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1 **A review of the abundance, behaviour and detection of clostridial pathogens in**
2 **agricultural soils**

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10 *Running title: Clostridial pathogens in soils: a review*
11

12 **Summary**

13 The soil is a reservoir for various clostridial pathogens, with agricultural soils representing a
14 major contamination source for overlying crops and grazing livestock. Understanding the
15 prevalence and behaviour of pathogens in these soils is fundamental to ascertaining and
16 mitigating disease risk from agroecosystems. This article reviews research pertaining to the
17 overall distribution and abundance of clostridial pathogens in the soil while identifying possible
18 environmental and soil factors influencing their behaviour. Large-scale soil screens have
19 identified pathogens across the globe, although some *Clostridium botulinum* toxinotypes are
20 more prevalent in certain geographic regions. Faecal inputs and organic waste amendments to
21 the soil can elevate the levels of enteric clostridial pathogens in the soil and the subsequent
22 disease risk, as highlighted by case-control studies. The ability of Clostridia to sporulate results
23 in their long-term persistence post-introduction, increasing the time period for disease
24 transmission. Regularly or permanently saturated soils may also enhance survival, or
25 potentially facilitate the regrowth of some indigenous or introduced Clostridia. This is
26 supported by the high prevalence of Clostridia in paddy soils, greater detection of pathogens in
27 flooded soils, and the higher onset of some clostridial diseases in regions with poorly-drained
28 soils. Future research should elucidate soil types and environmental conditions which can
29 enhance pathogen survival/regrowth. The adoption of molecular and sequencing technologies
30 for future diagnostics can facilitate more sensitive detection and a higher resolution of pathogen
31 typing, allowing a better understanding of pathogen population dynamics in farm soils and
32 disease epidemiology.

33

34 **Keywords:** *Clostridium;* soilborne pathogens; bacterial survival; livestock disease; anaerobes

35

36

37 **Highlights**

38 1) Understanding the behaviour of soilborne clostridial pathogens is key for disease
39 management.

40 2) Soil, environmental and management factors affecting pathogen survival/introduction are
41 discussed.

42 3) Soil waterlogging and application of organic soil amendments may increase the number of
43 soil pathogens.

44 4) More pathogen surveillance and standardisation of diagnostics to better understand
45 behaviour is needed.

46

47 **1. Introduction**

48 The link between soil and disease is well acknowledged, although not necessarily fully
49 understood (Oliver & Gregory, 2015). Researchers have discovered and isolated infectious
50 microbes from soils for well over a century (Noble, 1915; Schoenholz & Meyer, 1922), yet
51 soilborne diseases still cause significant loss to life, reductions in health and considerable
52 economic losses globally (World Health Organisation, 2016). While soil-related human disease
53 appears to have been curbed in many developed nations (Jeffery & Van der Putten, 2011),
54 many diseases are a continuing threat to human health in the developing world (Afshar *et al.*,
55 2011; Thwaites *et al.*, 2015) and also for wildlife, livestock and other domestic animals
56 (Songer, 1996; Lewis, 2011; Vidal *et al.*, 2013; Pirie *et al.*, 2014). Clostridial pathogens have
57 long since been affiliated with soil-borne disease, yet, compared to other soil-borne pathogens,
58 research pertaining to their behaviour and abundance in the soil is limited. *Clostridium*
59 *botulinum, C. perfringens* and *C. tetani* are species frequently reported as major soil pathogens,
60 responsible for debilitating and often fatal diseases (Hatheway, 1990). Many studies describe
61 these bacteria as euedaphic or geo-indigenous soil pathogens (Pepper *et al.*, 2009; Jeffery &
62 Van der Putten, 2011), meaning that they can grow, metabolise and reproduce in the soil. They
63 are also documented as being ubiquitous in soils, implying a uniform, pervasive threat is posed
64 to health from exposure to any soil. However, research into the prevalence of these bacteria in
65 soils, and their adaptation to environmental stressors, is limited. To better understand the
66 epidemiology of clostridial disease, it is imperative to elucidate the behaviour and distribution
67 patterns of these bacteria in the soil. This review explores historical research into the prevalence
68 and abundance of clostridial pathogens in the soil environment and compiles research from
69 various epidemiological and soil ecology studies with an aim to better understand the key soil
70 and environmental determinants affecting the behaviour of indigenous clostridial pathogens.

71 This paper also considers agricultural practices which could introduce pathogens into the soil
72 and discusses effective diagnostics for detecting and identifying pathogens from soil samples.
73

74 *1.1 Clostridial pathogens*

75 While most clostridial species are pathogenically benign, many species are known to induce
76 disease in humans or animals (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999; Popoff & Bouvet,
77 2013). Most of these pathogens belong to the genus *Clostridium,* a large genus characterised
78 by endospore-forming, rod-shaped, anaerobic bacteria. These pathogens contribute
79 significantly to the global burden of disease, in part due to the ubiquity of the organisms in
80 many environments, the potent toxins produced and the longevity of the environmentally-
81 persistent endospores (Hatheway, 1990). For example, despite highly effective vaccination
82 programs, tetanus (*C. tetani*) caused over 10 000 confirmed deaths in 2015 alone (World Health
83 Organisation, 2016), while *C. perfringens* is a leading cause of gastroenteritis in both the
84 United Kingdom and the United States. Other significant clostridial diseases include botulism
85 (*C. botulinum, C. baratii* and *C. butyricum*) (Fach *et al.*, 2011; Espelund & Klaveness, 2014)
86 and various gangrenous and necrotic diseases (*C. perfringens, C. novyi, C. septicum* and *C.*
87 *chauvoei*) (Sasaki *et al.*, 2000; Brynestad & Granum, 2002; Lindström *et al.*, 2011; Skarin &
88 Segerman, 2014). The formerly-assigned *Clostridium* species, *Clostridioides difficile,*
89 *Paeniclostridium sordellii* and *Paraclostridium bifermentans,* are genetically and
90 phenotypically akin to *Clostridium sensu stricto* species, and, due to their significant role in
91 disease mediation, will also be discussed within this review. Clostridial bacteria are also the
92 etiological agents for important veterinary diseases including: blackleg in cattle, sheep and
93 swine (*C. chauvoei*) (Sasaki *et al.*, 2002), enterocolitis in horses, lamb dysentery, necrotic
94 enteritis in piglets and poultry, enterotoxaemia in sheep, goats and foals (*C. perfringens*) (Van
95 Immerseel *et al.*, 2004; Songer, 2010), equine grass sickness (EGS) (*C. botulinum*) and various

96 other diseases (Hunter *et al.*, 1999; Mccarthy *et al.*, 2010). These diseases may lead to
97 substantial financial losses for the animal owners and can contribute to an overall reduction in
98 animal welfare (Bagge *et al.*, 2010).
99
100 Many clostridial pathogens can be differentiated into phylogenetically different strains and
101 toxinotypes which can demonstrate different growth preferences or virulence. Clostridial
102 diseases are mediated by the production of extracellular toxins. For example, there are eight
103 botulinum neurotoxin (BoNTs) serotypes (A-H), with different toxins and toxin combinations
104 affecting the disease host and virulence (Petit *et al.*, 1999; Persson *et al.*, 2008; Peck *et al.*,
105 2017). In *C. difficile,* nucleotide variations occur between strains in a genomic region called
106 the pathogenicity locus (PaLoc), effecting the expression of toxin and toxin-regulator genes
107 and causing significant variations in virulence (Griffiths *et al.*, 2010; Dingle *et al.*, 2011; Popoff
108 & Bouvet, 2013). Many species are classified by toxinotype, based on the major toxins
109 produced, such as with *C. botulinum, C. perfringens, C. novyi* and *C. difficile* (Hatheway, 1990;
110 Popoff & Bouvet, 2013). Some toxins can be produced by multiple pathogens; *C. baratii* can
111 produce BoNT type F like Group I and II *C. botulinum*, while *C. butyricum* can produce BoNT
112 type E, all being collectively termed botulinum-toxin producing Clostridia (BTPC) (Popoff &
113 Bouvet, 2013; Smith *et al.*, 2015). Recent research has demonstrated the ability of toxin genes
114 to be transferred to genetically related, non-toxigenic species (Skarin & Segerman, 2014;
115 Weigand *et al.*, 2015). Studies have observed different growth preferences and geographical
116 abundances between strains and toxinotypes, highlighting the complex yet poorly-understood
117 relationships between the strain physiology and genotype, soil growth and survival, and disease
118 acquisition (Hatheway, 1990). Determining the main drivers behind species and strain growth
119 in different soils is essential for understanding clostridial disease epidemiology.
120

