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# A review of the abundance, behaviour and detection of clostridial pathogens in agricultural soils

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

**Summary**

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

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

**Highlights**

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

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

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

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

## 1. Introduction

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

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

*1.1 Clostridial pathogens*

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

other diseases (Hunter *et al.*, 1999; Mccarthy *et al.*, 2010). These diseases may lead to substantial financial losses for the animal owners and can contribute to an overall reduction in animal welfare (Bagge *et al.*, 2010).

Many clostridial pathogens can be differentiated into phylogenetically different strains and toxinotypes which can demonstrate different growth preferences or virulence. Clostridial diseases are mediated by the production of extracellular toxins. For example, there are eight botulinum neurotoxin (BoNTs) serotypes (A-H), with different toxins and toxin combinations affecting the disease host and virulence (Petit *et al.*, 1999; Persson *et al.*, 2008; Peck *et al.*, 2017). In *C. difficile,* nucleotide variations occur between strains in a genomic region called the pathogenicity locus (PaLoc), effecting the expression of toxin and toxin-regulator genes and causing significant variations in virulence (Griffiths *et al.*, 2010; Dingle *et al.*, 2011; Popoff & Bouvet, 2013). Many species are classified by toxinotype, based on the major toxins produced, such as with *C. botulinum, C. perfringens, C. novyi* and *C. difficile* (Hatheway, 1990; Popoff & Bouvet, 2013). Some toxins can be produced by multiple pathogens; *C. baratii* can produce BoNT type F like Group I and II *C. botulinum*, while *C. butyricum* can produce BoNT type E, all being collectively termed botulinum-toxin producing Clostridia (BTPC) (Popoff & Bouvet, 2013; Smith *et al.*, 2015). Recent research has demonstrated the ability of toxin genes to be transferred to genetically related, non-toxigenic species (Skarin & Segerman, 2014; Weigand *et al.*, 2015). Studies have observed different growth preferences and geographical abundances between strains and toxinotypes, highlighting the complex yet poorly-understood relationships between the strain physiology and genotype, soil growth and survival, and disease acquisition (Hatheway, 1990). Determining the main drivers behind species and strain growth in different soils is essential for understanding clostridial disease epidemiology.

## 2. Pathogen diversity and prevalence in the soil

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

this pathogen across different soil types and agricultural regimes. Other pathogens, such as *C. chauvoei*, *C. novyi, C. septicum* and *C. difficile* were only detected in certain locations.

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

clostridial behaviour in soils would be of global impact. Despite the large body of research in clostridial pathogen presence, the studies have failed to identify the main soil determinants affecting the presence and abundance of these pathogens. Furthermore, these studies utilised a range of different microbiological techniques, which may limit the scope for integrating results.

## 3. Clostridia in agricultural soils

Agricultural soils are a reservoir for many foodborne pathogens (Newell *et al.*, 2010), including clostridial pathogens, and represent the first critical control point in the food-contamination pathway (Stefanis *et al.*, 2014). Pathogens may be acquired by humans and susceptible animals from soil or vegetation by wound infection, ingestion or inhalation (Haagsma, 1991; Baumgardner, 2012). Produce can be contaminated by both vegetative cells and endospores, both of which can induce disease (Tabaqchali & Jumaa, 1995). Moreover, some studies have found clostridial species as part of the endophytic plant population, suggesting possible mechanisms of internalisation within plant tissues (Miyamoto *et al*., 2004). The longevity of clostridial endospores in the soil also increases the time-window for bacterial transmission (Tabaqchali & Jumaa, 1995; Gessler & Böhnel, 2006). Girardin *et al*. (2005) demonstrated the longevity of *C. sporogenes* endospores in the soil after application (> 1 year), and their subsequent transfer to parsley plants growing in the soil. A better understanding of Clostridia survival and behaviour in agroecosystems is imperative to mitigating the risk of foodborne disease and grazing-livestock illness. Additionally, discrimination between endospore and vegetative forms is necessitated to allow the development of more accurate and effective microbial risk assessment tools.

