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Potential determinants of *Clostridium* spp. occurrence in Polish silage

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Abstract

Introduction: Silage quality deteriorates with *Clostridium* spp. contamination, and if consumed, such silage jeopardises herd health and productivity. Minimising its occurrence reduces economic and animal welfare risks. The study investigated the influence of environmental and technological determinants on the *Clostridium* genus' occurrence in silage. **Material and Methods:** Analyses were conducted on 305 silage samples directly collected from farms located in all Polish provinces. Cultures and isolates were evaluated phenotypically and examined for occurrence of *Clostridium* spp., particularly *C. perfringens* and *C. botulinum* using PCR techniques. The results were statistically analysed using the χ^2 test for continuous and Student's *t*-test for non-continuous values. **Results:** The most influential effect on *Clostridium* spp. occurrence is exerted by factors potentially associated with primary production, like the type of fertilisation and the contamination level of the ensiled feed material. *Clostridium* spp. was detected in 232 (76%) samples, and *C. perfringens* strains, predominantly toxinotype A, in 79 (26%). *C. botulinum* occurrence was not detected. **Conclusions:** Deterioration of silage by clostridia could be prevented by a properly conducted ensiling process with the addition of starter cultures, but the presence of spores mainly depends on primary production and the extent of contamination of the feed material.

Keywords: *Clostridium botulinum*, *Clostridium perfringens*, Poland, silage, toxins.

Introduction

Clostridium spp. occurrence in feed material could cause disturbances in the ensiling process leading to deterioration or constitute an epidemiological risk for cattle. Silage as roughage represents the basis of the beef cattle diet. To prepare good quality silage, all the technological procedures in best production practice should be carried out and all principles applied (5). The overarching ensiling principle consists in fast inactivation of enzyme systems and termination of microbial activity.

During harvesting of feed material, the topsoil layer could transfer undesirable microorganisms like bacteria from the *Clostridium* genus. Some pathogenic members of this genus could colonise the digestive tract of cattle and be shed in excrement, which may contaminate the environment and introduce clostridial pathogens into milk (13). Many experimental data on deterioration of silage by *Clostridium* spp. and these bacteria's influence on the ensiling process have been reported in literature (13, 18, 20, 23, 25). However, the influence of the risk factors which are the hygienic aspects of primary production, the fertilisers used, the storage period of silage, and the intensity of its production is rarely described in literature and is underestimated. Taking into account the epidemiological aspects, pathogenic species like *C. perfringens* and *C. botulinum* are of great importance. *C. perfringens* is one of the most widespread bacteria and ubiquitous in soil, sewage, food, and faeces. *C. perfringens* is able to produce up to 30 toxins, and its strains are classified into seven categories (A to G) according to the combination of the major toxins α, β, ι, and ε, *Clostridium perfringens* enterotoxin (CPE), and necrotic enteritis B-like toxin (NetB) they produce (9). Besides the listed major toxins, numerous minor ones can be produced by this microorganism. In animals, the six toxinotypes A–F

cause numerous forms of enteritis and this is associated with enterotoxaemia in cattle. The recently described toxinotype G is able to produce NetB toxin. It is supposed to play an important role in necrotic enteritis in broiler chickens (6). Dynamic growth of the opportunistic species *C. perfringens* in the small intestine can cause an acute or peracute syndrome with a fatality rate close to 100% (6, 9).

C. botulinum is the microorganism able to produce the most lethal toxins (botulinum neurotoxins – BoNTs) found in the environment. The most frequently seen botulism symptoms in cattle are caused by BoNT/C and D and their mosaic variants. According to Moeller *et al.* (14), the median toxic dose (MTD_{50}) of BoNT/C for cattle is 0.388 ng/kg (3.88 mouse lethal doses/kg). This dose suggests that cattle are 12.88 times more sensitive to BoNT/C than mice on a per-kilogram basis. Botulism cases in cattle are very rare, however, they are economically damaging. Most of them are associated with feeding silage contaminated by this microorganism, BoNT/C or D, or their mosaic variants (17).