121 **2. Pathogen diversity and prevalence in the soil**

122 Clostridia are considered a common constituent of soil microflora (Janssen, 2006; Russo *et al.*,
123 2012). They are part of a microbial consortium that plays a pivotal role in nutrient recycling,
124 improving soil fertility and other soil functions (Garbeva *et al.*, 2004; Ulrich & Becker, 2006).
125 For example, Clostridia are thought to be one of the main classes of bacteria responsible for
126 dissimilatory nitrate reduction to ammonium in the soil (Pett-Ridge & Firestone, 2005) and are
127 key in the degradation of cellulose in anaerobic soils (Leschine, 1995). In a survey of 16S
128 rDNA gene libraries, on average, 0.59% of the soil bacteria community belonged to Clostridia
129 in the 3398 gene clones examined across 32 gene libraries (Janssen, 2006). Soil is thought to
130 be the major reservoir for many pathogenic species, representing an important pathway of
131 disease transmission to human food products and grazing animals (Meng *et al.*, 1999; Li *et al.*,
132 2007; Mccarthy *et al.*, 2010), yet there is limited research on the abundance and diversity of
133 clostridial species in the soil. Previous research has focused on determining the prevalence of
134 individual, key pathogenic species or toxinotypes, with pertinent clostridial soil studies given
135 in Table 1. Species and toxinotype diversity of Clostridia can vary significantly between
136 locations from large-scale continental differences (Haagsma, 1991; Dodds, 1992) and regional
137 differences (Smith, 1978; Smith & Young, 1980; Lúquez *et al.*, 2005; del Mar Gamboa *et al.*,
138 2005), through to microscale differences within soil of the same sample (Kirk *et al.*, 2004).
139 Gamboa *et al*. (2005) studied clostridial prevalence in Costa Rican soils, isolating 54 different
140 species from 117 samples and averaging over seven species per sample. Eleven toxigenic
141 species were isolated, with *P. sordellii* and *C. perfringens* being the most prevalent (present in
142 42% and 38% of samples, respectively). *Clostridium tetani* (4% of samples), *C. difficile* (3%
143 of samples) and *C. botulinum* (1% of samples) were also identified. Kim *et al*. (2004) isolated
144 16 different *Clostridium* species from 152 South Korean soil samples spanning five locations.
145 *Clostridium perfringens* was common across all sampling locations, indicating the ubiquity of

146 this pathogen across different soil types and agricultural regimes. Other pathogens, such as *C.*
147 *chauvoei*, *C. novyi, C. septicum* and *C. difficile* were only detected in certain locations.
148
149 *Clostridium botulinum* prevalence has been examined more widely, particularly in American
150 and European soils. Some BTPC toxinotypes are endemic to geographical areas or
151 environments. Based on the literature, toxin type A is frequently isolated in North American
152 soils west of the Mississippi River and in uncultivated soils, whereas type B prevails in soils
153 to the east of the Mississippi, European soils and cultivated soils (Haagsma, 1991; Dodds,
154 1992; Espelund & Klaveness, 2014). Types C, D, G and C/D mosaic strains are also common
155 in European soils, with C, D and C/D strains frequently associated with environmental botulism
156 outbreaks. Type E is commonly isolated from marine environments, such as fish gut contents
157 and coastal sediments, with research indicating this toxinotype has a higher affinity for
158 permanently wet environments (Haagsma, 1991; Espelund & Klaveness, 2014). Lúquez *et al.*
159 (2005) identified BTPC in 23.5% of 2009 Argentinean soil samples, which is high compared
160 to the 5.7% and 16.5% prevalence found in British (Smith & Young, 1980) and Japanese soils
161 (Yamakawa *et al.*, 1988), respectively. *Clostridium perfringens* endospores were between 30-
162 65% prevalent in Greek soil samples depending on the type of overlying arable cultivation
163 (Voidarou *et al.*, 2011), with endospores (47.5%) more prevalent than the vegetative form
164 (11%). A study of Greek soils found *C. perfringens* endospores and vegetative cells in 36.4%
165 and 25.5% of soils, respectively (*n* =110) (Stefanis *et al.*, 2014). *Clostridium difficile* was
166 isolated in 21% of Welsh soil samples (Al Saif & Brazier, 1996) and in 37% of 147 soils
167 samples from a rural Zimbabwean homestead (Simango, 2006). *Clostridium tetani* prevalence
168 ranged from 25-42% in five different worldwide studies (total of 2491 soil samples) (Wilkins
169 *et al.*, 1988). These studies demonstrate how prevalent many clostridial pathogens are in
170 various intensive and extensive agricultural environments, and why a better understanding of

171 clostridial behaviour in soils would be of global impact. Despite the large body of research in
172 clostridial pathogen presence, the studies have failed to identify the main soil determinants
173 affecting the presence and abundance of these pathogens. Furthermore, these studies utilised a
174 range of different microbiological techniques, which may limit the scope for integrating results.
175
176 **3. Clostridia in agricultural soils**
177 Agricultural soils are a reservoir for many foodborne pathogens (Newell *et al.*, 2010), including
178 clostridial pathogens, and represent the first critical control point in the food-contamination
179 pathway (Stefanis *et al.*, 2014). Pathogens may be acquired by humans and susceptible animals
180 from soil or vegetation by wound infection, ingestion or inhalation (Haagsma, 1991;
181 Baumgardner, 2012). Produce can be contaminated by both vegetative cells and endospores,
182 both of which can induce disease (Tabaqchali & Jumaa, 1995). Moreover, some studies have
183 found clostridial species as part of the endophytic plant population, suggesting possible
184 mechanisms of internalisation within plant tissues (Miyamoto *et al*., 2004). The longevity of
185 clostridial endospores in the soil also increases the time-window for bacterial transmission
186 (Tabaqchali & Jumaa, 1995; Gessler & Böhnel, 2006). Girardin *et al*. (2005) demonstrated the
187 longevity of *C. sporogenes* endospores in the soil after application (> 1 year), and their
188 subsequent transfer to parsley plants growing in the soil. A better understanding of Clostridia
189 survival and behaviour in agroecosystems is imperative to mitigating the risk of foodborne
190 disease and grazing-livestock illness. Additionally, discrimination between endospore and
191 vegetative forms is necessitated to allow the development of more accurate and effective
192 microbial risk assessment tools.
193
194 **4. Environmental factors affecting clostridial survival and growth in the soil**

195 Investigations into soil microbial diversity using molecular techniques have indicated that the
196 soil type, environmental factors, and agricultural management of a soil can influence the
197 microbial activity (Roesch *et al.*, 2007; Baumgardner, 2012). The major environmental factors
198 influencing the behaviour and die-off of most bacterial pathogens are temperature, moisture
199 content, UV exposure, oxygen concentration, redox potential (*Eh*) and biotic interactions
200 (Venglovsky *et al.*, 2006). This section provides and overview of relevant studies of these
201 factors on Clostridia behaviour, or specifically clostridial pathogens where available, and the
202 interaction between these factors.#
203
204 *4.1 Temperature*
205 Clostridia demonstrate a wide range of optimal temperatures, with psychrophilic, mesophilic
206 and thermophilic representatives. The majority, including most key pathogenic species, grow
207 optimally between 30 °C and 40 °C, as summarised in Table 2. However, Brocklehurst and
208 Lund (1982) and Perry (1985) isolated various strains of Clostridia from UK soils capable of
209 significant growth at 10 °C or lower. The optimum temperature for growth, sporulation and
210 germination can vary between strains of the same species (Jensen *et al.*, 1987), making
211 behaviour and population dynamics in the soil difficult to predict (Evans *et al.*, 1997). Warmer
212 temperatures will lead to higher metabolic activity in the soil and increased oxygen demand,
213 which may generate anoxic conditions, particularly in wet soils (Pett-Ridge & Firestone, 2005).
214 Most *C. perfringens* strains have a generation time of less than 20 minutes at temperatures
215 between 33–49 °C, although the pathogen is capable of growing between 15–55 °C (Brynestad
216 & Granum, 2002; Albrecht, 2005). Different temperature optima between the *C. botulinum*
217 groups may explain differences in prevalence observed in different climatic areas. While
218 vegetative cells show species- and strain-dependent variation in response to temperature, one
219 key survival strategy of Clostridia is their ability to form endospores. Other bacteria, both Gram

