gdańsk, Poland). The pelleting conditions were monitored and maintained at a constant ampere draw of the load meter for the mill motor to the consistency of the pelleting conditions. The distribution of potentially pathogenic Clostridia was investigated using molecular biology methods. Before the microbiological analysis, samples were stored maximally for 2 to 3 weeks at room temperature (20 °C). According to previously published reports, this period should not significantly affect the Clostridial spores level stability in aerobic conditions [37–40].

2.2. Cultures

Each 10 g of representative IPAP or feed compound sample was inoculated into bottles with 90 mL of TPGY (Tryptone Peptone Glucose Yeast Extract Broth): 50-g/L casein enzymic hydrolysate, 5-g/L peptic digest of animal tissue, 20-g/L yeast extract, 4-g/L dextrose, and 1-g/L sodium thioglycolate, with a final pH of 7.0 ± 0.2 at 25 °C. After the inoculum preparation, the thermal treatment was conducted at 70 °C for 15 min in a water bath. The inoculated samples were subjected to incubation at 37 °C for 72 h. After this period, the growth of the anaerobes was assessed, and 1 mL of liquid culture from 90-mL tubes was inoculated to the tubes with 10-mL fresh TPGY broth for an additional one day of incubation.

Subsequently, 10 µL of these cultures were spread on Willis-Hobbs agar (10-g/L peptic digest of animal tissue, 10-g/L meat extract, 5-g/L sodium chloride, 12-g/L lactose, 0.032-g/L neutral red, 10-g/L skim milk powder, 2-g egg yolk powder, and 10-g/L agar, with a final pH of 7.0 ± 0.2 at 25 °C). The same aliquot of cultures were also spread on FAA (Fastidious Anaerobe Agar: 23-g/L peptone, 5-g/L sodium chloride, 1-g/L soluble starch, 0.4-g/L sodium bicarbonate, 1-g/L glucose, 1-g/L sodium pyruvate, 0.5-g/L L-cysteine HCl × H_2O, 0.25-g/L sodium pyrophosphate, 1-g/L L-arginine, 0.5-g/L sodium succinate, 0.01-g/L hemin, 0.001-g/L vitamin K, 2-g egg yolk powder, and 12-g/L agar, with a final pH of 7.2 ± 0.2 at 25 °C). The plates with Willis-Hobbs and FAA were incubated anaerobically at 37 °C for 48 h. The colonies were evaluated for their surface; shape; size; color and lipolytic, proteolytic, or lecitinolytic features. The isolates were additionally subjected to Gram staining.

Table 3. Recipes of feed compounds with and without the addition of mealworm IPAP.

	MCS-0%	**MCS-6.5%**	**MCS-13%**	**MCS-16%**	**MCS-19%**	**MCS-25%**	**MCG-0%**	**MCG-5.5%**	**MCG-11%**	**MCG-13.5%**	**MCG-16%**	**MCG-22%**	**MCF-0%**	**MCF-4.5%**	**MCF-9%**	**MCF-11%**	**MCF-13%**	**MM-17%**
Substitution of post-extraction soybean meal (%)	0	25	50	62.5	75	100	0	25	50	62.5	75	100	0	25	50	62.5	75	100
Feed Ingredients																		
Maize (%)	30	30	30	30	30	30	30	30	30	30	30	30	20	20	20	20	20	20
Wheat (%)	29.39	33.05	35.32	37.58	39.28	42.83	31.40	34.13	37.47	39.02	40.83	43.35	46.85	49.15	51.75	53.19	54.67	57.59
Post-extraction soybean meal (%)	34	25	17	12	8.5	0	30	22.5	15	11.25	7.5	0	24	18	12	9	6	-
Soybean oil (%)	2.5	2	1.5	0.9	0.6	-	4.5	4	3.5	3.2	3	2.5	5.5	5	4.5	4.2	4	3.5
Mealworm IPAP (%)	0	6.5	13	16	19	25	0	5.5	11	13.5	16	22	0	4.5	9	11	13	17
Monocalcium phosphate (%)	1.5	1.1	0.9	0.8	0.6	0.3	1.3	1.2	0.7	0.8	0.5	0.3	1.2	0.9	0.7	0.60	0.4	0.2
Fodder chalk (%)	1.25	0.8	0.7	0.6	0.4	0.2	1.25	1.2	0.8	0.7	0.6	0.2	1.25	1.1	0.80	0.7	0.5	0.3
Sodium bicarbonate (%)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Fodder salt (%)	0.2	0.15	0.1	0.1	0.05	0.05	0.2	0.12	0.1	0.05	-	-	0.2	0.15	0.1	0.05	0.05	0.05
Mineral and vitamin premix (%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lysine 78%	0.25	0.4	0.45	0.5	0.55	0.6	0.25	0.4	0.48	0.5	0.57	0.65	0.18	0.3	0.3	0.35	0.37	0.45
Methionine 99%	0.24	0.3	0.3	0.32	0.32	0.32	0.16	0.2	0.2	0.23	0.25	0.25	0.12	0.15	0.15	0.2	0.2	0.2
Threonine 98%	0.02	0.05	0.05	0.05	0.05	0.05	0.04	0.1	0.1	0.1	0.1	0.1	0.05	0.10	0.05	0.06	0.06	0.06
Choline chloride	0.09	0.09	0.09	0.90	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Total (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

MCS means the recipe of feed compound with mealworm IPAP addition; MCS 0%, 6.5%, etc. means a recipe with different percentages of mealworm IPAP additions.

Table 4. Recipes of feed compounds with and without the addition of black soldier fly IPAP.