## 4. Environmental factors affecting clostridial survival and growth in the soil

Investigations into soil microbial diversity using molecular techniques have indicated that the soil type, environmental factors, and agricultural management of a soil can influence the microbial activity (Roesch *et al.*, 2007; Baumgardner, 2012). The major environmental factors influencing the behaviour and die-off of most bacterial pathogens are temperature, moisture content, UV exposure, oxygen concentration, redox potential (*Eh*) and biotic interactions (Venglovsky *et al.*, 2006). This section provides and overview of relevant studies of these factors on Clostridia behaviour, or specifically clostridial pathogens where available, and the interaction between these factors.#

*4.1 Temperature*

Clostridia demonstrate a wide range of optimal temperatures, with psychrophilic, mesophilic and thermophilic representatives. The majority, including most key pathogenic species, grow optimally between 30 °C and 40 °C, as summarised in Table 2. However, Brocklehurst and Lund (1982) and Perry (1985) isolated various strains of Clostridia from UK soils capable of significant growth at 10 °C or lower. The optimum temperature for growth, sporulation and germination can vary between strains of the same species (Jensen *et al.*, 1987), making behaviour and population dynamics in the soil difficult to predict (Evans *et al.*, 1997). Warmer temperatures will lead to higher metabolic activity in the soil and increased oxygen demand, which may generate anoxic conditions, particularly in wet soils (Pett-Ridge & Firestone, 2005). Most *C. perfringens* strains have a generation time of less than 20 minutes at temperatures between 33–49 °C, although the pathogen is capable of growing between 15–55 °C (Brynestad & Granum, 2002; Albrecht, 2005). Different temperature optima between the *C. botulinum* groups may explain differences in prevalence observed in different climatic areas. While vegetative cells show species- and strain-dependent variation in response to temperature, one key survival strategy of Clostridia is their ability to form endospores. Other bacteria, both Gram

positive and Gram negative, can form endospores, however only the Gram positive genera of Clostridia and Bacillus and the visually Gram-variable Mycobacteria are widely known to have pathogenic species. Endospore formation is a complex developmental process, controlled by the expression of the master regulator Spo0A gene. The activity of Spo0A is mediated by five autophosphorylating histidine kinases (KinA-KinE) that respond to different environmental stresses, including nutrient depletion. The recalcitrance of clostridial endospores with respect to temperature is due to the structure and thickness of the bacterial endospore coat, which has proved particularly problematic for the food industry (Reddy *et al.*, 2003). Endospores can still remain viable after exposure to temperatures between -25 °C and 121 °C, and in some instances viability of endospores is enhanced after cold exposure (Mah *et al.*, 2009). Miwa (1975) identified *C. butyricum, C. perfringens*, *C. septicum* and *P. sordellii* in Antarctic soils exposed to temperatures between -38 °C and +3.2 °C, whereas Yang & Ponce (2011) isolated germinable-Clostridia endospores in 40 000-year-old Greenland ice cores, and up to 157 germinable endospores per gram in Atacama Desert soils, indicating pathogen persistence across extreme environmental conditions.

Seasonal changes in clostridial populations have been observed. For example, population decreases were reported in agricultural soils after cold periods, although populations recovered soon after (Brochier *et al.*, 2012). Some long-term field studies observed no seasonality in *C. botulinum* prevalence or abundance (Sandler *et al.*, 1993; Gessler & Böhnel, 2006) although seasonality is apparent in *C. botulinum* type C-related diseases such as avian botulism outbreaks and EGS (Sandler *et al.*, 1993; Mccarthy *et al.*, 2010; Espelund & Klaveness, 2014). Higher prevalence and abundance of clostridial pathogens in the soil can increase the frequency of disease (Wobeser *et al.*, 1987). Temperature increases above the lower bounds of growth 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

factor of $10^4$) (Neira *et al.,* 2015). Anaerobes begin to use inorganic and organic compounds as electron acceptors, pathways initiated by intracellular dehydrogenases and terminated in the soil solution. This anaerobic respiration causes a decrease of *Eh*, pH alteration and, after nitrate exhaustion, an increase in the concentration of such products as $Mn^{2+}$, $Fe^{2+}$, $S^{2-}$ and $CH_4$. Higher temperatures enhance the activity of dehydrogenases, further lowering *Eh* (Brzezińska *et al.,* 1998). Studies have indicated that some clostridial pathogens can only grow or sporulate within a range of *Eh*. For example, *C. perfringens* can grow at *Eh* < +200 mV in foods, whereas *C. botulinum* can only grow in foods <60 mV (Knechtges, 2011).