220 positive and Gram negative, can form endospores, however only the Gram positive genera of
221 Clostridia and Bacillus and the visually Gram-variable Mycobacteria are widely known to have
222 pathogenic species. Endospore formation is a complex developmental process, controlled by
223 the expression of the master regulator Spo0A gene. The activity of Spo0A is mediated by five
224 autophosphorylating histidine kinases (KinA-KinE) that respond to different environmental
225 stresses, including nutrient depletion. The recalcitrance of clostridial endospores with respect
226 to temperature is due to the structure and thickness of the bacterial endospore coat, which has
227 proved particularly problematic for the food industry (Reddy *et al.*, 2003). Endospores can still
228 remain viable after exposure to temperatures between -25 °C and 121 °C, and in some instances
229 viability of endospores is enhanced after cold exposure (Mah *et al.*, 2009). Miwa (1975)
230 identified *C. butyricum, C. perfringens*, *C. septicum* and *P. sordellii* in Antarctic soils exposed
231 to temperatures between -38 °C and +3.2 °C, whereas Yang & Ponce (2011) isolated
232 germinable-Clostridia endospores in 40 000-year-old Greenland ice cores, and up to 157
233 germinable endospores per gram in Atacama Desert soils, indicating pathogen persistence
234 across extreme environmental conditions.
235
236 Seasonal changes in clostridial populations have been observed. For example, population
237 decreases were reported in agricultural soils after cold periods, although populations recovered
238 soon after (Brochier *et al.*, 2012). Some long-term field studies observed no seasonality in *C.*
239 *botulinum* prevalence or abundance (Sandler *et al.*, 1993; Gessler & Böhnel, 2006) although
240 seasonality is apparent in *C. botulinum* type C-related diseases such as avian botulism
241 outbreaks and EGS (Sandler *et al.*, 1993; Mccarthy *et al.*, 2010; Espelund & Klaveness, 2014).
242 Higher prevalence and abundance of clostridial pathogens in the soil can increase the frequency
243 of disease (Wobeser *et al.*, 1987). Temperature increases above the lower bounds of growth
244 may allow favourable conditions for proliferation (Brochier *et al.*, 2012; Wolf *et al.*, 2017),

245 which could in part, explain the higher incidence of EGS in the late spring (Wood *et al.*, 1998;
246 Wylie & Proudman, 2009) and of blackleg between June and September in Europe (Wolf *et*
247 *al.*, 2017). Disease incidence may be increased during warm periods because of the indirect
248 effects of temperature; for instance, spring snowmelt leads to enhanced moisture which is
249 beneficial to Clostridia and higher temperatures enhance toxin production (Karlsson *et al.*,
250 2003). However, the risk of infection of human or animal receptors is determined both by their
251 susceptibility to disease and exposure to the organism in question. Thus, there are interactions
252 (often multi-way) between abundance of Clostridia in soil, farm management approaches (e.g.
253 when livestock are grazing outdoors vs. kept in barns; timing of manure applications), receptor
254 contact time with the soil and environmental factors (e.g. weather-related impacts on
255 dissemination of organisms such as rain-fall induced run-off).
256
257 *4.2 Soil moisture and redox potential (Eh)*
258 Soil moisture content has been recognised as a principal factor affecting the survival of enteric
259 bacteria in the soil, with increased survival of some pathogens observed in wetter soils
260 (Jamieson *et al.*, 2002). Water is essential for the optimal functioning of the cell membrane,
261 metabolic activity and providing an aqueous environment for nutrient transfer. Low water
262 activity (Aω) may induce cell desiccation (Knechtges, 2011), although endospores are resistant
263 to this effect. The effect of Aω on clostridial growth has been of great importance for the food
264 industry, and generally water activities below 0.9 are considered prohibitive to growth
265 (Knechtges, 2011). Increasing soil moisture content is also intrinsically associated with lower
266 *Eh* and increased oxygen depletion, creating reduced, anoxic soils which favour the growth of
267 anaerobes (Pett-Ridge & Firestone, 2005). Oxygen is the primary electron acceptor in aerated
268 soils, but as soils become increasingly saturated oxygen is rapidly exhausted due to the
269 increased biological demand and the far lower diffusion of oxygen into water than air (by a

270 factor of 10^4) (Neira *et al.,* 2015). Anaerobes begin to use inorganic and organic compounds
271 as electron acceptors, pathways initiated by intracellular dehydrogenases and terminated in the
272 soil solution. This anaerobic respiration causes a decrease of *Eh*, pH alteration and, after nitrate
273 exhaustion, an increase in the concentration of such products as Mn^{2+}, Fe^{2+}, S^{2-} and CH_4.
274 Higher temperatures enhance the activity of dehydrogenases, further lowering *Eh* (Brzezińska
275 *et al.,* 1998). Studies have indicated that some clostridial pathogens can only grow or sporulate
276 within a range of *Eh*. For example, *C. perfringens* can grow at *Eh* < +200 mV in foods, whereas
277 *C. botulinum* can only grow in foods <60 mV (Knechtges, 2011).
278
279 Båverud *et al.* (2010) identified a higher frequency of *C. difficile* in soil samples from water-
280 filled ditches. The affiliation between saturated soils and higher clostridial prevalence is
281 supported by numerous studies into bacterial populations in saturated (i.e. anaerobic) rice
282 paddy soils. Weber *et al*. (2001) attributed 20 out of 31 clones isolated from paddy soil to class
283 Clostridia. Additionally, they found that, after an eight-day anaerobic incubation of paddy soils,
284 55% of the active cells detected belonged to the *Clostridium* genus. Liesack *et al.,* (2000)
285 concluded that Clostridia and Clostridia-like lineages of bacteria are typical inhabitants of
286 flooded paddy soils. The prevalence of pathogenic species in sporadically waterlogged pasture
287 and arable (non-paddy) soils is an important, yet under-researched, line of enquiry. Pathogenic
288 strains are prevalent in many anoxic environments such as marshes, mudflats and water-bodies
289 (Smith *et al.*, 1978; Sandler *et al.*, 1993). *Clostridium botulinum* type C was isolated in over
290 half of 2200 sediment samples from 10 marshes in a study by Sandler *et al.* (1993), with a
291 higher pathogen prevalence in permanently vs. seasonally flooded marshes. Mccarthy *et al.*
292 (2010) suggest a decreased risk of EGS in pastures where soil drainage is utilised, supporting
293 the association between higher clostridial disease risk and wet or waterlogged soils.
294 Additionally, an association was described between regions with poor soil-drainage and

295 increased risk of blackleg (*C. chauvoei*) in Styria, Austria (Wolf *et al.*, 2017). Localised areas
296 of perpetually waterlogged soil could act as contamination "hotspots" in both arable and
297 livestock regimes. Highly-poached, poorly-drained areas in grazed fields, such as around
298 feeders or drinking troughs could be at particularly high risk of contamination.
299
300 *4.3 Light Exposure*
301 On the soil surface, bacteria pathogens may be inactivated due to exposure to UV radiation
302 from sunlight. This causes DNA/RNA damage, preventing cellular processes such as
303 translation and transcription, and inhibiting multiplication (Gehr *et al.*, 2003; Hijnen *et al.*,
304 2006). UV-induced DNA damage can trigger cell SOS-responses causing induction of
305 prophages which can result in cell lysis (Meessen-Pinard *et al.*, 2012; Nanda *et al.*, 2015). Cell
306 lysis may increase bacterial fitness as the released extracellular polymeric substances aid
307 biofilm formation and accumulation of extracellular DNA promotes horizontal gene transfer
308 (Nanda *et al.*, 2015). Whilst the focus of these authors was on clinical isolates, Hargreaves *et*
309 *al*. (2013) reported an abundance of diverse prophages within environmental isolates of *C.*
310 *difficile*. Any prophage-related improvement in fitness or competitive advantage in soil will
311 depend on the strains present and on the phage infectivity. *Clostridium perfringens*
312 demonstrates greater UV light resistance than other pathogenic indicators (Gehr *et al.*, 2003),
313 and endospores are more resistant than vegetative cells (Hijnen *et al.*, 2006). Endospore
314 resistance to UV light (in addition to other environmental factors) is mainly due to the high
315 concentration (5–10%) of α/β-type small, acid-soluble spore proteins (SASP) in the endospore
316 core. This general feature is also common to other endospore-forming genera (Bacillus and
317 Thermoactynomycetes) and protects the DNA backbone from damage (Setlow, 2007). Lanao
318 *et al.* (2010) observed a 1.2 log inactivation of *C. perfringens* vegetative cells in river water
319 after a 5-minute exposure to light (λ 320–800 nm), whereas endospores were only inactivated