The aim of this study was evaluation of the occurrence of the *Clostridium* genus and its pathogenic species *C. perfringens* and *C. botulinum* in silage samples collected from all Polish provinces. The study focused on the potential environmental and technological determinants of the presence of these microorganisms in silage samples.

Material and Methods

Silage samples. The study was carried out on 305 silage samples which had been collected randomly and directly from farms located in all Polish provinces. Silage was collected during official surveillance of feeding stuffs in Poland carried out in the years 2016–2017. A total of 207 corn, 75 grass, 13 alfalfa, 2 beet pulp, 2 rye, 2 crushed grain, and 4 shamrock silage samples were gathered and examined (Table 1).

Approximately two thirds (207/305; 68%) of the samples were prepared with starter culture addition (Table 2) and 115/305 (38%) were prepared from feed material harvested from fields fertilised with manure or slurry (organic fertilisers) (Table 3). The storage period of collected samples varied from 1 to 24 months (Table 4). Samples were collected from farms with herd sizes ranging from 12 to 2,300 cows (Table 5).

Culture process. A 10 g portion of each sample was inoculated in two bottles with 90 mL of deoxygenated (10 min/100°C) tryptone peptone glucose yeast extract broth (TPGY; 50 g/L of casein enzymic hydrolysate, 5 g/L of peptic digest of animal tissue, 20 g/L of yeast extract, 4 g/L of dextrose, and 1 g/L of sodium thioglycolate with a final pH of 7.0 ±0.2) at 25°C and one of them was pasteurised at 70°C for 15 min. Subsequently, the samples were incubated at 37°C for 48 h under anaerobic conditions. After this time, a few drops of liquid culture were spread onto the surface of Willis–Hobbs agar (10 g/L of peptic digest of animal tissue, 10 g/L of meat extract, 5 g/L of sodium chloride, 12 g/L of lactose, 0.032 g/L of neutral red, 12 mL of egg yolk emulsion, and 10 g/L of agar with a final pH of 7.0 ±0.2) at 25°C and incubated anaerobically at 37°C for 48 h. Aerobic control of each sample was provided. Simultaneously, the *Clostridium* spp. contamination level and pH value of the silage samples were determined according to the Polish PN-R-64791 (19) and European EN-ISO 7218:2007 (8) standards. The contamination level was expressed as colony-forming units (cfu) per gram of silage in subsequent decimal dilutions of each sample (*e.g.* 10^1, 10^2, and 10^3 cfu/g).

Table 1. Examined silage samples

Province	Silage							
	Corn	Grass	Alfalfa	Beet pulp	Rye	Crushed grain	Shamrock	Total in provinces
Greater Poland	11	4	1	-	1	-	-	17
Holy Cross	14	4	3	-	-	-	-	21
Kuyavian-Pomeranian	12	3	2	2	-	1	-	20
Lesser Poland	5	4	-	-	-	-	-	9
Lower Silesian	10	5	1	-	1	-	2	19
Lublin	19	1	-	-	-	-	-	20
Lubuskie	12	8	-	-	-	-	1	21
Łódź	16	3	-	-	-	-	-	19
Masovian	18	4	-	-	-	-	-	22
Opole	14	4	2	-	-	-	-	20
Podlasie	16	4	-	-	-	-	-	20
Pomeranian	15	6	-	-	-	-	-	21
Silesian	17	2	-	-	-	-	-	19
Subcarpathian	10	7	2	-	-	-	1	20
Warmian-Masurian	13	6	1	-	-	-	-	20
West Pomeranian	5	10	1	-	-	1	-	17
Total	207	75	13	2	2	2	4	305