	BSFCS-0%	**BSFCS-20%**	**BSFCS-30%**	**BSFCS-40%**	**BSFCG-0%**	**BSFCG-17%**	**BSFCG-25%**	**BSFCG-34%**	**BSFCF-0%**	**BSFCF-13%**	**BSFCF-20%**	**BSFCF-27%**
Substitution of Post-Extraction Soybean Meal (%)	0	50	75	100	0	50	75	100	0	50	75	100
Feed Ingredients												
Maize (%)	30	30	30	30	30	30	30	30	20	20	20	20
Wheat (8%)	28.75	30.36	29.16	27.78	31.46	33.36	40.68	41.21	46.51	50.51	49.66	48.61
Post-extraction soybean meal (%)	34	17	8.5	0	30	15	7.5	0	24	12	6	0
Soybean oil (%)	3	-	-	-	4.5	2.5	3	2.5	5.5	3.5	3	2.5
Black soldier fly IPAP (%)	0	20	30	40	0	17	25	34	0	13	20	27
Monocalcium phosphate (%)	1.5	0.4	0.3	0.2	1.3	0.4	0.5	0.3	1.2	0.3	0.2	0.2
Fodder chalk (%)	1.25	0.5	0.3	0.2	1.25	0.5	0.6	0.2	1.25	0.4	0.3	0.3
Sodium bicarbonate (%)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Fodder salt (%)	0.2	0.05	0.05	0.05	0.2	0.1	-	-	0.2	0.05	-	-
Mineral and vitamin premix (%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Lysine (%)	0.25	0.42	0.45	0.60	0.25	0.35	0.57	0.65	0.18	0.30	0.35	0.4
Methionine (%)	0.24	0.28	0.3	0.3	0.16	0.25	0.19	0.19	0.12	0.15	0.15	0.15
Threonine (%)	0.02	0.15	0.15	0.15	0.04	0.15	0.1	0.1	0.05	0.1	0.1	0.1
Choline chloride (%)	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
Total (%)	100	100	100	100	100	100	100	100	100	100	100	100

BSFCS means a recipe of feed compound with black soldier fly IPAP addition; BSFCS 0%, 20%, etc. means a recipe with different percentages of mealworm IPAP addition.

Table 5. Nutritional composition of feed compounds with and without the addition of mealworm IPAP.

	MCS-0%	**MCS-6.5%**	**MCS-13%**	**MCS-16%**	**MCS-19%**	**MCS-25%**	**MCG-0%**	**MCG-5.5%**	**MCG-11%**	**MCG-13.5%**	**MCG-16%**	**MCG-22%**	**MCF-0%**	**MCF-4.5%**	**MCF-9%**	**MCF-11%**	**MCF-13%**	**MM-17%**
EM, kcal/kg	2990	2992	2990	3000	3000	3013	3120	3120	3128	3120	3138	3120	3180	3180	3180	3180	3180	3180
EM, MJ/kg	12.35	12.53	12.51	12.56	12.56	12.62	13.12	13.12	13.14	13.12	13.17	13.12	13.28	13.28	13.28	13.28	13.23	13.23
Total proteins (%)	22.00	21.96	22.11	22.02	22.08	22.00	20.00	20.17	20.00	20.13	20.00	20.00	18.30	18.45	18.21	18.39	18.26	18.29
Lysine (%)	1.35	1.38	1.35	1.36	1.35	1.35	1.25	1.29	1.26	1.27	1.27	1.26	1.03	1.08	1.00	1.04	1.00	1.00
Methionine (%)	0.58	0.60	0.59	0.60	0.62	0.62	0.47	0.48	0.54	0.49	0.51	0.53	0.45	0.46	0.47	0.46	0.50	0.52
Met+ Cys (%)	1.04	1.07	1.02	1.03	1.08	1.03	0.90	0.90	0.90	0.90	0.90	0.90	0.82	0.84	0.81	0.82	0.83	0.83
Threonine (%)	0.80	0.82	0.80	0.82	0.84	0.88	0.80	0.81	0.80	0.80	0.80	0.80	0.67	0.73	0.66	0.67	0.66	0.66
Tryptophan (%)	0.25	0.25	0.23	0.26	0.23	0.23	0.22	0.23	0.21	0.24	0.22	0.21	0.21	0.22	0.20	0.20	0.20	0.20
Crude fiber (%)	2.88	3.88	3.69	3.94	3.71	3.98	2.78	3.67	3.22	3.70	3.50	3.65	2.78	3.51	3.19	3.56	3.37	3.55
Raw fat (%)	5.96	5.16	5.44	5.29	5.37	5.54	6.31	6.93	7.83	7.15	7.38	8.60	7.67	7.47	7.55	7.53	7.57	7.18
Linoleic acid (%)	0.83	2.63	4.14	5.24	6.07	7.73	0.86	2.37	3.89	4.57	5.26	6.89	0.73	1.96	3.20	3.76	4.31	5.41
Crude ash (%)	5.86	5.06	4.65	4.45	3.99	3.44	5.52	4.91	4.38	3.88	3.98	3.30	5.26	4.77	4.17	3.50	3.51	3.10
Total Ca (%)	1.10	1.08	1.18	1.31	1.25	1.30	1.00	1.08	1.00	1.08	1.00	1.00	0.86	0.90	0.90	0.91	0.87	0.86
Total phosphorus (%)	1.52	1.58	1.65	1.68	1.66	1.79	1.51	1.57	1.57	1.64	1.63	1.70	1.17	1.19	1.25	1.27	1.26	1.30
Absorbable phosphorus (%)	0.47	0.52	0.60	0.63	0.67	0.68	0.50	0.50	0.51	0.58	0.57	0.60	0.40	0.41	0.47	0.48	0.48	0.52
Na (%)	0.17	0.22	0.24	0.27	0.28	0.30	0.17	0.18	0.20	0.21	0.23	0.25	0.17	0.18	0.19	0.19	0.20	0.23

Table 6. Nutritional composition of feed compounds with and without the addition of black soldier fly IPAP.