320 by <0.5 log after 30 minutes light exposure. Importantly, the light penetration depth (LPD)
321 (depth at which surface light intensity is reduced by 99%) in soils can be as little as 300 μm
322 (Ciani *et al.*, 2005). Therefore, solar inactivation may represent a small, but effective, method
323 of vegetative cell destruction at the soil surface (Moynihan, 2012). Tillage may enhance
324 clostridial die-off by exposing deeper soil layers to UV radiation.
325
326 *4.4 Oxygen availability*
327 Clostridia are frequently described as obligatory anaerobic organisms, although many species
328 can exhibit varying degrees of aerotolerance (Hill & Osterhout, 1972; Tally *et al.*, 1975;
329 Brioukhanov & Netrusov, 2007). Pathogenic species including *C. botulinum*, *C. perfringens*,
330 *C. septicum* and *C. tetani* can survive in temporarily microoxic environments in a growth-
331 arrested stage (Hill & Osterhout, 1972; Dezfulian, 1999; Briolat & Reysset, 2002; Brüggemann
332 *et al.*, 2004). Both *C. septicum* and *Pr. bifermentans* were more resistant to oxygen than other
333 clostridial pathogens (Hill & Osterhout, 1972), although Tally *et al.* (1975) demonstrated that
334 pathogens *C. botulinum* and *P. sordellii* could grow at 10 and 7.5% oxygen concentrations,
335 respectively. This is within the range of expected soil oxygen concentrations. Ioannou *et al.*
336 (1976) found that soil oxygen concentrations varied from 1.5-20% depending on the irrigation
337 regime. When flooded, oxygen concentrations remained consistently low at around 2%
338 (Ioannou *et al.,* 1976). The adaptive response to oxidative stress is thought to be dependent on
339 a range of specialised genes, some which are permanently expressed, and others which are
340 transcribed under oxidative stress (Jean *et al.*, 2004; Brüggemann *et al.*, 2004; Brioukhanov &
341 Netrusov, 2007; Hillmann *et al.*, 2008). These mechanisms increase clostridial resistance to
342 oxygen-exposure whilst in a vegetative form. It has been shown that 18 hours of oxygen
343 exposure (100% O_2) results in almost complete inactivation of *C. perfringens, C. histolyticum,*
344 *C. novyi* and *C. tetani* vegetative cells, although *Pr. bifermentans*, *C. butyricum* and *C.*

345 *septicum* were slightly more aerotolerant (Hill & Osterhout, 1972; Brioukhanov & Netrusov,
346 2007). Variable oxygen concentrations in soil micropores may provide niches for the vegetative
347 cells to survive within most soils, including strict anaerobes. More aerotolerant pathogens such
348 as *C. botulinum* and *P. sordellii* may be able to grow closer to the soil-air interface. Oxidative
349 stress (and other stressors) has also been shown to increase expression of toxin genes, such as
350 the *pfoA* gene in *C. perfringens* (Abo-Remela & Shimizu, 2012). However, clostridial
351 endospores are highly resistant to oxygenic species, which is partly due to high α/β-type SASP
352 concentrations in the endospore core (Setlow, 2007), protecting DNA against damage.
353 Clostridia also contain oxygen-sensitive enzymes that are required for anaerobic metabolism.
354 Oxygenic species disrupt the specialised cell metabolic pathways (Imlay, 2006). As endospores
355 are not metabolically active, the lack of such enzymes naturally lends itself to oxygen
356 resistance. Hill & Osterhout (1972) found virtually no inactivation of *C. perfringens, C.*
357 *histolyticum* and *Pr. bifermentans* endospores that were exposed to 100% O_2 for 18 hours. The
358 authors also suggest that heightened oxygen resistance seen in some species may relate to their
359 ability to rapidly transition to endospore-form. Additionally, the presence of some exogenous
360 enzymes such as catalases and peroxidases had a protective effect of vegetative cells against
361 oxygen inactivation (Hill & Osterhout, 1972).
362
363 *4.5 Biotic interactions*
364 Biotic interactions with other soil-microbes will affect pathogen population dynamics,
365 although the size of this effect is intrinsically difficult to determine *in situ* due to the various
366 combinations of mutualistic, commensal and antagonistic ecological interactions (Moynihan,
367 2012). One such interaction is the predation of pathogens by bacteriophages (phages), which
368 are likely to be present in the same natural habitats as the bacteria (Ogata & Hongo, 1980;
369 Minton & Clarke, 1989). Numerous phages have been identified for most pathogenic

370 *Clostridium* species, and they are likely to play an important role in population control. A study
371 of prophage (phage DNA integrated into bacterium DNA) carriage in estuarine sediment
372 showed that 74% of *C. difficile* carried phage particles, likely playing a key role in the bacterial
373 life cycle (Hargreaves *et al.*, 2013). In contrast, an earlier study failed to identify any *C. difficile*
374 phages in soil, animal faeces or sewage samples (Goh *et al.*, 2005). They argued that *C. difficile*
375 is often found in endospore form in environmental samples, and phages require a host
376 bacterium to be in a vegetative stage of growth for phage multiplication. Additionally,
377 endospores lack the cell surface structures such as pili required for phage-reception which may
378 infer a higher resistance of endospores to phage infection. Phage induction can be mediated by
379 various stressors including UV, oxygen and heat exposure. It has also been demonstrated that
380 phages can influence the toxin production of some pathogenic *Clostridium* (Minton & Clarke,
381 1989; Sekulovic *et al.*, 2011).
382
383 Competitive inhibition by other microorganisms is also an important factor for clostridial die-
384 off. Soil microbes have been shown to produce antimicrobial products, such as bacteriocins,
385 which kill or inhibit the growth of other bacteria (He *et al.*, 2006). These antimicrobial
386 compounds can show either inter- or intra-specific inhibition. Potential soil bacteria such as
387 *Enterococcus faecalis*, *E. faecium*, *Bacillus badius, B. mycoides, B. cereus* and several
388 *Streptococcus* species have been shown to inhibit growth and toxin production of various *C.*
389 *botulinum* strains (Smith, 1975b; Sandler *et al.*, 1998; Shehata *et al.*, 2013). Smith (1975a) also
390 demonstrated the inhibitory effect of *C. perfringens* isolated from the soil on *C. botulinum* and
391 *C. sporogenes* growth. A study of 10 Californian marshes revealed that 32% of samples
392 (*n*=1600) contained bacteria inhibitory to *C. botulinum* type C (Sandler *et al.*, 1998).
393 *Clostridium botulinum* may be reduced or absent in soils with *C. perfringens* populations.
394 Conversely, Sandler *et al.* (1998) suggest that the presence of antagonistic bacteria has a

