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Food Safety Implications of Potentially Pathogenic Clostridia

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EXECUTIVE SUMMARY

Background

The microbiological safety of food depends on ensuring that pathogenic microbes are eliminated from food or prevented from growing. For most of the well-known microbial pathogens the conditions necessary to achieve this have been worked out in considerable detail over many years. However from time to time new hazards emerge and information about the properties of the new organisms becomes necessary. In recent years certain strains of *C. butyricum* and *C. barati* have been isolated that produce neurotoxins very similar to those produced by *C. botulinum* types E and F respectively. Initially these organisms were associated with infant botulism but *C. butyricum* has also been implicated in foodborne illness. A different example of a possible new hazard is illustrated by a product recall based on high numbers of *C. tertium* and *C. bifermentans* in pate. Though neither of these organisms is associated with foodborne illness the counts were sufficiently high to cause concern. With all three organisms there is a need for more information about their growth characteristics and properties in relation to food safety.

Aims

The aims of this project were (i) to establish the proportion of *Clostridium butyricum* strains isolated from natural sources that carry the botulinum neurotoxin gene, (ii) to determine whether *C. tertium* and *C. bifermentans* produce toxins and (iii) to define the conditions of temperature, pH and solute concentration that will prevent growth of *Clostridium butyricum, C. tertium, C. bifermentans* and *C. barati.*

Approach

The experimental plan for the first objective was to develop and use a selective enrichment medium to isolate *C. butyricum* from food and environmental samples, and then to screen isolates for the presence of the type E toxin gene using PCR. Testing for possible toxicity in *C. tertium* and *C. bifermentans* was based on screening culture filtrates for toxic effects against cultured Vero cells. For the final objective, limiting pH values and solute concentrations (NaCl, sucrose) and temperatures for growth were determined by inoculating replicate tubes of growth medium with spore inocula of test organisms derived from a cocktail of non-toxigenic strains or toxigenic ones.

Key findings

Just under one thousand food samples were examined for *C. butyricum* by enrichment in a minimal medium with lactate and acetate as a source of carbon and energy. Selective antibiotics are present in the medium to favour growth of *C. butyricum.* The foods examined were mainly fresh vegetables but also included milk, cream, yoghurt and pâté. Ninety three isolates were tested for the presence of the gene encoding type E botulinal toxin by PCR but no toxin positive samples were detected. A nested PCR approach was tested as a means of distinguishing between *C. butyricum* and the physiologically similar *C. beijerinckii* but the primers were sufficiently specific for the unequivocal identification of *C. butyricum.*

When culture supernatants of *C. tertium* and *C. bifermentans* were tested against Vero cells three out of five *C. tertium* strains and five out of six *C. bifermentans* strains showed a very weak toxic effect but, in most cases, it could only be demonstrated by incubating Vero cells for 48h rather than 24h and was quickly lost on moderate dilution.

Growth limits for *C. butyricum, C. barati, C. tertium* and *C. bifermentans* were determined in broth incubated anaerobically at 30°C for up to 42d. The minimum pH values permitting growth depended on the acidulant and strain. Organic acids were more effective at inhibiting growth than HCl as expected. In general the toxigenic strains appear to be less tolerant of acid conditions than the non-toxigenic ones.

The lowest pH values at which growth of toxigenic and non-toxigenic strains of *C. butyricum* was observed in broth acidified with HCl were 4.1 and 4.2 respectively. The minimum water activities for growth of toxigenic and non-toxigenic strains of *C. butyricum* were 0.95 and 0.96 respectively. The lowest pH values for growth of *C. bifermentans* and *C. tertium* in broth with HCl as acidulant were 4.1 and 4.2 respectively whilst in the presence of organic acids the minimum pH for both species was between 4.4 and 4.9. The minimum water activity for growth of *C. tertium, C. bifermentans and C. barati* was 0.95 for all. The minimum growth temperatures of the toxigenic strains of *C. butyricum* (ca 10 -11°C) was somewhat higher than for non-toxigenic ones (7-8°C). The minimum growth temperature for the other clostridia was about 8°C.

Conclusions

Growth of *C. butyricum, C. bifermentans, C tertium* and *C. barati* was prevented by water activities that will prevent growth of proteolytic strains of *C. botulinum*. However these organisms can grow at pH values below those that prevent growth of *C. botulinum*. Control of *C. butyricum* in the food industry needs to allow for the greater pH tolerance of this species compared with proteolytic *C. botulinum*. *C. tertium* and *C. bifermentans* showed some toxic activity against Vero cells but the effect was so weak that it was concluded that growth of these organisms in food is unlikely to be a significant risk factor in food safety.