	BSFCS-0%	**BSFCS-20%**	**BSFCS-30%**	**BSFCS-40%**	**BSFCG-0%**	**BSFCG-17%**	**BSFCG-25%**	**BSFCG-34%**	**BSFCF-0%**	**BSFCF-13%**	**BSFCF-20%**	**BSFCF-27%**
EM, kcal/kg	2990	2990	3000	3013	3100	3100	3100	3100	3160	3160	3160	3160
EM, MJ/kg	12.35	12.56	12.56	12.62	12.98	12.98	12.98	12.98	13.22	13.22	13.22	13.22
Total proteins (%)	22.00	22.11	22.08	22.00	20.07	20.00	20.00	20.00	18.00	18.11	18.16	18.19
Lysine (%)	1.35	1.35	1.35	1.35	1.17	1.18	1.18	1.18	1.03	1.00	1.00	1.00
Methionine (%)	0.58	0.64	0.67	0.67	0.47	0.62	0.60	0.60	0.45	0.49	0.50	0.52
Met + Cys (%)	1.04	1.02	1.02	1.03	0.95	0.89	0.88	0.88	0.82	0.87	0.87	0.88
Threonine (%)	0.80	0.80	0.84	0.88	0.80	0.84	0.84	0.84	0.67	0.70	0.70	0.71
Tryptophan (%)	0.25	0.23	0.23	0.23	0.22	0.21	0.22	0.21	0.21	0.20	0.20	0.20
Crude fiber (%)	2.88	3.81	4.23	4.78	2.78	3.62	4.00	4.44	2.78	3.45	3.78	4.12
Raw fat (%)	6.96	8.15	10.99	13.84	6.31	9.66	10.99	13.07	7.67	9.20	10.70	12.23
Linoleic acid (%)	0.83	6.29	9.00	11.27	0.86	3.89	5.26	6.89	0.73	3.20	4.31	5.41
Crude ash (%)	5.86	4.87	4.98	5.23	5.52	4.38	3.98	3.30	5.26	4.17	3.51	3.10
Total Ca (%)	1.10	1.30	1.57	1.84	1.00	1.00	1.00	1.00	0.86	0.90	1.00	1.25
Total phosphorus (%)	1.52	1.65	1.66	1.79	1.51	1.65	1.78	1.83	1.17	1.25	1.26	1.30
Absorbable phosphorus (%)	0.47	0.66	0.87	0.88	0.50	0.58	0.70	0.80	0.40	0.47	0.59	0.64
Na (%)	0.17	0.31	0.32	0.33	0.17	0.24	0.29	0.30	0.17	0.20	0.25	0.26

The contamination level of Clostridia was evaluated according to PN-R-64791:1994 [41] by preparation serial decimal dilutions (1:10; 1:100; 1:1000, etc.) of the analyzed samples, which were inoculated in aliquots of 1 mL into two tubes of TPGY broth. One was subjected to thermal treatment at 70 °C for 15 min. The tubes were incubated at 37 °C for 72h, and, after this time, about 10µl of liquid cultures were spread on Willis-Hobbs and FAA agar surfaces.

After incubation at 37 °C for 48 h, the characteristic phenotypical features were evaluated, and Gram staining was applied. The level of contamination was adequate to the dilution on which the Clostridia growth was observed. The obtained results were expressed as log(cfu/g). The limit of detection corresponded to 0 log(cfu/g). The range of detection was from 0 log(cfu/g) up to 6 log(cfu/g). All the cultures were prepared in duplicate, and anaerobic conditions were obtained by using an anaerogen Oxoid system (Thermo, USA). All media were prepared in the Department of Media Preparation of the National Veterinary Research Institute in Pulawy (Pulawy, Poland). All the procedures were previously described and validated [36,42,43].

2.3. DNA Isolation

Genomic DNA was isolated from 1 mL of liquid cultures, and several colonies were obtained from agar plates. The DNA was extracted with a Genomic Mini AX Bacteria kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. The amount of DNA used in the PCR reaction varied between 1 and 25 ng. The DNA amount was estimated using a Nicolet Evolution 300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNA was frozen at −20 °C or directly subjected to PCR analysis.

2.4. Characterization of Clostridia Strains by Amplification and Sequencing of 16S rDNA

For the characterization of the conservative fragments of 16S rDNA in unidentified anaerobic strains (cultured on FAA and Willis-Hobbs agar), the PCR method with primers according to Vaneechoutte et al. [44] was used. The reaction volume was 25 µL, and the reagent constituents were 5 µL of DNA matrix, 2.5 µL of 10× Taq buffer with KCl (Thermo Fisher Scientific, Waltham, MA, USA), 4-mM $MgCl_2$, 200-µM dNTP, 0.3 µM of each primer, and 1.25-U/25 µL Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The reaction was staged as follows: after the initial denaturation at 95 °C for 5 min, there were 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min.

Finally, a 10-min extension period at 72 °C was included. The obtained length of the products was about 1500 bp. A sequence analysis of the PCR products was commissioned from Genomed (Warsaw, Poland). The FASTA (text-based format for representing either nucleotide sequences or amino acid sequence) files produced thereby were analyzed with the BLAST (NCBI—National Center for Biotechnology Information, Bethesda, MD, USA) algorithm against the nucleotide collection database. A BLAST search was performed for the comparison of amplicons with the highest sequence similarity scores corresponding to species from NCBI.

2.5. Detection of BoNT-Producing Clostridia Genes

Analyses were performed using methods enabling *ntnh* and *bont/A–F* gene detection. The DNA extracted from liquid cultures and suspected isolates previously characterized by the culture method (showing lipolytic properties and the subterminal location of spores) and 16S rDNA analysis was examined.

The *ntnh* gene is common in all BoNT-producing Clostridia toxin types and was detected using a set of seven primers and the TaqMan probe described by Raphael and Anreadis in 2007 [45] and with the reagent concentrations, as previously described [34].