Båverud *et al.* (2010) identified a higher frequency of *C. difficile* in soil samples from water-filled ditches. The affiliation between saturated soils and higher clostridial prevalence is supported by numerous studies into bacterial populations in saturated (i.e. anaerobic) rice paddy soils. Weber *et al*. (2001) attributed 20 out of 31 clones isolated from paddy soil to class Clostridia. Additionally, they found that, after an eight-day anaerobic incubation of paddy soils, 55% of the active cells detected belonged to the *Clostridium* genus. Liesack *et al.,* (2000) concluded that Clostridia and Clostridia-like lineages of bacteria are typical inhabitants of flooded paddy soils. The prevalence of pathogenic species in sporadically waterlogged pasture and arable (non-paddy) soils is an important, yet under-researched, line of enquiry. Pathogenic strains are prevalent in many anoxic environments such as marshes, mudflats and water-bodies (Smith *et al.*, 1978; Sandler *et al.*, 1993). *Clostridium botulinum* type C was isolated in over half of 2200 sediment samples from 10 marshes in a study by Sandler *et al.* (1993), with a higher pathogen prevalence in permanently vs. seasonally flooded marshes. Mccarthy *et al.* (2010) suggest a decreased risk of EGS in pastures where soil drainage is utilised, supporting the association between higher clostridial disease risk and wet or waterlogged soils. 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

by <0.5 log after 30 minutes light exposure. Importantly, the light penetration depth (LPD) (depth at which surface light intensity is reduced by 99%) in soils can be as little as 300 μm (Ciani *et al.*, 2005). Therefore, solar inactivation may represent a small, but effective, method of vegetative cell destruction at the soil surface (Moynihan, 2012). Tillage may enhance clostridial die-off by exposing deeper soil layers to UV radiation.

*4.4 Oxygen availability*

Clostridia are frequently described as obligatory anaerobic organisms, although many species can exhibit varying degrees of aerotolerance (Hill & Osterhout, 1972; Tally *et al.*, 1975; Brioukhanov & Netrusov, 2007). Pathogenic species including *C. botulinum*, *C. perfringens*, *C. septicum* and *C. tetani* can survive in temporarily microoxic environments in a growth-arrested stage (Hill & Osterhout, 1972; Dezfulian, 1999; Briolat & Reysset, 2002; Brüggemann *et al.*, 2004). Both *C. septicum* and *Pr. bifermentans* were more resistant to oxygen than other clostridial pathogens (Hill & Osterhout, 1972), although Tally *et al.* (1975) demonstrated that pathogens *C. botulinum* and *P. sordellii* could grow at 10 and 7.5% oxygen concentrations, respectively. This is within the range of expected soil oxygen concentrations. Ioannou *et al.* (1976) found that soil oxygen concentrations varied from 1.5-20% depending on the irrigation regime. When flooded, oxygen concentrations remained consistently low at around 2% (Ioannou *et al.,* 1976). The adaptive response to oxidative stress is thought to be dependent on a range of specialised genes, some which are permanently expressed, and others which are transcribed under oxidative stress (Jean *et al.*, 2004; Brüggemann *et al.*, 2004; Brioukhanov & Netrusov, 2007; Hillmann *et al.*, 2008). These mechanisms increase clostridial resistance to oxygen-exposure whilst in a vegetative form. It has been shown that 18 hours of oxygen exposure (100% $O_2$) results in almost complete inactivation of *C. perfringens, C. histolyticum, 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

negligible role in the prevalence of *C. botulinum*. Further research is needed to identify other antagonistic relationships between *Clostridium* pathogens and other bacteria, and their significance in pathogen population dynamics.

## 5. Soil physicochemical factors

### *5.1 Soil type and structure*

Soil type and structure strongly affects the prevalence, survival and movement of bacteria in all soils. Mawdsley *et al.* (1995) stated that as bacterial populations are confined to the aqueous phase and solid-liquid interface of soils, soil water content and water movement are of utmost importance to bacteria survival and movement. The soil parent material affects the composition of the mineral components clay, silt and sand and organic matter, which influences various soil properties including soil chemistry, texture, porosity and nutrient availability. Finer-grained or organic soils have been shown to enhance survival of some enteric bacteria, due to the increased ability to retain water and nutrients (Jamieson *et al.*, 2002); this is also likely to be the case with clostridial pathogens.