395 negligible role in the prevalence of *C. botulinum*. Further research is needed to identify other
396 antagonistic relationships between *Clostridium* pathogens and other bacteria, and their
397 significance in pathogen population dynamics.
398
399 **5. Soil physicochemical factors**
400
401 *5.1 Soil type and structure*
402 Soil type and structure strongly affects the prevalence, survival and movement of bacteria in
403 all soils. Mawdsley *et al.* (1995) stated that as bacterial populations are confined to the aqueous
404 phase and solid-liquid interface of soils, soil water content and water movement are of utmost
405 importance to bacteria survival and movement. The soil parent material affects the composition
406 of the mineral components clay, silt and sand and organic matter, which influences various soil
407 properties including soil chemistry, texture, porosity and nutrient availability. Finer-grained or
408 organic soils have been shown to enhance survival of some enteric bacteria, due to the increased
409 ability to retain water and nutrients (Jamieson *et al.*, 2002); this is also likely to be the case
410 with clostridial pathogens.
411
412 The movement of water through a soil profile is strongly influenced by soil pore micro- and
413 macro-structure. Micropores of < 1-1.5 μm diameter may severely restrict the translocation of
414 large, rod-shaped bacteria in the soil (Mawdsley *et al.*, 1995). Studies indicate that increased
415 vertical translocation of bacteria can be expected in more macroporous soils, such as in
416 structured clay or sandy soils (Mosaddeghi et al., 2009; Safadoust et al., 2011; Natsch et al.,
417 1996). Soils with a predominately microporous structure, such as unstructured, compacted,
418 silty or sandy clays, are more efficient at filtering bacteria by physical obstruction, reducing
419 vertical translocation of bacteria in percolating water (Mosaddeghi *et al.*, 2009; Safadoust *et*

420 *al.*, 2011). In mechanically disturbed soils (i.e. ploughed, tilled or repacked) water can readily
421 infiltrate immediately after tillage but over time the macrostructure may be lost and vertical
422 translocation is reduced (van Elsas *et al.*, 1991; Safadoust *et al.*, 2011). In saturated soils,
423 preferential flow occurs through macropores and channels, increasing vertical translocation of
424 bacteria due to the reduced filtering effect of the soil (van Elsas *et al.*, 1991; Mawdsley *et al.*,
425 1995; Safadoust *et al.*, 2011). Gessler and Böhnel (2006) found evidence for vertical
426 translocation of *C. botulinum* from upper to lower soil horizons after the introduction of
427 endospore-contaminated compost to a loess soil. Newton *et al.* (2010) found that premises with
428 a case history of EGS located on sandy or loam soils (which should have higher rates of vertical
429 translocation) had a higher associated recurrence risk of EGS than comparable premises on
430 clay soils. This conflicts with the translocation mechanism described above, although this may
431 be due to confounding variables such as soil pH. The importance of vertical translocation is
432 emphasised after organic waste application. When pathogens are introduced to the topsoil
433 horizons, vertical translocation may be a key mechanism for dispersion of these pathogens to
434 lower horizons, meaning contamination of crops or ingestion by livestock is less likely to occur.
435
436 *5.2 Soil chemistry*
437 Limited significant associations have been identified between clostridial abundance/behaviour
438 and soil chemistry. An association was identified between higher incidence of EGS and higher
439 soil nitrogen content (Mccarthy *et al.*, 2010), although it is likely that this is due to increased
440 pasture growth or pasture nitrogen content, and the resulting dietary change is the prevailing
441 factor for illness. Dorr de Quadros *et al.* (2012) found a comparatively higher abundance of
442 Clostridia in an oat/maize rotation without added nitrogen. As Clostridia are often diazotrophic
443 organisms under anaerobic conditions, nitrogenase activity would be inhibited by increasing
444 soil nitrogen, such as by ammonium or nitrate additions, possibly favouring growth in nitrogen-

445 limited soils (Dorr de Quadros *et al.*, 2012). As fertiliser addition is a common practice on both
446 arable land and improved pasture, the growth response of clostridial pathogens to increased
447 nutrients should be ascertained.
448
449 Many studies have found significant relationships between soil organic matter content and
450 occurrence of *C. botulinum*. *Clostridium botulinum* type A is more prevalent in soils with low
451 organic matter content, whereas types B and C show a strong association with higher organic
452 contents (Smith, 1978; Dodds, 1992; Espelund & Klaveness, 2014). This may just be due to
453 variations in soil pH rather than organic content, although Böhnel & Lube (2008) postulate that
454 the general lack of microporous aeration, or raised nutrient contents in decaying organic matter,
455 may assist in triggering *C. botulinum* growth.
456
457 *5.3 Soil pH*
458 Smith (1975b) identified a statistically significant relationship between higher counts of *C.*
459 *botulinum* types A & B in neutral to alkaline soils. However, in a later study, Smith (1978)
460 found a higher prevalence of *C. botulinum* type A in neutral-alkaline soils (average pH 7.5),
461 whereas type B prevailed in slightly more acidic soils (average pH 6.25). Environmental
462 botulism outbreaks have also been associated with water of pH 7.5–9 (Espelund & Klaveness,
463 2014). Both *C. perfringens* and *P. sordellii* were prevalent across a wide soil pH range in Costa
464 Rican soils (del Mar Gamboa *et al.*, 2005). This is coincident with other studies which have
465 isolated both vegetative and endospore forms of *C. perfringens* across a range of acidities (Li
466 *et al.*, 2007; Stefanis *et al.*, 2014), including the acidic soils (pH 4.5–6.5) surveyed by Voidarou
467 *et al.* (2011). These studies are all in agreement with laboratory-determined pH growth
468 conditions (Table 2), and collectively indicate that soil pH is not a suitable predictor for *C.*
469 *perfringens* and *P. sordellii* prevalence.

470

471 **6. Agricultural management**

472 Land management and land use practices can alter the bacterial biomass, diversity and
473 community structure of the agricultural soils (Roesch *et al.*, 2007; Acosta-Martínez *et al.*,
474 2008). Changes in management regimes could prove the most effective and realistic approach
475 for reducing or preventing high-risk soils, once the remedial or risk-enhancing practices have
476 been determined.

477 *6.1 Tillage*

478 Landowners are increasingly adopting sustainable management practices which are intended
479 to minimise the negative impacts of agriculture on the environment. No-till farming can
480 increase the microbial diversity and biomass of soils (Ibekwe *et al.*, 2002; Dorr de Quadros *et*
481 *al.*, 2012). The incorporation of crop residues into the soil increases organic carbon, which in
482 turn increases the oxygen demand and decreases *Eh* (Kusliene, 2010), which may encourage
483 clostridial growth. The complex relationship between the effect of tillage/no-tillage practices
484 on soil structure and the resulting air and water capacity, air conductivity and permeability, and
485 pore continuity is well-detailed elsewhere, such as by Mentges *et al.* (2016). Mentges *et al.*
486 (2016) describe how no-till alters the physical parameters of the soil in a manner which favours
487 anaerobic growth. In general, no-till soils are more compacted, which may increase bulk
488 density and lower porosity, especially that of macropores, although this can be attenuated by
489 increased bioturbation from higher earthworm populations. Compacted soils have less airspace
490 and air-filled pores, and gas-permeability was reduced. Reduced pore continuity, a major
491 structural property of clay soils, reduces airflow and gas permeability, and may be a feature of
492 no-till soils. Application of organic matter to no-till soil can further block the passage of air
493 through pores, although increased soil organic matter associated with no-till will generally
494 reduce bulk density. Linn & Doran (1984) identified that in no-till soils, anaerobic organisms

495 were found to comprise a greater proportion of the total bacterial population than in
496 conventionally tilled soils. Using 16S rDNA analysis, Dorr de Quadros *et al.* (2012) found that
497 the relative abundance of Firmicutes showed a positive association with no-till systems, with
498 *Clostridium* species and other anaerobic bacteria dominating. In conclusion, it is likely that the
499 reduced aeration and increased water-retention in no-till soils enhance the formation of anoxic
500 soil microsites. Deep and conventional-ploughing will bring unexposed and protected
501 clostridial cells to the surface, where they may be inactivated by the sunlight and oxygen,
502 although Clostridia may profilerate in compacted soils caused from repeated wheeling or loss
503 of soil organic matter. However, there is a paucity of empirical evidence linking agricultural
504 practices that aerate the soil to changes in clostridial disease frequency (Jeffery & Van der
505 Putten, 2011).
506
507 *6.2 Organic soil amendments*
508 Historically, the application of organic soil amendments (OSA) to agricultural land has been
509 used as an efficient method of replenishing soil nutrients and managing organic wastes
510 (Venglovsky *et al.*, 2006). This practice has become an increasingly attractive option due to
511 heightened environmental and economic concerns regarding the use of conventional chemical
512 fertilisers. Moreover, other bio-wastes such as compost and anaerobic digestates are now
513 frequently applied to soils. Organic soil amendments such as manure, slurry, sewage and other
514 bio-wastes can contain high pathogenic loads, including many clostridial pathogens, as
515 indicated in Table 3 (Bagge *et al.*, 2005, 2010; Sahlström *et al.*, 2008; Torniainen *et al.*, 2011).
516 Incorporating unsanitised organic wastes into agricultural soils can induce artificially high
517 pathogen populations, increasing the risk of pathogen transmission into the food chain or to
518 livestock (Bagge *et al.*, 2010; Brochier *et al.*, 2012). Furthermore, Clostridia have been shown
519 to survive heat treatment processes, such as pasteurisation and thermophilic digestion, which