After obtaining positive results, the DNA was subjected to determination of the *bont/A–F* genes with primers, probes, and a temperature profile described by Kirhner et al., 2010 [46].

The reactions were conducted using a version of singleplex real-time PCR and prepared with the subsequent reagents: 5 µL of DNA, 4 µL of LightCycler TaqMan Master (Roche, Basel, Switzerland), 0.7 µM of each primer, and 0.24 µM of TaqMan probe. The real-time PCR was performed using a LightCycler 2.0 thermocycler (Roche, Basel, Switzerland) on the following thermal cycling profile: activation of the *Taq* DNA polymerase at 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 40 s.

All reactions were conducted on a LightCycler 2.0 instrument (Roche, Basel, Switzerland).

2.6. Statistical Analysis

A statistical analysis was applied to the *Clostridium* spp. level results, expressed as log(cfu/g). In order to verify a normal distribution of collected data from all experimental and control groups, the Shapiro-Wilk test [47] was used. On the basis of the obtained results, the analysis of the statistical influence of IPAP's addition to animal feed on the *Clostridium* spp. level was examined using the Mann-Whitney *U* test [48]. During the statistical evaluation of the IPAP influence on the *Clostridium* spp. level, three null hypotheses were tested:

(1) The addition of IPAP increases the *Clostridium* spp. level.

This hypothesis was investigated using all samples from the control groups and all samples from the experimental groups independently of the IPAP origin (derived from mealworms or black soldier flies).

(2) The addition of IPAP derived from mealworms significantly increases the *Clostridium* spp. level in experimental feed compounds.

This hypothesis was investigated using samples from the control groups (adequate in composition to feed compounds with a mealworm IPAP addition) and the experimental group with the mealworm PAP addition.

(3) The addition of insect IPAP derived from black soldier flies significantly increases the *Clostridium* spp. level.

This hypothesis was investigated using samples from the control groups (adequate in composition to feed compounds with a black soldier fly PAP addition) and the experimental group with the black soldier fly IPAP addition.

The results were expressed as *p*-values and considered to be significant when the *p*-value was <0.05.

3. Results

3.1. Contamination Level and Statistical Analysis Results

The differences of the contamination levels by *Clostridium* spp. between the control and experimental groups were significant. The level of contamination ranged from 0 log(cfu/g) to 2 log(cfu/g) in the control groups, whilst in the experimental groups, from 1 log(cfu/g) to 4 log(cfu/g). The contamination of insect meals varied from 0 log(cfu/g) to 5 log(cfu/g). The majority of samples from the experimental groups showed higher levels of Clostridia contamination, independently of the percentage of IPAP inclusion level (Figure 1).

This graph showed the differences between the contamination levels by the Clostridia of PAP and compound feed samples from the control and experimental groups. A higher level of contamination was observed for samples with the addition of insect PAP. The statistical data regarding the significance of the observed differences are included in Table 7.

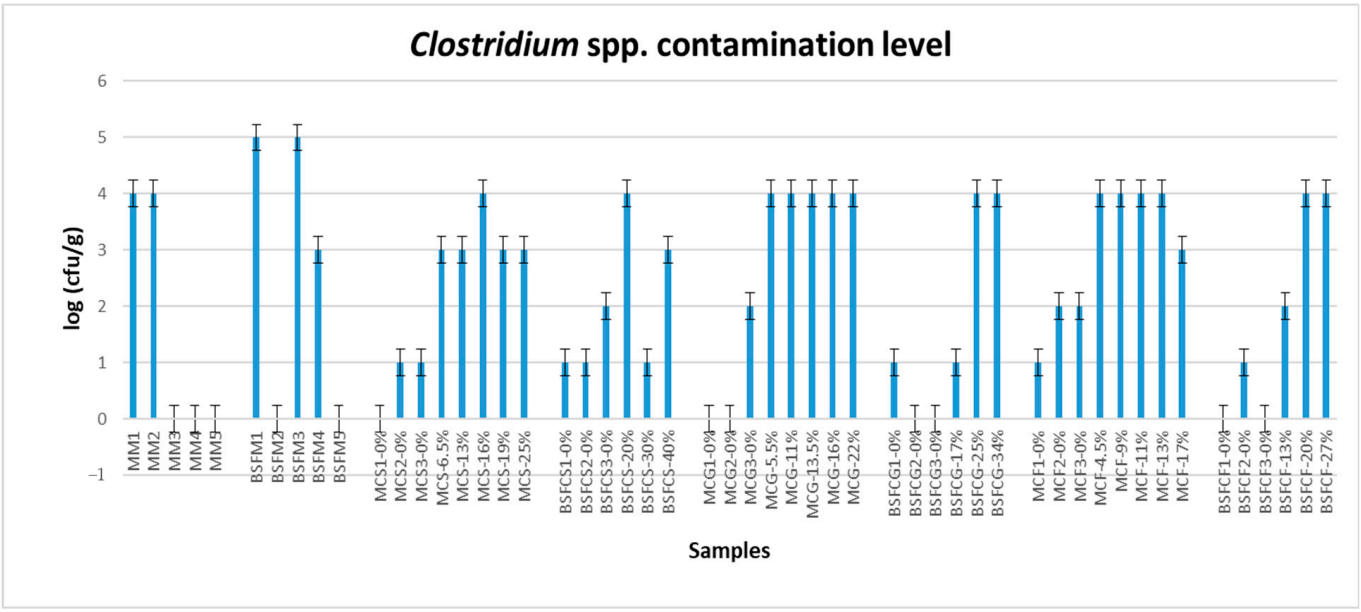

Figure 1. Contamination level of the processed animal protein (PAP) and experimental feed samples by *Clostridium* spp. cfu: colony-forming units.