Table 2. Silage enriched with starter culture

Province	Silage (enriched/total by material)							
	Corn	Grass	Alfalfa	Beet pulp	Rye	Crushed grain	Shamrock	Enriched/total in provinces (%)
Greater Poland	7/11	3/4	0/1	-	0/1	-	-	10/17 (59)
Holy Cross	11/14	4/4	2/3	-	-	-	-	17/21 (81)
Kuyavian-Pomeranian	8/12	2/3	1/2	0/2	-	0/1	-	11/20 (55)
Lesser Poland	5/5	4/4	-	-	-	-	-	9/9 (100)
Lower Silesian	6/10	2/5	0/1	-	0/1	-	0/2	8/19 (42)
Lublin	17/19	0/1	-	-	-	-	-	17/20 (85)
Lubuskie	9/12	6/8	-	-	-	-	1/1	16/21 (76)
Łódź	12/16	1/3	-	-	-	-	-	13/19 (68)
Masovian	15/18	2/4	-	-	-	-	-	17/22 (77)
Opole	12/14	0/4	1/2	-	-	-	-	13/20 (65)
Podlaskie	14/16	4/4	-	-	-	-	-	18/20 (90)
Pomeranian	11/15	3/6	-	-	-	-	-	14/21 (67)
Silesian	11/17	2/2	-	-	-	-	-	13/19 (68)
Subcarpathian	6/10	3/7	1/2	-	-	-	1/1	11/20 (55)
Warmian-Masurian	12/13	2/6	1/1	-	-	-	-	15/20 (75)
West Pomerania	2/5	2/10	0/1	-	-	1/1	-	5/17 (29)
Enriched/total (%)	158/207 (76)	40/75 (53)	6/13 (46)	0/2 (0)	0/2 (0)	1/2 (50)	2/4 (50)	207/305 (68)

Table 3. Samples of silage made of material harvested from areas fertilised organically (manure and slurry)

Province	Silage (organically fertilised/total by material)							
	Corn	Grass	Alfalfa	Beet pulp	Rye	Crushed grain	Shamrock	Organically fertilised/total in provinces (%)
Greater Poland	5/11	1/4	1/1	-	0/1	-	-	7/17 (41)
Holy Cross	5/14	2/4	2/3	-	-	-	-	9/21 (43)
Kuyavian-Pomeranian	5/12	2/3	1/2	0/2	-	0/1	-	8/20 (40)
Lesser Poland	1/5	0/4	-	-	-	-	-	1/9 (11)
Lower Silesian	7/10	3/5	0/1	-	0/1	-	0/2	10/19 (53)
Lublin	10/19	0/1	-	-	-	-	-	10/20 (50)
Lubuskie	5/12	2/8	-	-	-	-	0/1	7/21 (33)
Łódź	3/16	1/3	-	-	-	-	-	4/19 (21)
Masovian	5/18	0/4	-	-	-	-	-	5/22 (23)
Opole	9/14	1/4	2/2					12/20 (60)
Podlaskie	3/16	0/4	-	-	-	-	-	3/20 (15)
Pomeranian	7/15	3/6	-	-	-	-	-	10/21 (48)
Silesian	7/17	0/2	-	-	-	-	-	7/19 (37)
Subcarpathian	7/10	2/7	1/2	-	-	-	0/1	10/20 (50)
Warmian-Masurian	0/13	0/6	1/1	-	-	-	-	1/20 (5)
West Pomeranian	4/5	5/10	1/1	-	-	1/1	-	11/17 (65)
Organically fertilised/total (%)	83/207 (40)	22/75 (29)	9/13 (69)	0/2	0/2	1/2(50)	0/4	115/305 (38%)

Table 4. Storage periods of collected silage

Number of silage samples	Storage period (months)
109 (36%)	1–4
100 (33%)	5–8
81 (27%)	9–12
15 (5%)	13–24