CONTENTS

INTRODUCTION

The main species of clostridia associated with foodborne illness are *Clostridium botulinum* and *C. perfringens* and much effort has been directed towards understanding the factors controlling their growth, toxin production and the resistance of their spores (Labbé, 2000, Lund and Peck, 2000). Other clostridia occur in certain types of food and, whilst they may cause serious spoilage problems, are not normally regarded as dangerous. Examples include *Clostridium tyrobutyricum* and other butyric clostridia in cheese (Fowler, 1979; Senyk *et al.*, 1989; Ryser, 1999), *C. pasteurianum* and *C. butyricum* in canned fruit and pasteurised acid sauces and the clostridia that cause soft rot of vegetables (Lund, 2000). Most strains of *C. butyricum* are harmless to humans and culture filtrates are not lethal to mice; in fact some strains have been investigated as probiotics for preventing diarrhoea (Sato and Tanaka, 1996; Kamiya *et al.*, 1997; Sato and Tanaka, 1997) *Helicobacter pylori* infection (Takahashi *et al.,* 2000) and inflammatory bowel disease (Kanauchi *et al.,* 2003). However some strains have acquired the type E botulinum neurotoxin gene (BoNT E) and have caused both infant and classical botulism. The first recorded incident of *C. butyricum* type E botulism was a case of infant botulism in Italy (Aureli *et al.*, 1986). More recently there has been an outbreak of classical type E botulism in China associated with *C. butyricum* (Meng *et al.*, 1997, Meng *et al.*, 1999). The food implicated was a salted and fermented paste made from soybeans and gourds. There has also been a recent suspected outbreak *C. butyricum* type E classical botulism in India associated with Sevu a crisp made gram flour (Chaudhry *et al.*,1998).

It is generally assumed that toxigenic strains of *C. butyricum* are rare but very little information is available on their occurrence in the environment or in food materials (Lucchini *et al.,* 1998; de Jong, 1989). Following the Chinese outbreak, soil specimens were collected from near the home of patients and also from five separate locations near a lake about 50 miles away (Meng *et al.,* 1999). Toxigenic *C. butyricum* were isolated from four of the six sample sites, suggesting that these organisms may be more common than previously supposed. There are no data available for the UK. The potential for non-botulinum clostridia to harbour botulinum genes and cause

disease is further illustrated with *C. barati* (Hall, 1985, McCroskey *et al.*, 1991) Some strains of this common saccharolytic species have acquired genes for type F toxin. This organism is phenotypically similar to *C. perfringens* and many strains were deposited in culture collections as *C. paraperfringens*, but there is almost no information on the conditions limiting its growth.

In routine examinations of food products for the presence *C. perfringens* the presence of *C. tertium* and *C. bifermentans* was discovered in some pate samples. Though neither of these organisms is associated with foodborne illness the counts were sufficiently high to cause concern leading to a product recall. *Clostridium bifermentans* is not uncommon in foods and in a survey of processed meats in Japan, 20% of products tested contained culturable *C. bifermentans* (Kokubo *et al.*, 1986). It was also the clostridium most commonly isolated from unprocessed meats by Baltzer and Wilson (1965) and Matsuda *et al.* (1975). *Clostridium bifermentans* forms a single genospecies with *C. sordellii* though there are certain phenotypic differences between them (Nakamura *et al.*,1975). *Clostridium bifermentans* produces a phospholipase C and a protease - properties that are often associated with the more pathogenic species- and contains genes with sequences similar to those encoding *Bacillus thuringiensis* toxins (Barloy *et al.*, 1998). *Clostridium tertium* has not been isolated from foodstuffs with the same frequency, but has been identified as a spoilage organism in feta cheese (El-Fatal *et al.*, 1998). *C. tertium* does not normally cause disease in healthy individuals but can cause bacteraemia in immunocompromised patients, can infect wounds and is associated with some other medical conditions (Gosbell *et al.*, 1996).

Implementation of HACCP in the food chain requires knowledge of both the hazard and the risk posed by foodborne microbes. In the case of *C. butyricum* the hazard is the possible production of botulinum toxin in food or in the gut of infants. The risk depends on the frequency with which toxigenic strains occur in food materials and the likelihood that they are able to survive processing and grow and produce toxin. Although some information is available in the literature on minimum growth temperatures and maximum salt concentrations permitting growth of *C. butyricum*, the data are very limited and, where available, not precise (Morton, 1998). In the cases of *C. barati*, *C.*

tertium and *C. bifermentans* there is even less information available on the limiting conditions for growth.