Table 7. Statistical significance of the IPAP addition origin on the *Clostridium* spp. level in feed compounds.

Sample Types	Groups	Starter		Grower		Finisher		Mann-Whitney U Test Result (*p*-Value)
		Sample Name	Results (log cfu/g)	Sample Name	Results (log cfu/g)	Sample Name	(log cfu/g)	
Feed compounds with and without addition of mealworm and black soldier fly IPAP	Control group	MCS1-0%	0	MCG1-0%	0	MCF1-0%	1	$p < 0.001$
		MCS2-0%	1	MCG2-0%	0	MCF2-0%	2	
		MCS3-0%	1	MCG3-0%	2	MCF3-0%	2	
		BSFCS1-0%	0	BSFCG1-0%	0	BSFCF1-0%	1	
		BSFCS2-0%	1	BSFCG2-0%	0	BSFCF2-0%	2	
		BSFCS3-0%	2	BSFCG3-0%	2	BSFCF3-0%	2	
	Experimental group	MCS-6.5%	3	MCG-5.5%	4	MCF-4.5%	4	
		MCS-13%	3	MCG-11%	4	MCF-9%	4	
		MCS-16%	4	MCG-13.5%	4	MCF-11%	4	
		MCS-19%	3	MCG-16%	4	MCF-13%	4	
		MCS-25%	3	MCG-22%	4	MCF-17%	3	
		BSFCS-20%	4	BSFCG-17%	1	BSFCF-13%	2	
		BSFCS-30%	1	BSFCG-25%	4	BSFCF-20%	4	
		BSFCS-40%	3	BSFCG-34%	4	BSFCF-27%	4	
Feed compounds with and without addition of mealworm IPAP	Control group	MCS1-0%	0	MCG1-0%	0	MCF1-0%	1	$p = 0.001$
		MCS2-0%	1	MCG2-0%	0	MCF2-0%	2	
		MCS3-0%	1	MCG3-0%	2	MCF3-0%	2	
	Experimental group	MCS-6.5%	3	MCG-5.5%	4	MCF-4.5%	4	
		MCS-13%	3	MCG-11%	4	MCF-9%	4	
		MCS-16%	4	MCG-13.5%	4	MCF-11%	4	
		MCS-19%	3	MCG-16%	4	MCF-13%	4	
		MCS-25%	3	MCG-22%	4	MCF-17%	3	
Feed compounds with and without addition of black soldier fly IPAP	Control group	BSFCS1-0%	0	BSFCG1-0%	0	BSFCF1-0%	1	$p = 0.003$
		BSFCS2-0%	1	BSFCG2-0%	0	BSFCF2-0%	2	
		BSFCS3-0%	2	BSFCG3-0%	2	BSFCF3-0%	2	
	Experimental group	BSFCS-20%	4	BSFCG-17%	1	BSFCF-13%	2	
		BSFCS-30%	1	BSFCG-25%	4	BSFCF-20%	4	
		BSFCS-40%	3	BSFCG-34%	4	BSFCF-27%	4	

MM 1, 2, and 3, etc.—mealworm PAP of different batches. BSFM 1, 2, and 3, etc.—black soldier PAP of different batches. MCS, MCG, and MCF—different types of feed compounds (S: starter, G: Grower, and F: Finisher) with an adequate percentage of soy meal substitution by mealworm PAP. BSFCS, BSFCG, and BSFCF—different types of feed compounds (S: starter, G: Grower, and F: Finisher) with an adequate percentage of soy meal substitution by black soldier PAP. cfu: colony-forming units.

The obtained data from the *Clostridium* spp. level examination were not normally distributed according to the results of the Shapiro-Wilk test; therefore, further analyses were conducted using the nonparametric Mann-Whitney *U* test for independent groups. The statistical analysis by using the Mann-Whitney *U* test showed a strong influence of the

addition of insect PAP independently of the origin (mealworm or black soldier fly) on the *Clostridium* spp. level increase ($p < 0.001$; Table 7).

The statistical analysis of the results obtained for feed compounds with the mealworm IPAP addition showed a significant influence on the *Clostridium* spp. level increase ($p < 0.001$; Table 7). The statistical significance of the black soldier IPAP addition on the *Clostridium* spp. level increase was also strong; however, this was lower than in the case of IPAP independently of its origin and mealworm PAP addition ($p = 0.003$; Table 7).

3.2. Results of the 16S rDNA Analysis

Clostridia strains were isolated from 40 samples, and 50 isolates were subjected to the 16S rDNA analysis. In the tables below (Tables 8–10), the results corresponding to the adequate species with the highest score and percentage of similarity were performed. The results of the 16S rDNA analysis suggested the occurrence of saprophytic and potentially pathogenic isolates, also strains genetically and phenotypically similar to *C. botulinum* species.

Table 8. Sequencing analysis results for the IPAP samples.

MM *		**BSFM ***	
Sequencing Results According to BLAST Analysis	**Sequence ID; % Similarity**	**Sequencing Results According to BLAST Analysis**	**Sequence ID; % Similarity**
Clostridium tepidum	NR_157639.1; 97%	*Peaniclostridium sordelli*	LC15549.1; 97%
Clostridium botulinum, group I	CP046450.1; 95%	*Paraclostridium bifermentans*	MF510818; 97%
		Peaniclostridium sordelli	LC15549.1; 97%
		Paraclostridium bifermentans	MF510818; 97%
		Clostridium tepidum	NR_157639.1; 98%
		Clostridium botulinum group I	CP046450.1; 97%

* MM—mealworm IPAP and BSFM—black soldier fly IPAP.

Table 9. Sequencing results for the mealworm feed compound samples.