The movement of water through a soil profile is strongly influenced by soil pore micro- and macro-structure. Micropores of < 1-1.5 μm diameter may severely restrict the translocation of large, rod-shaped bacteria in the soil (Mawdsley *et al.*, 1995). Studies indicate that increased vertical translocation of bacteria can be expected in more macroporous soils, such as in structured clay or sandy soils (Mosaddeghi et al., 2009; Safadoust et al., 2011; Natsch et al., 1996). Soils with a predominately microporous structure, such as unstructured, compacted, silty or sandy clays, are more efficient at filtering bacteria by physical obstruction, reducing 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-

limited soils (Dorr de Quadros *et al.*, 2012). As fertiliser addition is a common practice on both arable land and improved pasture, the growth response of clostridial pathogens to increased nutrients should be ascertained.

Many studies have found significant relationships between soil organic matter content and occurrence of *C. botulinum*. *Clostridium botulinum* type A is more prevalent in soils with low organic matter content, whereas types B and C show a strong association with higher organic contents (Smith, 1978; Dodds, 1992; Espelund & Klaveness, 2014). This may just be due to variations in soil pH rather than organic content, although Böhnel & Lube (2008) postulate that the general lack of microporous aeration, or raised nutrient contents in decaying organic matter, may assist in triggering *C. botulinum* growth.

*5.3 Soil pH*

Smith (1975b) identified a statistically significant relationship between higher counts of *C. botulinum* types A & B in neutral to alkaline soils. However, in a later study, Smith (1978) found a higher prevalence of *C. botulinum* type A in neutral-alkaline soils (average pH 7.5), whereas type B prevailed in slightly more acidic soils (average pH 6.25). Environmental botulism outbreaks have also been associated with water of pH 7.5–9 (Espelund & Klaveness, 2014). Both *C. perfringens* and *P. sordellii* were prevalent across a wide soil pH range in Costa Rican soils (del Mar Gamboa *et al.*, 2005). This is coincident with other studies which have isolated both vegetative and endospore forms of *C. perfringens* across a range of acidities ( Li *et al.*, 2007; Stefanis *et al.*, 2014), including the acidic soils (pH 4.5–6.5) surveyed by Voidarou *et al.* (2011). These studies are all in agreement with laboratory-determined pH growth conditions (Table 2), and collectively indicate that soil pH is not a suitable predictor for *C. perfringens* and *P. sordellii* prevalence.

## 6. Agricultural management

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

### *6.1 Tillage*

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

were found to comprise a greater proportion of the total bacterial population than in conventionally tilled soils. Using 16S rDNA analysis, Dorr de Quadros *et al.* (2012) found that the relative abundance of Firmicutes showed a positive association with no-till systems, with *Clostridium* species and other anaerobic bacteria dominating. In conclusion, it is likely that the reduced aeration and increased water-retention in no-till soils enhance the formation of anoxic soil microsites. Deep and conventional-ploughing will bring unexposed and protected clostridial cells to the surface, where they may be inactivated by the sunlight and oxygen, although Clostridia may profilerate in compacted soils caused from repeated wheeling or loss of soil organic matter. However, there is a paucity of empirical evidence linking agricultural practices that aerate the soil to changes in clostridial disease frequency (Jeffery & Van der Putten, 2011).

*6.2 Organic soil amendments*

Historically, the application of organic soil amendments (OSA) to agricultural land has been used as an efficient method of replenishing soil nutrients and managing organic wastes (Venglovsky *et al.*, 2006). This practice has become an increasingly attractive option due to heightened environmental and economic concerns regarding the use of conventional chemical fertilisers. Moreover, other bio-wastes such as compost and anaerobic digestates are now frequently applied to soils. Organic soil amendments such as manure, slurry, sewage and other bio-wastes can contain high pathogenic loads, including many clostridial pathogens, as indicated in Table 3 (Bagge *et al.*, 2005, 2010; Sahlström *et al.*, 2008; Torniainen *et al.*, 2011). Incorporating unsanitised organic wastes into agricultural soils can induce artificially high pathogen populations, increasing the risk of pathogen transmission into the food chain or to livestock (Bagge *et al.*, 2010; Brochier *et al.*, 2012). Furthermore, Clostridia have been shown 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 $1.5 \times 10^4$ 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

cultivations have been documented in other studies (Stefanis *et al.*, 2014). *C. perfringens* endospores and vegetative cells occurred in 67% and 17% of samples, respectively, under maize cropping, yet both forms were absent under cabbage cropping. It is unclear whether these variations can be attributed to the cropping regime alone, emphasising the need for more controlled experiments where confounding variables can be minimised.