Aims

The scientific objectives of this work were as follows:

1. To establish the proportion of *Clostridium butyricum* strains isolated from natural sources that carry the botulinum neurotoxin gene.
2. To determine whether *C. tertium* and *C. bifermentans* produce toxins.
3. To define the conditions of temperature, pH and solute concentration that will prevent growth of *Clostridium butyricum, C. tertium, C. bifermentans* and *C. barati*

The experimental approach was to isolate strains of *C. butyricum* from soil and food materials and test their ability to produce toxin by probing for the presence of toxin genes. Testing for toxin in *C. tertium* and *C bifermentans* is more problematic because the nature of the toxin is completely unknown. Toxins represent a large class of very different molecules and there is no general test that will detect all types. For example testing for fluid accumulation in ligated ileal loops can be used to detect many diarrhoeal toxins but will not detect the emetic toxin of *B. cereus*. This toxin is also not antigenic or cytotoxic (Granum and Baird-Parker, 2000). However a number of toxins from both Gram-positive and -negative organisms give a positive result when tested against cell lines. For example the *C. perfringens* enterotoxin and the *B. cereus* diarrhoeal toxin cause cytopathic changes in Vero and CaCo2 cells and the *B cereus* emetic toxin causes vacuolation in cultured Hep-2 cells (Labbé, 2000). It was therefore decided to to screen culture filtrates for toxic effects using cultured Vero cell lines.

The growth limits for *C. butyricum, C. tertium* and *C. bifermentans* will be defined in broth incubated under anaerobic conditions at different temperatures and pH values and with increasing concentrations of solute (NaCl and sucrose representing ionic and non-ionic solutes). The limiting pH will be determined for different acidulants (HCl, lactic, acetic acids). There is some evidence that the growth properties and heat resistance of toxigenic

strains of *C. butyricum* are different from the non-toxigenic types (Morton *et al.*, 1990) and these will therefore be tested separately.

This work will provide information on the incidence of neurotoxigenic *C. butyricum* in food and the environment and will clarify the capacity for toxin production in *C. tertium* and *C. bifermentans*. Information on the limiting growth conditions of temperature, pH and water activity for these organisms will allow better control in the food chain. The Food Standards Agency will receive new information on clostridia in food on which it can base its decisions when providing food safety advice to the public and to the Government.

Tasks

Task number	Delivery date	Description
01/01	31/12/02	Assemble a collection of strains of *Clostridium butyricum*, *C. bifermentans* and *C. tertium*.
01/02	30/09/04	Isolate *C. butyricum* strains from natural sources (soil, food raw materials etc) using elective enrichment methodology.
01/03	31/03/05	Screen *C. butyricum* isolates for toxin genes
01/04	30/09/05	Develop and test a gene-probe method for identifying *C. butyricum* that is able to distinguish it from *C. beijerinckii*.
02/01	31/03/04	Screen culture filtrates of *C. bifermentans* and *C. tertium* for toxic effects against cultured cell lines
03/01	30/09/03	Define the growth limits for *C. butyricum*, in broth under anaerobic conditions
03/02	30/09/03	Define the growth limits for *C. tertium* in broth under anaerobic conditions.
03/03	30/09/03	Define the growth limits for *C. baratii* in broth under anaerobic conditions.
03/04	30/09/03	Define the growth limits for *C. bifermentans* in broth under anaerobic conditions.
03/05	30/09/03	Examine growth behaviour in food substrates.

Glossary

16S rRNA. Ribonucleic acid of the small ribosomal subunit. Comparison of the base sequences of variable regions provides information on the genetic relatedness of microorganisms. Unique regions can be used for identification

Anaerobe cabinet. Large perspex cabinet kept under an anaerobic atmosphere (85%Nitrogen, 10% carbon dioxide, 5% hydrogen) for growth and manipulation of oxygen-sensitive microbes. Manipulations are carried out using arm-length butyl rubber gloves that protrude into the cabinet. Samples are introduced via an air-lock.

BoNT toxin. Botulinum neurotoxin

dNTP Deoxyribonucleotide, where N stands for any of the four bases adenine, guanine, cytosine, thymine. Used in PCR reaction.

Eppendorf tube. Disposable small conical plastic tube (0.5 – 1.5ml volume). Designed to fit in bench-top centrifuges

Ethidium bromide. Fluorescent dye used for staining nucleic acids

Hungate tubes. Glass tubes sealed with butyl rubber stoppers for growth of anaerobes.