Feed Type	**Control Group, Mealworm Feed Compounds: MCS, MCG, and MCF-0%**		**Experimental Group, Mealworm Feed Compounds: MCS, MCG, and MCF**	
	Sequencing Results According to BLAST Analysis	**Sequence ID; % Similarity**	**Sequencing Results According to BLAST Analysis**	**Sequence ID; % Similarity**
Starter	*Paraclostridium bifermentans*	MN75886.1; 97%	*Clostridium tepidum*	NR_157639.1; 98%
			Paraclostridium bifermentans	MN758863.1; 97%
			Paraclostridium benzoelyticum	MN999977.1; 98%
			Clostridium sporogenes	KY962939.1; 90%
Grower	*Paraclostridium bifermentans*	MN75886.1; 97%	*Paraclostridium bifermentans*	MK894870.1; 98%
			Clostridium tepidum	NR_157639.1; 98%
Finisher	*Paraclostridium bifermentans*	MN75886.1; 97%	*Paraclostridium sulfidigenes*	MF967245.1; 96%
			Paraclostridium benzoelyticum	LC515632.1; 97%
			Paraclostridium bifermentans	MF510818.1; 98%

Table 10. Sequencing results for the black soldier fly feed compound samples.

Feed Type	Control Group, Black Soldier Fly Feed Compound: HCS, HCG, and HCF-0%		Experimental Group, Black Soldier Fly Feed Compound: BSFCS, BSFCG, and BSFCF	
	Sequencing Results According to BLAST Analysis	**Sequence ID; % Similarity**	**Sequencing Results According to Blast Analysis**	**Sequence ID, % Similarity**
Starter	*Clostridium sporogenes*	MT356160.1; 95%	*Clostridium botulinum* group I	(CP031097.1; 93%),
	Clostridium sartagoforme	MN646980.1; 90%	*Paraclostridium benzoelyticum*	(MT510437.1; 100%)
Grower	*Clostridium sporogenes*	MT356160.1; 95%	*Paraclostridium benzoelyticum*	(MT510437.1, 97%)
			Clostridium sporogenes	(MT356160.1; 95%)
Finisher	*Clostridium sporogenes*	MT356160.1; 95%	*Clostridium bifermentans*	(DQ680018.1; 97%),
			Paraclostridium benzoelyticum	(MT510437.1; 97%)
			Clostridium tepidum	(NR_157639.1, 97%)
			Clostridium sporogenes	(MT356160.1; 100%)

According to the BLAST analysis, in the samples from the mealworm PAP (MM), the obtained sequences were similar to the *C. tepidum* and *C. botulinum* species (Table 8). The isolates from Black soldier fly PAP (BSFM) showed sequences similar to the *P. sordelli*, *P. bifermentans*, *C. tepidum*, and *C. botulinum* strains (Table 8).

The isolates from the control group of mealworm feed compounds, according to BLAST, were classified as *Paraclostridium bifermentans* species (Table 9). This sequence appeared in all strains isolated from the samples of the mentioned group. The experimental group of mealworm feed compounds showed the presence of strains with sequences characteristic for *C. tepidum*, *P. bifermentans*, *P. benzoelyticum*, *C. sporogenes*, and *Paraclostridium sulfidigenes* (Table 9).

The isolates from the control group of black soldier fly feed compounds were classified as *C. sporogenes* and *C. sartagoforme* species (Table 10). Isolates from the experimental group of black soldier fly feed compounds contained 16S rDNA sequences characteristic for *C. botulinum*, *P. benzolyticum*, *C. sporogenes*, *C. bifermentas*, and *C. tepidum* (Table 10).

Sequences characteristic for *C. tepidum* were detected in all PAP samples and experimental groups (Tables 8–10).

3.3. Occurrence of BoNT-Producing Clostridia

The analysis of DNA isolated from the liquid cultures in TPGY broth and strains phenotypically similar to *C. botulinum* species showed the occurrence the *ntnh* and *bont/D* genes only in one sample of BSFM meal. The phenotypically similar strain isolated from the same sample, however, did not possess the mentioned genes. According to the 16S rDNA analyses, the sequence was similar to the *C. tepidum* species (97% similarity). The lack of isolates does not exclude the potential occurrence of the *C. botulinum* group III strain and BoNT/D. During the PCR analysis, two genes of botulinum cluster were considered to increase the probability of botulinum cluster detection. However, the positive result was obtained only at the step of the liquid culture.

4. Discussion

Insects are considered as evolutionarily adapted in animal diets and, therefore, can be a possible commercial feed source. However, the potential biological threat should be considered before enabling their use in animal feeds. The insect microbiome varies and depends on the rearing, diet, and farming conditions. The time of the digestive passage of insects depends on their species and type of diet. The processing of insects is conducted after emptying the intestinal contents; however, it is possible to find some remains of frass and surface containing microorganisms that could be pathogenic for

humans and animals [3]. These bacteria could be transferred from insects through PAP to feed and, subsequently, to food. The genera of insect microbiota can contain *Staphylococcus, Streptococcus, Bacillus, Proteus, Pseudomonas, Escherichia, Micrococcus, Lactobacillus,* and *Acinetobacter* [49–51]. Researchers noticed that unprocessed insects showed a high level of Enterobacteriaceae and the occurrence of spore-forming bacteria [52]. According to a report prepared by the Scientific Committee of Belgian Federal Agency for Safety of The Food Chain [53], the insects specifically farmed for food production exhibited high contamination values (up to 10^7 cfu/g) of the total bacterial count and, also, high Enterobacteriacae and anaerobic bacteria levels for mealworms (*T. molitor*), locusts (*Locusta migratoria*), and Morio worms (*Zophobas morio*).