## 7. Methods of detection

A more comprehensive understanding of clostridial prevalence, abundance and behaviour in soils could be obtained with extensive, geographically-widespread soil-surveys covering the complete range of soil types, agricultural regimes and climates. This, in combination with laboratory microcosm experiments manipulating the key environmental and physicochemical factors mentioned, would help elucidate the complex behaviour of Clostridia in soil. Suitable diagnostic techniques are needed to facilitate the following objectives (1) generation of accurate pathogen prevalence and abundance data, (2) differentiation between vegetative growth and endospore cell forms, and (3) easy reproducibility and application in microbiology laboratories. The scientific concept behind the various diagnostic methods is discussed in depth elsewhere, therefore this paper overviews the applicability of various techniques for meeting these objectives.

### *7.1 Culture-based assays*

Culture assays can be used to identify and enumerate some clostridial species in samples using the plate count or the most probable number (MPN) methods. Culture-based identification and enumeration is still a widely used technique (Sonnabend *et al.*, 1987; Vijayavel & Kashian, 2014), and samples are commonly cultured in enrichment media prior to using other 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

between the clostridial species/toxinotypes using one protocol, such as with denaturing gradient gel electrophoresis (DGGE), temperature-GGE (TGGE) (Marzorati *et al.*, 2008), amplified fragment length polymorphism (AFLP) (Keto-Timonen *et al.*, 2006), ARISA (Dahllöf, 2002; Feligini *et al.*, 2015), T-RFLP (Khoruts *et al.*, 2010) and single strand conformation polymorphism (SSCP) (Smalla *et al.*, 2007; Marzorati *et al.*, 2008).

Polymerase chain reaction-based approaches have high applicability for pathogen detection, although the methods are not without their limitations. Wintzingerode *et al.* (1997) detail the common pitfalls associated with soil microbial analysis using PCR-based approaches. The sensitivity of these methods is heavily dependent on the design of the primers, the efficacy of the DNA extraction methods and various other considerations; these biases can impair the sensitivity and accuracy of results (Acosta-Martínez *et al.*, 2008). The exceptionally high but poorly understood soil microbial diversity creates another issue with PCR-based diagnostics. In what is commonly referred to as the "black-box" of soil ecology, it is unknown as to what effect the many undiscovered species could have on interpreting PCR-based data (Tiedje *et al.*, 1999). Many rare species may remain undetected, and unknown species showing high genetic homology could lead to the generation of false-positives (Culman *et al.*, 2008).

*8.2 Sequencing*

Nucleotide sequencing is now the standard technique for confirmative detection of pathogens. This tool also provides an increasingly cost-effective way to identify, survey and compare bacterial communities across different environments (Burke & Darling, 2014). Gene fragments are sequenced using a number of techniques, such as pyrosequencing (Roesch *et al.*, 2007; Acosta-Martínez *et al.*, 2008) or Illumina sequencing (Dorr de Quadros *et al.*, 2012; Burke & 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

predicting Clostridia behaviour due to the persistence of endospores which can survive in soil for several years. Highly variable physiologies result in different geographic distributions between species and strains, although further work is needed to identify the genetic adaptations which affect strain prevalence in certain regions.

The major environmental, soil physicochemical and agricultural management variables likely to influence pathogen presence and behaviour were identified. Although only tentative links could be made, increased soil moisture is likely to enhance survival, and even promote the regrowth of clostridial pathogens. Soil type will also influence the water retention and nutrient status of a soil, subsequently affecting survival rates, while the structure will dictate the ability of a soil to retain pathogens in the uppermost soil horizons. Common agricultural practices, such as manure, slurry and other biowaste application, intensive grazing, and no-tillage systems could lead to elevated pathogen levels, heightening the risk of clostridial disease in livestock, and transfer of contaminated soil onto overlying crops.