Table 5. Silage samples collected from farms by herd size

Province	Herd size						
	< 50	< 100	< 250	< 500	< 1,000	< 2,000	< 2,500
Greater Poland	3	4	2	6	-	1	1
Holy Cross	10	6	4	1	-	-	-
Kuyavian-Pomeranian	3	8	2	3	4	-	-
Lesser Poland	6	-	3	-	-	-	-
Lower Silesian	3	2	3	5	2	4	-
Lublin	8	4	7		1	-	-
Lubuskie	4	3	9	3	-	1	1
Łódź	11	6	1	-	1	-	-
Masovian	11	6	4	1	-	-	-
Opole	4		4	5	4	2	1
Podlaskie	5	12	3	-	-	-	-
Pomeranian	6	2	4	7	2	-	-
Silesian	8	8	1	1	1	-	-
Subcarpathian	7	7	4	1	1	-	-
Warmian-Masurian	5	8	4	3	-	-	-
West Pomeranian	1	4	3	6	3	-	-
Total	95	80	58	42	19	8	3

The occurrence of *Clostridium* spp. was proved by morphological evaluation of growth and Gram staining. Characteristic colonies with lipolytic properties (a pearl layer) were subjected to further analysis using a real-time PCR method for *C. botulinum ntnh* gene detection. Protuberant yellow colonies with smooth surfaces surrounded by a lecithinolytic layer were subjected to multiplex PCR (mPCR) analysis for *C. perfringens* detection and toxinotype determination.

DNA preparation for *Clostridium botulinum* detection. The DNA for *C. botulinum* detection was extracted from 1 mL of liquid culture in TPGY broth and from suspected lipolytic colonies grown on Willis–Hobbs agar plates. DNA was isolated and purified using a GenomicMini AX Bacteria kit (A&A Biotechnology, Poland), according to the manufacturer's instructions. The amount of DNA used in the PCR reaction varied between 1 and 25 ng.

DNA preparation for *Clostridium perfringens* detection. The DNA of strains suspected of being *C. perfringens* was extracted from characteristic colonies grown on Willis–Hobbs agar according to the method described by Kukier and Kwiatek (10). To perform a reliable multiplex PCR with a heat-lysed bacterial suspension, material from an overnight culture of suspected isolates on Willis–Hobbs agar was resuspended with a plastic disposable inoculating loop in 2 mL of PBS to obtain a 3.5 McFarland turbidity, and then bacterial suspensions were incubated in 1.5 mL disposable Eppendorf tubes at 95°C for 15 min. Next, the tubes were subjected to heat lysis at 95°C for 15 min and after that were placed on ice for 5 min and centrifuged at 11,000 × g for 8 min.

PCR methods for *Clostridium perfringens* detection. The isolates were examined for the presence of *cpa* (α toxin), *cpb* (β), *cpb2* (β2), *etx* (ε), *iap* (ι), and *cpe* (enterotoxin) toxin genes by mPCR according to the method by Baums *et al.* (1). The reaction was performed according to Kukier and Kwiatek's procedure (10) in a volume of 50 µL with 2.5 mM of $MgCl_2$, 0.25 mmol of each deoxyribonucleotide triphosphate, and 2.5 U of Taq polymerase (Thermo Fisher Scientific, USA) as reagents and with the primers used by Baums *et al.* (1).

Real-time PCR method for *C. botulinum* detection. A set of seven primers and a TaqMan probe accredited to Raphael and Andreadis (21) were used for detection of the *ntnh* gene common to all *C. botulinum* toxinotypes. The reactions were conducted with reagents comprising 5 µL of DNA, 4 µL of LightCycler TaqMan Master (Roche Diagnostics, Germany), 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, Switzerland).

Statistical tests. The relationships and statistical significance were investigated between the contamination level of *Clostridium* spp. and the following factors: pH value, addition of starter culture, organic fertilisation, storage period of silage, herd size, and *C. perfringens* occurrence. The same were also investigated between *C. perfringens* occurrence and pH of silage The χ^2 test average values were compared to those of continuous and non-continuous Student's *t*-tests. All significant results were validated by their P value of < 0.05, and tendency (close-to-significant results) was validated by its P value ε (0.051, 0.1). All statistical analyses were conducted with Statistica 13.1 software (Tibco, USA).