PCR. Polymerase chain reaction. Reaction in which a target sequence of DNA is amplified exponentially. In the first part of the process, the two DNA chains in the double helix are separated by heating the reaction vial to 90-95°C. The vial is then cooled to around 55°-65° C to allow short oligonucleotide `primers' to bind or `anneal' to specific sequences on the DNA strands. The final step of the reaction is to make a complete copy of the target templates. Since the Taq polymerase works best at around 72° C, the temperature of the vial is raised. The Taq polymerase adds nucleotides to the primer and eventually makes a complementary copy of the template. This completes one PCR cycle. At the end of a cycle, each piece of DNA in the vial has been duplicated. The cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template, so after 30 cycles, 1 billion copies of a single piece of DNA can be produced.

Primers. A pair of oligonucleotides that bind to the two ends of the target sequences of the DNA. These provide the recognition points for the Taq polymerase enzyme

Selective agar. Agar plating medium containing ingredients to suppress the growth of non-target organisms. Selective agars often contain `diagnostic' reagents to indicate characteristic biochemical activities of the target organisms e.g. production of hydrogen sulphide or fermentation of particular sugars.

Selective enrichment. Incubation in a liquid medium containing inhibitors or selective agents to suppress the growth of non-target organisms

Taq enzyme. Heat resistant DNA polymerase enzyme obtained from Thermus aquaticus

Template DNA. DNA to be amplified

Thermocycler, Biometra Thermoblock. Apparatus designed to perform the heating and cooling cycles during amplification of DNA sequences by PCR

Vero cell. Cell Line originally derived from Monkey kidneys. Grown in monolayers in clear plastic flat-sided sterile plastic containers. Used for testing possible toxic effects of culture supernatants.

N.B. Abbreviations used for microbiological media are given in Annex 2.

MATERIALS AND METHODS

Organisms

The *Clostridium* species used and checks of identity are described in the Results Section under Task 1. A full list of strains and their origins is given in Annex 1.

Media

The compositions of the media used are described in Annex 2. Fluid Media were produced in 500 ml volumes and reduced by heating to 121˚C for 15 min. They were transferred to an anaerobe cabinet containing a $CO_2/H_2/N_2$ (5:10:85 *v/v*) atmosphere to cool overnight. Aliquots (9.5 ml) of each medium were transferred to Hungate tubes and sealed within the anaerobe cabinet. The tubes were then removed from the anaerobe cabinet and sterilised by autoclaving at 121˚C for 15 minutes. Agar Media were sterilised at 121˚C for 15 min and cooled to 50˚C before addition of growth supplements or selective agents (see Annex 2). Plates were poured under aseptic conditions, cooled, and stored at 4°C until required. Before use plates were incubated for 24 h in an anaerobe cabinet under an atmosphere of 10% H_2, 10% CO_2, 80% N_2 (Don Whitley Scientific, Shipley, UK)

Maintenance of cultures

Freeze-dried cultures were suspended in 0.5 ml of sterile reduced PYGS medium and mixed gently with a pipette. Each cell suspension was inoculated into 10 ml pre-reduced Growth and Storage Medium (0.1ml inoculum); onto PYGS agar and onto selective blood agar plates containing neomycin supplement (Oxoid SR 163). Inoculated media were incubated for 48 h under anaerobic conditions. Cultures were sub-cultured anaerobically to obtain single colonies which were Gram-stained and examined under a 100 x phase-contrast oil immersion objective (total magnification x 1000) to confirm purity. Cultures prepared from single colonies were inoculated into Microbank vials (ProLab Diagnostics, Neston, Cheshire) and stored at -70°C. Cultures were also inoculated into sterile reduced augmented cooked meat medium, incubated at 30˚C overnight and subsequently stored at 4˚C.

Production of spore stocks

Spore suspensions of *C. barati* strains were produced using Duncan and Strong sporulation medium as previously described for *C. perfringens* (Harmon and Kautter, 1986; Garcia-Alvarado *et al.*, 1992). Spores of other species were produced by growth in PYGS broth or on PYGS plates. Growth from overnight cultures in PYGS broth was inoculated into PYGS broth or onto PYGA agar and incubated anaerobically at 30°C for 3-4 days (broth) or 7 days (agar). In some initial experiments spores were produced using the two-phase Robertson's Cooked Meat Medium system as described by Peck *et al.* (1992).