## 10. Future research

Future research should identify the role of key soil, environmental and management factors on pathogen behaviour, using both microcosm and field studies. Pathogen behaviour in saturated, poorly-drained and fine-grained soils should be determined, as these soil types may permit the proliferation or enhanced survival of pathogens. Additionally, the long-term effect of applying pathogen-containing biowastes to farm soils should be ascertained, as this could create elevated contamination levels, especially with some underlying soil types. This knowledge will allow improved pathogen modelling and mapping, development of better risk management strategies, with the aim of reducing the incidence of clostridial disease. Fundamental to the validity of 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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1276
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 |

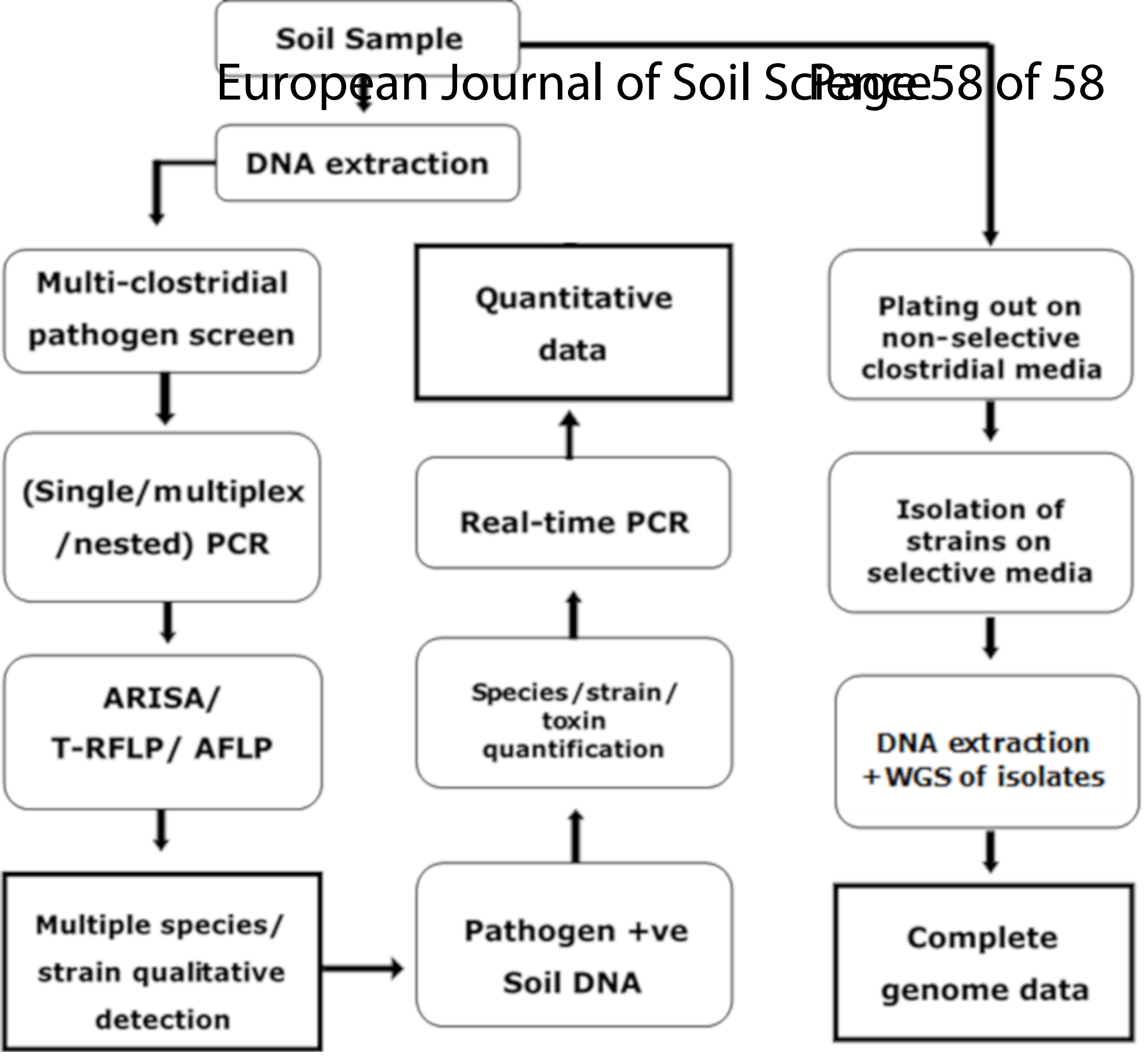
1283 [a]Restriction enzyme analysis; [b]Municipal soil waste

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