Results

***Clostridium* spp. occurrence.** *Clostridium* spp. was detected in 232 (76%) examined samples and occurrence of this genus was observable at least at the level of 10^1cfu/g in each positive silage sample. The contamination level ranged from 10^1cfu/g to 10^4cfu/g and in most of the samples was 10^1 cfu/g (31%) or 10^2 cfu/g (27%). A higher level of 10^3 cfu/g was found in 12% and the highest of 10^4 in 6% of samples (Table 6). The highest number of *Clostridium* spp. positive samples was noted among corn silage samples (157/305) which constituted about 50.5% of all examined samples.

***Clostridium perfringens* occurrence.** *C. perfringens* strains were isolated from 26% (79/305) of samples. Among them, isolates of toxinotype A able to produce α toxin were detected in 42 samples and the same type of isolates with genes determining the production of α and β2 toxins were detected in 33 samples. The strains isolated from two samples showed the presence of genes for α, ε, and β2 toxins and were classified to toxinotype D. One sample showed the presence of type E possessing the α, ι, and β2 toxin genes. Enterotoxic *C. perfringens* was not detected. The highest number of *C. perfringens*-positive isolates was noted in corn silage samples (51/305) which constituted about 17% of the total tested.

Table 6. Contamination level by *Clostridium* spp.

Number of silage samples	Level				
	10^1 cfu/g	10^2 cfu/g	10^3 cfu/g	10^4 cfu/g	Total (%)
Corn	71	56	23	7	157/207 (76)
Grass	22	18	11	9	60/75 (80)
Alfalfa	-	2	2	3	7/13 (54)
Beet pulp	-	1	-	-	1/2 (50)
Rye	-	1	-	-	1/2 (50)
Crushed grain	1	1	-	-	2/2 (100)
Shamrock	1	3	-	-	4/4 (100)
Total (%)	95/305 (31)	82/305 (27)	36/305 (12)	19/305 (6)	232/305 (76)

Table 7. pH values of examined silages samples

Silage	pH				
	3.50–3.99	4.00–4.49	4.50–4.99	5.00–5.99	6.0–7.99
Corn	74	98	7	8	1
Grass	3	13	19	30	8
Alfalfa		3	3	4	2
Beet pulp	1	1		-	-
Rye	-	1	1	-	-
Crushed grain	-	1	-	-	1
Shamrock	-	-	-	3	-
Total (%)	78/282 (28)	117/282 (41)	30/282 (11)	45/282 (16)	12/282 (4)

Table 8. Statistical significance and tendency of factors determining *Clostridium* spp. occurrence

Relationship	Tendency (P value)	Significance (P value)
Clostridium spp. (level 10^3 and 10^4 cfu/g) *vs* pH > 4.5	-	0.007
Clostridium spp. *vs* organic fertilisation	-	0.0024
Clostridium spp. (level 10^3 cfu/g) *vs* herd size (12–800 cows)	0.019	-

***Clostridium botulinum* occurrence.** No *C. botulinum ntnh* genes were detected in any examined samples.

Acidity of examined silages. The observed pH value varied from 3.50 to 7.99. Considerably more samples showed pH in the range of 4.00–4.49 (41%) than in other ranges. The majority of corn silage samples had pH from 3.50 to 4.49, whilst most grass silage samples ranged from 4.00 to 5.99 (Table 7).

Statistical analysis results. Statistical analysis showed significance in the correlation between pH > 4.5 and *Clostridium* spp. contamination level of 10^4 cfu/g (P = 0.007). No significant correlation between the other contamination levels and pH values was found.

A statistical tendency close to significance was noted in the relationship between starter culture omission and *Clostridium* spp. contamination levels of 10^3 and 10^4 cfu/g (P = 0.095). Significance also emerged in the relationship between *Clostridium* spp. occurrence and organic fertilisation (P = 0.0024), but no statistical significance or tendency was discerned when *Clostridium* spp. occurrence and the storage period of silage were compared. Significant was the relationship between the relatively high contamination level of 10^3 cfu/g and herd head counts from 12 to over 800 (P = 0.019) (Table 8). Statistical significance was not observed in the relationship between feed material used for silage production and *Clostridium* spp. occurrence.