520 are designed to remove or reduce pathogen loads (Sahlström *et al.*, 2008; Bagge *et al.*, 2010).
521 The application of OSA to agricultural land is governed by both European and individual
522 member state directives, which are well-detailed by Moynihan (2012). These legislative
523 measures are designed to reduce the likelihood of pathogen transmission to crops and grazing
524 livestock, and infiltration into groundwater. However, current legislation is aimed at
525 minimising survival of key pathogenic, non-endospore forming bacteria such as *Escherichia*
526 *coli* O157 and *Salmonella*. Endospore formation may further enhance the longevity of
527 clostridial pathogens, meaning populations remain higher in the soil for a longer duration after
528 introduction *via* OSA. Therefore, current legislation may provide insufficient controls on the
529 transfer of clostridial pathogens from organic wastes to agricultural soils.
530
531 Post-OSA application, swine, fresh and aged cattle manure amendments induced significantly
532 higher concentrations of *C. perfringens* in field run-off water after three simulated rainfall
533 events (Thurston-Enriquez *et al.*, 2005). Brochier *et al*. (2012) found no statistical difference
534 in *C. perfringens* abundance between amended and unamended soils over a 33-month field
535 study, despite two additions of various OSA (at 0 and 24 months) with concentrations of up to
536 1.5×10^4 CFU g^{-1}. They suggested that environmental stressors, particularly temperature, play
537 a more pivotal role than OSA application in the abundance of *C. perfringens* in soil. Enteric
538 Clostridia, including *C. difficile* and *P. sordellii*, are more likely to be enhanced by OSA
539 additions (Simango, 2006). Increased nutrient availability from OSA may also affect the
540 growth of both indigenous and introduced clostridial pathogens.
541
542 *6.3 Grazing regime*
543 The pathogen load in animal faecal matter is known to vary considerably based on the social,
544 nutritional and immunological (i.e. age, stress, diet, disease) status of the animal (Waggett *et*

545 *al.*, 2010). Waggett *et al.* (2010) used culture and enzyme-linked immunosorbent assays
546 (ELISA) to demonstrate the higher prevalence of *C. perfringens* in the faeces of horses
547 suffering from EGS compared to healthy horses. *Clostridium perfringens* and *C. difficile* are
548 likely to be more prevalent in faeces of young foals than mature horses (Tillotson *et al.*, 2002;
549 Newton *et al.*, 2010), as well as many other young livestock (Songer, 1996). Kim *et al.* (2004)
550 attributed high *C. perfringens* prevalence in Korean soils to contamination by domestic
551 animals. A case-control study indicated that the EGS incidence rate was significantly higher
552 on recurrent grazing land, suggesting that soil harbours *C. botulinum* type C. The same study
553 found that co-grazing with ruminants reduced the risk of EGS in horses (Newton et al., 2010).
554 Similarly, the incidence of blackleg in cattle (*C. chauvoei*) is thought to increase year-on-year
555 with heavily contaminated pastures (Hang'ombe *et al*., 2000; Bagge *et al*., 2009). Simango
556 (2006) isolated *C. difficile* in the faeces of various domesticated animals, with chicken faeces
557 showing the highest prevalence (17%, n=115), which were thought to be the major source of
558 soil contamination. Additionally, mechanised manure removal, such as harrowing, could
559 spread clostridial pathogens across a wider area of soil.
560
561 *6.4 Cropping regime*
562 Crop type can affect the underlying soil microbial communities (Garbeva *et al.*, 2004),
563 including pathogenic bacteria (Reed-Jones *et al.*, 2016). Carbon-rich root exudates and
564 expansive root growth will alter the physicochemical environment of the rhizosphere and
565 surrounding soil, whereas oxygen uptake into roots by respiration from the rhizosphere will
566 alter the redox potential. Voidarou *et al*. (2011) found significant differences in the occurrence
567 of *C. perfringens* endospores and vegetative cells under 10 different bulb-forming crops,
568 although this may be due to antimicrobial effects of pesticides applied to the crops or variations
569 in pH or soil organic matter. Similar variations in *C. perfringens* prevalence under different

570 cultivations have been documented in other studies (Stefanis *et al.*, 2014). *C. perfringens*
571 endospores and vegetative cells occurred in 67% and 17% of samples, respectively, under
572 maize cropping, yet both forms were absent under cabbage cropping. It is unclear whether these
573 variations can be attributed to the cropping regime alone, emphasising the need for more
574 controlled experiments where confounding variables can be minimised.
575
576 **7. Methods of detection**
577 A more comprehensive understanding of clostridial prevalence, abundance and behaviour in
578 soils could be obtained with extensive, geographically-widespread soil-surveys covering the
579 complete range of soil types, agricultural regimes and climates. This, in combination with
580 laboratory microcosm experiments manipulating the key environmental and physicochemical
581 factors mentioned, would help elucidate the complex behaviour of Clostridia in soil. Suitable
582 diagnostic techniques are needed to facilitate the following objectives (1) generation of
583 accurate pathogen prevalence and abundance data, (2) differentiation between vegetative
584 growth and endospore cell forms, and (3) easy reproducibility and application in microbiology
585 laboratories. The scientific concept behind the various diagnostic methods is discussed in depth
586 elsewhere, therefore this paper overviews the applicability of various techniques for meeting
587 these objectives.
588
589 *7.1 Culture-based assays*
590 Culture assays can be used to identify and enumerate some clostridial species in samples using
591 the plate count or the most probable number (MPN) methods. Culture-based identification and
592 enumeration is still a widely used technique (Sonnabend *et al.*, 1987; Vijayavel & Kashian,
593 2014), and samples are commonly cultured in enrichment media prior to using other
594 diagnostics. One benefit is that high-temperature short time (HTST) pasteurisation (typically

595 70 °C for 2-10 minutes prior to incubation) can be applied to destroy all vegetative cells,
596 providing a simple method for discriminating between endospore and vegetative-cell forms.
597 Although cost-effective, culture-based assays face several limitations, most notably the time-
598 consuming and labour-intensive nature of tests. Furthermore, not all cells are culturable; those
599 that are injured will not be detected, although may still be capable of pathogenesis. Atypical
600 colonial morphology can also lead to misidentification. Selective or differential growth media
601 have not been developed for many clostridial species, meaning closely related species in co-
602 contaminated samples are difficult to distinguish between.
603
604 *7.2 Biochemical assays*
605 Traditional biochemical identification of anaerobic bacteria comprises a multi-step
606 methodology typically culminating in the analysis of metabolic end-products using gas-liquid
607 chromatography (Burlage & Ellner, 1985; Perry, 1985). Biochemical assays allow the
608 definitive identification of anaerobic bacteria, as opposed to just the presumptive identification
609 using culture-based approaches (Head & Ratnam, 1988). However, the slow growth of obligate
610 anaerobic bacteria in comparison to aerobic or facultative bacteria also delays identification. A
611 range of proprietary biochemical kits which utilise constitutive enzymes on specific substrates
612 have been developed to identify anaerobic bacteria including Clostridia such as the RapID™
613 ANA II (Hang'ombe *et al.*, 2000) and API® systems (Cordoba *et al.*, 2001; Lindström *et al.*,
614 2001; Kim *et al.*, 2004). Despite their rapidity, studies have shown that these biochemical kits
615 lack both the sensitivity and specificity to accurately identify some pathogenic Clostridia, such
616 as *C. botulinum* (Lindström *et al.*, 1999) and *C. difficile* (Head & Ratnam, 1988). More
617 recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
618 (MALDI-TOF MS) has become the gold standard for bacterial identification and has shown
619 some promise for typing applications and with the development of fully-automated workflows,