Our results showed that the level of *Clostridium* spp. turned out to be significantly higher in the experimental groups after the addition of the insect PAP. A statistical analysis showed a strong significance of mealworm PAP ($p < 0.001$) and black soldier PAP ($p = 0.003$) additions considered separately and simultaneously ($p < 0.001$) (Table 7). It means that each stated null hypothesis was proved by the obtained results. Although the genus *Clostridium* mainly consists of nonpathogenic species, a high contamination may be associated with pathogenic Clostridia. We can assume that a higher probability of pathogenic Clostridia occurrence could be observed after the IPAP addition. Of the about 200 *Clostridium* spp. known to exist, approximately 30 have been associated with human and animal diseases. Commonly found in soil, marine sediment, and mammalian intestinal tracts, these Gram-positive bacilli are known to cause infections ranging from cellulitis to septicaemia [54], and some of them are able to produce botulinum toxins—the etiological factor of botulism in humans and animals [50].

The characteristic strains for *Clostridium* spp. were explored by us more thoroughly, and the 16S rDNA results with the highest scores showed similarity to sequences belonging to *C. botulinum, C. sporogenes, C. tepidum, C. sartagoforme, P. bifermentans* (the most frequently obtained result), *P. sulfidigenes, P. benzoelyticum,* and *P. sordelli*, as listed in Tables 8–10. Besides *C. botulinum*, among the mentioned species, the strains of *C. sporogenes*, *P. bifermentans*, and *P. sordelli* are known to be pathogenic and cause serious health complications in humans and animals [54–61]. Some more information about the potential pathogenicity of these strains is mentioned below.

According to 16S rRNA PCR, we noticed the occurrence of *C. sporogenes* in the experimental group of feed compounds (Tables 9 and 10). This microorganism is relative to the *C. botulinum* group I strains. *C. botulinum* group I usually harbor botulinum neurotoxin (*bont*) genes on their chromosomes, while some carry these genes on large plasmids. Recent findings, however, show that *C. sporogenes* is not a nontoxic form of *C. botulinum* Group I [62,63]. Most strains of *C. botulinum* Group I form botulinum neurotoxin, whilst some *C. sporogenes* strains also form botulinum neurotoxin [59]. The transfer of plasmids possessing *bont* genes to resistant *Clostridium* spp. such as *C. sporogenes* could impact the biological safety for animals and humans. Strains of *C. botulinum* Group I (and, to a lesser extent, *C. sporogenes*) are a major cause of the three most frequent types of botulism in humans (foodborne, infant, and wound botulism) and are also responsible for botulism in animals [64,65]. *C. sporogenes* was also considered as the causative agent of secondary bacteraemia in immunocompetent patients [54].

Another species, that could be potentially pathogenic and, according to our results (Table 8), was detected in IPAP is *P. sordellii*. Infections caused by *P. sordellii* pose difficult clinical challenges and are usually fatal. Generally, a *P. sordellii* infection in animals can result in enteritis, omphalitis, and equine atypical myopathy [58]. In humans, a range of *P. sordellii* diseases arise after trauma; childbirth; and routine gynecological procedures, medically induced abortions, and injected drug use [56–58].

Another strain identified during our experiment was *P. bifermentas* (Tables 8 and 9). It is closely related to *P. sordelli* and is usually considered nonpathogenic; some reports indicates that it plays an important role in the pathology of human ulcerative colitis (UC) [66]. Kutsuna et al. [66] investigated the influence of the *P. bifermentans* PAGU1678

strain on the pathology of a UC mouse model and found it increased the UC pathosis scores, such as loose and bloody stools; the reduced diversity of fecal flora; the disappearance of the crypt structure of the distal colon tissue; destruction of the intestinal epithelial cells; atrophy of the colon; an increase of inflammation-related factors and inflammatory cytokines; a decrease in the concentration of short-chain fatty acids (acetic acid, propionic acid, and butyric acid) in feces; and an increase of the intestinal mucosal myeloperoxidase activity. Cases of pneumonia with associated empyema, septic arthritis, osteomyelitis, soft tissue infection, abdominal infections, brain abscess, bacteremia, and endocarditis were also reported [59–61].

The most interesting from the epidemiological aspect are the results suggesting the occurrence of *C. botulinum* strains and phenotypically similar strains. These results were verified using PCR methods for the detection of the *ntnh* and *bont* genes. The PCR results did not prove the occurrence of the genes determining botulinum toxin production. However, the 16S rDNA results did not exclude the potential occurrence of BoNT-producing Clostridia in the examined samples.

Simultaneously, real-time PCR results of the DNA analysis extracted from the TPGY broth suggested the occurrence of *ntnh* and *bont/D* genes in one sample of BSFM meal. From the mentioned sample, we were not able to isolate the *C. botulinum* strain. However, we isolated a strain with lipolytic properties, which, according to the 16 rDNA results, revealed 98% similarity to *C. tepidum* (NR_157639.1) (Table 8). The *C. tepidum* species was described by Dobritsa et al. in 2017 [20]. The *C. tepidum* species is considered to be phenotypically similar to *C. botulinum* species group I. According to Dobritsa et al. [20], *C. tepidum* is closely related to *C. sporogenes* and *C. botulinum* Group I based on the 16S rRNA gene sequence similarities. Our results of the 16S rDNA sequencing suggested that *C. tepidum* could be transferred from IPAP into the feed compounds, because it was only detected in the experimental and not in control groups (Tables 8–10).

Considering BoNT-producing Clostridia as a single species is now only a historical notion.