Discussion

The results of this study indicated that *Clostridium* spp. was present in 76% of silage samples, in most cases at 10^1 and 10^2 cfu/g. According to Rammer *et al.* (20), in a freshly harvested crop the minimum level of spores determined did not usually exceed 100/g. It was observed that the storage period had no statistically significant influence on *Clostridium* spp. occurrence in silage samples and this fact could be explained by high contamination of the feed material used to produce it. Primary production could account for most of the presence of clostridia in the examined samples. Tabacco *et al.* (25) found that after a properly executed ensiling process using a *Lactobacillus buchneri* starter culture, the clostridia spore count did not increase in maize silage after 342 h of incubation. The same authors also observed that use of a *Lactobacillus plantarum* starter culture also had an inhibitory effect on clostridia growth, limiting the increase in the number of spores to a minimum. Our results showed only one correlation between starter culture omission and high contamination levels of 10^3 and 10^4 cfu/g (P = 0.095). It could be concluded that starter culture addition might inhibit the growth of *Clostridium* spp.; however, the microbiological condition of feed material exerts the most significant influence on the silage occurrence of this species.

The highest contamination by *Clostridium* spp. was revealed in the samples with pH between 4 and 5 (most of the examined samples), which is considered the optimal pH for good quality silage. Severe deterioration by clostridia is observed in silage with pH above 7.0. This pH was recorded only in an insignificant number of examined samples. An interesting finding is the high contamination with *Clostridium* spp. in the samples with pH between 3.5 and 3.99. It could be explained by a high influence of anaerobic microflora transferred to silage from primary production processes. Low pH could have a fixative effect for *Clostridium* spp. spores derived from feed material (13). Statistical significance between high contamination with *Clostridium* spp. of 10^4 cfu/g and pH > 5 was noticed. It indicated possible deterioration of silage if the contamination level is high and pH favourable to clostridia growth. Cai *et al.* (3) reported that if silage pH does not decline to <4.0, the growth of coliform and aerobic bacteria is not inhibited. Below this pH, growth of pathogenic microflora is inhibited, however, spores of *Clostridium* spp. are still likely to occur. Statistical significance was observed in the relationship between organic fertilisation with slurry or manure and *Clostridium* spp. occurrence. Instances of clostridia in silage are principally considered to be connected with contamination from soil and farmyard manure (18, 20). However, in a properly conducted ensiling process maintaining low pH, their significance

is limited, because of the inhibitory pH level and activity of lactic acid microflora. No statistically significant relationship between *Clostridium* spp. occurrence and type of silage (corn or grass) was discovered. Statistical analysis showed significance between relatively high contamination with *Clostridium* spp. of 10^3 spores/g and herd size ranging from 12 to 800 cows. It is difficult to interpret if occurrence of clostridia is determined by the intensity of dairy or beef production; however, on the bigger farms (with herd sizes over approximately 1,000 cows), hygienic practices attain a far higher standard and different equipment is used.

C. perfringens strain occurrence was found in 79 (26%) examined samples. Mainly type A strains were isolated, and among them 33 (11%) were able to produce β2 toxin. Toxinotype D and E occurrence was noticed only in three silage samples. The infective dose of *C. perfringens* for cattle is undetermined. The occurrence of *C. perfringens* type A in the digestive tract of cattle is physiological, but its pathogenesis depends on physiological conditions. *C. perfringens* type A has been associated with several diseases in cattle, such as clostridial enteritis in neonates, haemorrhagic abomasitis or abomasal ulceration in affected calves, haemorrhagic enteritis in adult cattle and calves, or type A haemorrhagic enteritis and sudden death in veal calves during the suckling period (6, 12). Some *C. perfringens* type A isolates with the ability to produce β2 toxin could cause haemorrhagic lesions in the small intestine in cases of bovine enterotoxaemia (along with α toxin). Isolates of *C. perfringens* type A have also been suggested as a cause of jejunal haemorrhage syndrome (JHS) in beef and dairy cattle. However, further experiments attempting to reproduce JHS using this microorganism did not prove the theory thoroughly, suggesting the possibility of other contributing or predisposing factors in the aetiology of this disease (4, 6).