620 including for Clostridia (Grosse-Herrenthey *et al.,* 2008; Schubert & Kostrzewa, 2016). The
621 high initial investment and maintenance costs of MALDI-TOF MS may prove prohibitive for
622 some laboratories, but with sufficient access to reference databases and strains, the technique
623 provides a high-throughput and sensitive method for strain identification.
624
625 *7.3 Immunological assays*
626 Immunological assays are readily used for identification of *Clostridium* pathogens and for
627 detection of specific toxins. The method of detection of clostridial toxins, such as BoNTs, was
628 typically a microbiological method combined with the *in vivo* standard mouse bioassay (SMB)
629 (Fenicia *et al.*, 2011). Although sensitive and highly specific, the approach is expensive, time-
630 consuming and now discouraged in some jurisdictions for ethical reasons regarding animal
631 experimentation (Cordoba *et al.*, 2001; Lindström *et al.*, 2001; De Medici *et al.*, 2009; Fenicia
632 *et al.*, 2011).
633
634 Enzyme immunoassays (EIAs) are diagnostic methods that are sensitive and high-throughput
635 approaches for toxin detection (Paulie *et al.*, 2006; Peterson *et al.*, 2011), and include ELISA
636 and immuno-fluorescent assays (IFAs). These assays, and similar variations, are used to detect
637 and quantify specific antigens, such as clostridial toxins; a good overview of the methods was
638 given by Paulie *et al.* (2001). These methods have major drawbacks in the identification and
639 enumeration of bacterial cells. Different target pathogens can produce identical toxins, which
640 are indistinguishable using ELISA, and some pathogens will not produce toxins, meaning the
641 true pathogen abundance may be underestimated. Separate tests would need to be conducted
642 for each possible toxinotype, increasing the cost and complexity of the experiment. Other
643 immunological diagnostic kits utilise the same scientific principles as ELISA, such as reversed
644 passive latex agglutination (RPLA) test kits, and can be useful for toxin identification, although

645 the same limitations apply, and poor sensitivity and specificity in comparison to the SMB has
646 been documented (Head & Ratnam, 1988; Peterson *et al.*, 2011).
647

648 8. Molecular Techniques

649 *8.1 Polymerase chain reaction (PCR)-based techniques*

650 In recent decades, advances have been made in the development of molecular diagnostic tools.
651 These techniques, in addition to constantly-growing genetic databases, allow increasingly fast
652 and sensitive bacterial strain detection and characterisation (Cordoba *et al.*, 2001). Polymerase
653 chain reaction (PCR) permits microbial analysis on relatively small and/or rare environmental
654 samples and has facilitated a more thorough taxonomic assessment of the Clostridia class
655 (Collins *et al.*, 1994). Protocols have been designed to target highly-specific genetic loci, such
656 as toxin-coding genes, or highly-conserved regions such as the 16S rDNA gene, with successful
657 amplification of the target region indicating the presence of the particular pathogen strain in
658 the sample (Lindström *et al.*, 2001; Fach *et al.*, 2009). Techniques such as multiplex, nested
659 and semi-nested PCR can enable simultaneous and specific detection of different target genes,
660 species or toxinotypes (Lindström *et al.*, 2001; De Medici *et al.*, 2009). The use of
661 fluorescently-labelled primers and probes allow real-time visualisation of fragment
662 amplification during PCR, through real-time quantitative PCR (RT-qPCR), or the automated
663 sizing of amplicons, such as with automated ribosomal intergenic spacer analysis (ARISA) or
664 terminal-restriction fragment length polymorphism (T-RFLP). This permits a quantitative
665 approach to microbial diagnostics, providing an alternative to the SMB or other toxin-detecting
666 immunological assays (Fach *et al.*, 2009; Fenicia *et al.*, 2011). Many authors have
667 demonstrated the usefulness and sensitivity of using PCR protocols to detect clostridial
668 pathogens in various samples, including soil (Fach *et al.*, 2009; De Medici *et al.*, 2009; Fenicia
669 *et al.*, 2011). Downstream molecular fingerprinting techniques can be used to differentiate

670 between the clostridial species/toxinotypes using one protocol, such as with denaturing
671 gradient gel electrophoresis (DGGE), temperature-GGE (TGGE) (Marzorati *et al.*, 2008),
672 amplified fragment length polymorphism (AFLP) (Keto-Timonen *et al.*, 2006), ARISA
673 (Dahllöf, 2002; Feligini *et al.*, 2015), T-RFLP (Khoruts *et al.*, 2010) and single strand
674 conformation polymorphism (SSCP) (Smalla *et al.*, 2007; Marzorati *et al.*, 2008).
675
676 Polymerase chain reaction-based approaches have high applicability for pathogen detection,
677 although the methods are not without their limitations. Wintzingerode *et al.* (1997) detail the
678 common pitfalls associated with soil microbial analysis using PCR-based approaches. The
679 sensitivity of these methods is heavily dependent on the design of the primers, the efficacy of
680 the DNA extraction methods and various other considerations; these biases can impair the
681 sensitivity and accuracy of results (Acosta-Martínez *et al.*, 2008). The exceptionally high but
682 poorly understood soil microbial diversity creates another issue with PCR-based diagnostics.
683 In what is commonly referred to as the "black-box" of soil ecology, it is unknown as to what
684 effect the many undiscovered species could have on interpreting PCR-based data (Tiedje *et al.*,
685 1999). Many rare species may remain undetected, and unknown species showing high genetic
686 homology could lead to the generation of false-positives (Culman *et al.*, 2008).
687
688 *8.2 Sequencing*
689 Nucleotide sequencing is now the standard technique for confirmative detection of pathogens.
690 This tool also provides an increasingly cost-effective way to identify, survey and compare
691 bacterial communities across different environments (Burke & Darling, 2014). Gene fragments
692 are sequenced using a number of techniques, such as pyrosequencing (Roesch *et al.*, 2007;
693 Acosta-Martínez *et al.*, 2008) or Illumina sequencing (Dorr de Quadros *et al.*, 2012; Burke &
694 Darling, 2014), and compared to known sequences on databases for identification and genetic

695 comparison. While the identification of multiple species from one sample is technically
696 feasible, the method then becomes more expensive and requires a higher level of bioinformatics
697 skill to analyse sequence reads. Whole genome sequencing (WGS) is now considered the
698 ultimate tool for isolate identification and genetic analysis (Salipante *et al.*, 2015). The falling
699 cost of sequencing (Burke & Darling, 2014) and the comprehensive range of bioinformatics
700 software make this approach increasingly suitable for identifying clostridial pathogens in the
701 soil. However, the cost may still be prohibitive for large pathogen-surveillance studies, and
702 selective media is still needed to isolate and differentiate some pathogen species. Whole
703 genome sequencing would be the most sensitive, informative approach to generate important
704 data on genetic and ecological function diversity of Clostridia in the soil. However, a high-
705 throughput, inexpensive molecular assay to screen for pathogens (such as T-RFLP, ARISA),
706 would allow for a comprehensive survey of agricultural soils. Contaminated soils can be
707 identified, pathogenic strains isolated, and WGS used to elucidate how genotypic
708 characteristics allow adaption to the soil environment and changes in virulence. An overview
709 of a suitable methodology is shown in Figure 1.
710

711 **9. Conclusions**

712 Studies have demonstrated the prevalence of clostridial pathogens, as either vegetative cells or
713 as endospores, in most soil environments including agricultural soils. Quantifying the
714 prevalence and abundance of pathogens in agricultural soils is of key importance, as this
715 environment represents the first critical control point in the food contamination pathway, and
716 route of infection for susceptible grazing animals. Numerous studies have identified key
717 pathogenic species and toxinotypes in farm soils, although the findings of these studies differ
718 significantly. This may reflect the high variability in pathogen populations or the differences
719 in diagnostic techniques used. Existing soil pathogenic indicator species are not suitable for