Significant progress has been made in understanding the structure and function of Clostridial neurotoxins. As demonstrated by the scattered phyletic distribution of neurotoxin-producing clostridia and the sequence similarities between different neurotoxin gene clusters, they appear to have undergone significant horizontal transfers between different species of Clostridium [67–69]. A horizontal transfer was also proven by the evidence of plasmid-encoded neurotoxin genes in numerous *C. botulinum* strains [68], as well as the existence of putative insertion sequences flanking the neurotoxin gene cluster [69]. The horizontal *bont* genes transfer often causes challenges in the laboratory diagnostics of botulism [22,70,71]. Insect larvae are considered to be vectors of *C. botulinum* group III. In the literature, many reports have described avian botulism cases caused by insects. Avian botulism is a serious problem in European countries, leading to significant economic losses [72]. The number of botulism outbreaks has been increasing in several European countries during the past two decades [72–74]. The factors behind this upsurge of avian botulism have not been identified, and currently, little is known about the epidemiology of the disease. However, one of them could be the occurrence of Clostridia spores in maggots. *C. botulinum* spores can actually survive for decades in the environment, and botulism can be recurrent in affected farms, implying an effect on the poultry industry [73]. Poultry manure, after processing in biogas plants, used as a fertilizer could cause the transmission of pathogenic Clostridia spores to the environment and epidemiological problems not only for poultry [75].

The literature data indicate a possible public health risk associated with botulism in animals. Rasetti-Escargueil et. al. [76] emphasized in their review that the high prevalence of botulism types C, D, and variants DC and CD in farmed and wild birds and, to a lower extent, in cattle, raises the risk of transmission to human beings. However, human botulism is much rarer than animal botulism, and botulism types C and D are exceptional in humans. Only 15 cases or suspected cases of botulism type C and one outbreak of botulism type D have been reported in humans to date [76].

The hypothesis that adult blowflies may carry *C. botulinum* cells between carcasses was suggested by Anza et al. [18]. They carried out a field experiment in which bird carcasses free of *C. botulinum* types C/D were kept in containers available only to necrophagous flying insects. The experiment was conducted in the wetlands, where avian botulism cases are frequent. They noticed that maggots bearing *C. botulinum* types C/D occurred in 27% of the examined carcasses during the period when botulism outbreaks were observed the most often [18]. A laboratory trial proved that adult necrophagous flies were vectors in spreading the botulism outbreaks [50]. In our study, we noticed the *ntnh* and *bont/D* genes responsible for botulinum protoxin type D production in the liquid culture of the BSFM PAP sample. Our results showed that an IPAP addition to the compound feed could significantly increase the level of Clostridia and raise the probability of pathogenic species occurrence.

A significant level of Clostridia and other microbiological contaminants in insect products was also observed by Garofalo et al. [16] They conducted experiments on different batches of eligible insect products marketed in the European Union and purchased from a company located in the Netherlands. These products contained powdered and whole mealworm larvae (*Tenebrio molitor*), small crickets (*Acheta domesticus*), and locusts (*Locusta migratoria*). They noticed the occurrence of Enterobacteriaceae, *Clostridium* spp., and *C. perfringens* species in all the analyzed samples at the level of <2 log(cfu/g).

The legislation of insect feed usage in Europe is stated by Regulation (EU) 2017/893 [34] amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and the Council and Annexes X, XIV, and XV to Commission Regulation (EU) No 142/2011 (implementing the regulation to 1069/2009) [33] regarding the provisions on processed animal protein. Only seven species of insects are currently authorized—among them, yellow mealworms (*Tenebrio molitor*) and black soldier flies (*Hermetia illucens*) are listed.

Frequently, PAP producers declare that their products are processed under low temperatures. However, potentially low temperature-processing could lead to spore germination and possible toxin production by pathogenic species, e.g., *C. botulinum* [77]. According to a description of method seven in the 142/2011 EU Regulation [33], any processing could be applied if it was authorized by a competent authority and the producer demonstrated that the relevant hazards were identified in the starting material. Additionally, if the capacity of the processing method was sufficient to reduce those hazards to a level that did not pose any significant risk to the public and animal health and the microbiological requirements were met regarding the absence of *C. perfringens* and *Salmonella* and limited the Enterobacteriaceae number on a daily basis over a period of 30 production days. We did not identify *C. perfringens* strains in all examined PAP samples. As it was mentioned previously, the absence of this microorganism and usage of 65 °C temperature (cold pelleting) does not exclude the occurrence of other pathogenic Clostridia, e.g., *C. botulinum* group I and phenotypically similar strains: *C. tepidum*, *C. sporogenes*, *C. bifirmentans*, etc., all strains described in this study. *Clostridium perfringens* is one of the easiest strains from *Clostridium* species to control and isolate.

The general hygiene requirements of PAP are authorized by Regulation No. 1069/2009 [78]. According to Regulation No 142/2011, insects are considered as category III and are treated in accordance with Section 1 of Chapter II of Annex X with the amendments included in Commission Regulation (EU) 2017/893 [32], and particular treatment methods are described in chapter III Annex IV of this regulation.

5. Conclusions

Feed products with the addition of insect PAP are likely to be considered as a sustainable animal protein source fulfilling the needs of the growing consumption of animal products in the near future. Our study showed preliminary, signalizing results of the potential pathogenic Clostridia distribution for experimental feed compounds. The significant increase in the Clostridia level indicates the occurrence of saprophytic and pathogenic species as more probable.

The literature reports and our results indicated the potential incidence of pathogens from *C. botulinum* species or genetically and phenotypically similar strains to this species (which could potentially acquire the ability of botulinum toxin production). New microbiological requirements are still under discussion. Taking into account the microbiological safety of insect PAP and expanding the possibility of its use in livestock animal feeding, these discussions should include a complex risk assessment on the potential distribution of Clostridia to assure the safety and sustainable development of the insect PAP industry and to decide whether insect PAP presents an epidemiologically significant problem.

Author Contributions: Conceptualization, investigation, methodology, and original draft preparation—T.G.; writing—review and editing and project supervision—K.K.; writing—review and editing and investigation—M.G. and M.S.; investigation—N.K.; and statistical analysis and investigation—P.D. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the National Centre for Research and Development 2018–2020 in the project frame GOSPOSTRATEG 1/385141/16/NCBIR/2018.

Data Availability Statement: The analyzed datasets are available from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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