C. perfringens type A strains are natural inhabitants of normal intestinal microbiota, and isolation is not sufficient for proper diagnosis. It is very difficult to clearly associate the presence of this toxinotype with disease. Also, the major α toxin can be detectable in the faeces of healthy animals and has little diagnostic value (6). We also found toxinotypes D and E, which, in contrast, can be mooted as causative disease agents in animals. *C. perfringens* type E is considered an infrequent cause of haemorrhagic enteritis and sudden death in neonatal calves (17); however, one report also describes type E enterotoxaemia in adult cows (22). Literature reports are mainly focused on the type D strain pathology. *C. perfringens* types A and D are both often described as enterotoxaemia causative agents, however, they show completely different pathologies (6). Various diseases caused by *C. perfringens* are determined as enterotoxaemia, however, this term should only be used for symptoms mainly associated with systemic actions of the toxins. Characteristic for type D enterotoxaemia is the occurrence of neurological signs without the major intestinal lesions, in contrast to the disease caused by type A strains, where the characteristic symptoms are intestinal haemorrhages and necrosis and neurological effects are only sporadic (6, 15).

C. botulinum occurrence was not evident and none of the examined samples was associated with a botulism case in cattle. *C. botulinum* comprises microorganisms which are characterised by huge genetic variability, especially those classified to group III, which causes most of the botulism outbreaks in cattle. Outbreaks were associated with the use of poultry litter as a dietary supplement and ingestion of silage and water contaminated by carcasses of small ruminants or birds. It is nevertheless quite improbable that this could occur on properly managed farms. Botulism cases were linked to feeding with insufficiently acidified silage contaminated with *C. botulinum* from soil, or ingestion of silage made from polluted brewers' grain. In the last few decades, an increasing number of D- and mosaic D/C-type botulism outbreaks associated with direct contact with poultry litter have been reported, caused by spores of type D or D/C (which possess genes coding for two-thirds of the type D toxin and one-third of the type C toxin). It is worth considering the hypothesis that *C. botulinum* of these types could have been present in the litter due to asymptomatic carriage in the poultry gut, as has been observed with *C. botulinum* type B and C in pigs (16). Particles of litter containing the spores or the BoNTs could then be dispersed in the silage pasture by wind, runoff water or scavengers. Poultry is less susceptible than cattle to type D botulism (7, 11) and the mosaic D/C form. Further research on the asymptomatic carriage of type D and D/C *C. botulinum* by poultry is needed in order to understand the risk of exposure of cattle to *C. botulinum* spores associated with poultry litter. Therefore it is reasonable to recommend that farmers ensure the safe storage and proper disposal of poultry and ruminant litter in order to prevent further cattle botulism outbreaks (22).

The majority of these cases were attributed to the ingestion of BoNTs from poultry or mammal carcasses or litter when the material was used as fertiliser or stacked on pastures, although this association was not always proved (23). In the last decade, some reports were published of botulism cases in cattle associated with biogas plant waste and digestants (2, 24).

The high genetic variability of *C. botulinum* group III, loss of toxinogenic abilities and problems with cultivation of this microorganism make laboratory diagnosis of this disease extremely difficult. In our experiments we used the methods enabling the detection of this microorganism directly from liquid culture, which increased the chance of detection of this microorganism before potential toxinogenicity was lost. However, samples showed too few *C. botulinum* spores for these to be a probably causative agent of botulism in herds fed the silage examined. We do not have information about any usage of poultry manure or biogas plant digestants as fertilisers.

Summarising, the conducted statistical analysis shows that the most influential effect on *Clostridium* spp. occurrence is felt from factors potentially associated with primary production like fertilisation and the contamination level of feed material used for silage preparation. Deterioration of silage by clostridia could be stopped by a properly conducted ensiling process with addition of starter cultures; however, the presence of spores is highly probable and could pose a biological threat to animals.

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