720 predicting Clostridia behaviour due to the persistence of endospores which can survive in soil
721 for several years. Highly variable physiologies result in different geographic distributions
722 between species and strains, although further work is needed to identify the genetic adaptations
723 which affect strain prevalence in certain regions.
724
725 The major environmental, soil physicochemical and agricultural management variables likely
726 to influence pathogen presence and behaviour were identified. Although only tentative links
727 could be made, increased soil moisture is likely to enhance survival, and even promote the
728 regrowth of clostridial pathogens. Soil type will also influence the water retention and nutrient
729 status of a soil, subsequently affecting survival rates, while the structure will dictate the ability
730 of a soil to retain pathogens in the uppermost soil horizons. Common agricultural practices,
731 such as manure, slurry and other biowaste application, intensive grazing, and no-tillage systems
732 could lead to elevated pathogen levels, heightening the risk of clostridial disease in livestock,
733 and transfer of contaminated soil onto overlying crops.
734
735 **10. Future research**
736 Future research should identify the role of key soil, environmental and management factors on
737 pathogen behaviour, using both microcosm and field studies. Pathogen behaviour in saturated,
738 poorly-drained and fine-grained soils should be determined, as these soil types may permit the
739 proliferation or enhanced survival of pathogens. Additionally, the long-term effect of applying
740 pathogen-containing biowastes to farm soils should be ascertained, as this could create elevated
741 contamination levels, especially with some underlying soil types. This knowledge will allow
742 improved pathogen modelling and mapping, development of better risk management strategies,
743 with the aim of reducing the incidence of clostridial disease. Fundamental to the validity of
744 future research is the standardisation of appropriate diagnostics allowing differentiation

745 between vegetative and endospore forms and clinically different species and strains. Suitable
746 methodologies allow a high-throughput, cost-effective and widely accessible application. A
747 variety of suitable approaches were critiqued, although the falling cost and sensitivity of PCR
748 and sequencing techniques make them attractive tools for clostridial soil diagnostics.
749 Techniques such as ARISA, T-RFLP and RT-qPCR, in combination with culture and
750 sequencing-based approaches, are recommended as the most appropriate technologies for
751 multi-pathogen species and strain identification, enumeration and even toxin detection with the
752 use of suitably designed primers.
753

754 **Data Availability Statement**

755 Data sharing is not applicable to this article as no new data were created or analysed in this
756 study.
757

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761

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1277

1278 **Table 1** Summary of pertinent studies of Clostridia soil prevalence

Species	Author	Sample area(s)	Samples (n)
Clostridium argentinense	Sonnabend *et al*., 1987	Switzerland	41
C. botulinum	Creti *et al*., 1990	Rome	520
	Huss, 1980	Denmark, Faroe Islands & Iceland	118
	Lúquez *et al.,* 2005	Argentina	2009
	Serikawa *et al*., 1977	Japan	230
	Smith & Milligan, 1979	London	60
	Smith & Young, 1980	Great Britain	74
	Yamakawa *et al*., 1988	Japan & Shinkiang Province, China	286
C. butyricum	Meng *et al*., 1999	Jiangsu Province, China	60
C. difficile	Al Saif & Brazier, 1996	South Wales, UK	104
	Båverud *et al*., 2010	Sweden	598
C. perfringens	Kuske *et al*., 2006	USA	129
	Li *et al.,* 2007	Pittsburgh, USA	502
	Voidarou *et al*., 2011	Greece	750
C. tetani	Smith, 1978	USA	260
	Wilkins *et al*., 1988	South Africa	60
Multiple	Gamboa *et al.,* 2005	Costa Rica	117
	Hang'ombe *et al.,* 2000	Zambia	46
	Kim et al., 2004	South Korea	152
	Miwa, 1975	Antarctica	31
	Sathish & Swaminathan, 2009	Southern India	115

1280 **Table 1** Summary of optimum pH and temperature growth conditions for selected *Clostridium* pathogens

Organism	Temperature optima	Temperature range	pH optima	Reference
	(°C)	(°C)		
Clostridium botulinum				
Group I	35–40	10–48	4.6–9	McLauchlin & Grant, 2007; Stringer *et al.,* 2013
Group II	18–30	2.5–45	5.0–9	McLauchlin & Grant, 2007; Stringer *et al.*, (2013)
C. butyricum	n.a.	8	4.22[a]	Ghoddusi *et al.*, (2013)
C. histolyticum	30–37	25–45	8.5	Whitman & Parte (2009)
C. novyi	45	>25	< 8.5	Whitman & Parte (2009)
C. perfringens	43–47	15–55	5–9	Albrecht (2005)
C. tetani	37	14–43	7.4	Chessbrough (2002)
C. difficile	30–37	25–45	6·5–7·5 [b]	Wheeldon *et al.*, 2008; Whitman & Parte, 2009
Paeniclostridium sordellii	37†	25–40 [b]	5.7–6.5 [b]	Ramirez & Abel-Santos (2010)

1281 [a]Minimum pH for growth; [b]Optimal conditions for endospore germination

1282 **Table 3** Examples of pathogenic Clostridia isolated from organic wastes.

Organic soil amendment	**Species isolated**	**Method**	**Reference**
Anaerobic digestates	*Clostridium perfringens*	Culture based	Bagge *et al.,* 2005
Bio-compost	*C. botulinum*	Culture based + mouse bioassay	Böhnel & Lube, 2008
Bovine manure	*C. butyricum, C. perfringens*	Sequencing and R.E. analysis[a] of 16S rDNA	Ouwerkerk & Klieve, 2001
Dairy manure	*C. baratii, C. botulinum, C. butyricum, C. novyi, C. perfringens, Paeniclostridium sordellii, Paraclostridium bifermentans, C. sordellii*	Biochemical analysis + sequencing of 16S rDNA	Bagge *et al.*, 2010
Farmyard manure	*C. perfringens*	Culture based	Brochier *et al.*, 2012
MSW[b] compost	*C. perfringens*	Culture based	Brochier *et al.*, 2012
Sewage sludge	*C. perfringens*	Culture based	Dudley *et al.*, 1980
Swine manure	*C. butyricum*	DGGE of 16S rDNA	Leung & Topp, 2001

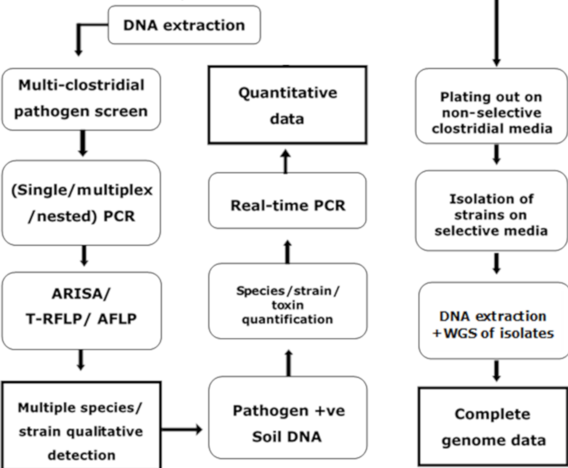
1283 [a]Restriction enzyme analysis; [b]Municipal soil waste

1284

1285 **Figure Legend**

1286

1287 **Figure 1** Schematic demonstrating a suitable workflow for the detection, quantification and
1288 confirmative identification of clostridial species, strains and toxins in the soil. Boxes indicate
1289 the potential outputs of specific procedures. PCR: Polymerase chain-reaction, ARISA:
1290 Automated ribosomal intergenic spacer analysis, T-RFLP: Terminal-restriction fragment
1291 length polymorphism, AFLP: Amplified fragment length polymorphism, WGS: Whole-
1292 genome sequencing.


Soil Sample
DNA extraction
Multi-clostridial pathogen screen
(Single/multiplex /nested) PCR
ARISA/ T-RFLP/ AFLP
Multiple species/ strain qualitative detection
Quantitative data
Real-time PCR
Species/strain/ toxin quantification
Pathogen +ve Soil DNA
Plating out on non-selective clostridial media
Isolation of strains on selective media
DNA extraction +WGS of isolates
Complete genome data