Sporulated growth on agar plates was suspended in sterile 0.85% (w/v) NaCl (saline). Spore suspensions or sporulated cultures were harvested by centrifugation at 15,000 x *g* at 4°C for 15 min. Each pellet was washed 15 times with ice-cold sterile saline. The resultant pellet was suspended in 5 -10 ml saline and stored at 4°C. For enumeration spore suspensions were heat-shocked at 60°C for 10 minutes and plated onto reduced PYGS agar in triplicate. Inoculated plates were incubated for 48 h under anaerobic conditions. The numbers of viable spores were then estimated by counting the colony-forming units present on each plate.

Viable counts

Samples were serially diluted in maximum recovery diluent and plated on Blood Agar or Reinforced Clostridial Agar. Plates were incubated anaerobically at 30°C for 24 or 48h.

Methods for defining growth limits in *C. butyricum, C. baratii, C. tertium* and *C. bifermentans*

An aliquot of each spore suspension was heat-shocked at 60°C for 15 min, cooled on ice and diluted in pre-cooled deoxygenated PYGS broth. Spore suspensions of non-toxigenic strains of individual species were mixed to give a final concentration of 10^4 to $2x10^6$ spores ml^{-1}. The toxigenic strains *C. butyricum* ATCC 43755 and ATCC 43181 were studied individually. These strains also had a final concentration of 10^4 to $2x10^6$ spores ml^{-1}. Aliquots

(500µl) of these suspensions were used to inoculate 9.5ml of PYGS broth in anaerobic Hungate tubes to give a final volume of 10 ml and a final spore concentration of 10^2-10^5 spores ml^{-1}. The lid and septum of each Hungate tube were treated with Bioguard immediately before inoculation to prevent contamination. All experiments were conducted alongside negative controls inoculated with sterile PYGS broth and positive controls, where standard PYGS broth (pH 7.0 and 30°C incubation) was inoculated with spores. Each tube was examined daily for visible turbidity. Experiments were conducted over 42 days. Each spore suspension was checked by phase contrast microscopy (x100 oil immersion objective, total magnification x 1000) prior to use to ensure they had not germinated during storage.

Detection of cryptic growth. Visible turbidity occurs when cells reach a concentration of approximately 10^7 cells ml^{-1} medium. Growth below this threshold would not be detectable by visual estimation. In order to determine the presence of growth below the visual threshold, 0.1 ml volumes were taken from tubes at each concentration at time zero and after 1, 3, 5, 7, 14, 21, 28, 35 and 42 days incubation. This aliquot was suspended in 9.9 ml reinforced clostridial medium, mixed by vortexing and plated out onto Columbia blood agar. Plates were incubated under anaerobic conditions at 30°C for 48 h, and viable cell numbers estimated by counting the colony forming units.

Determination of culture purity in growth limitation studies. A 0.1ml aliquot was taken from every tube testing positive for growth and subcultured onto Columbia blood agar (48 hours at 30°C in anaerobic conditions). Cells were then identified using biochemical methods. The parameters tested were gelatin liquefaction, lecithinase activity, motility, spore position, catalase activity, Gram stain and ability to grow aerobically.

Adjustment of pH. The pH of PYGS medium was adjusted with HCl (1M), citric acid (1M), acetic acid (5M) or lactic acid (5M) as required. An excess of this medium was prepared to allow pH to be measured in sample tubes of media immediately after autoclaving, immediately before use, and at the end

of each 42 day experimental period. The pH meter was calibrated before the preparation of each batch of PYGS broth.

Adjustment of NaCl concentration. In initial experiments sodium chloride was added to PYGS medium on a percentage weight to volume basis. This was later converted from w/v to w/w using a calibration curve. In later experiments a weighed amount of salt was added to weighed medium to give w/w values. In all cases, the pH was adjusted to 7.0 prior to autoclaving and checked after autoclaving. All incubation was at 30°C. Corrections were made for the dilution effect of adding inoculum to the adjusted medium. Water activity was measured as described below.

Adjustment of sucrose concentration. Sucrose was added to PYGS medium on a percentage weight to volume basis, corrected for the dilution factor of adding 0.5ml of PYGS medium without sucrose as described for NaCl (above). When preparing media containing sucrose the sucrose and the solid constituents of PYGS medium were weighed out into a receptacle with volume gradations. Salt and antifoam solutions were then added, followed by distilled water to the required volume. Media were weighed before and after the addition of media solutions and distilled water. This ensured media concentration remained constant for the volume of medium prepared.

Measurement of water activity

Water activity was measured using an Aqualab water activity meter (Labcell Ltd. Basingstoke Hants), calibrated with saturated salt solutions of known water activity.

Temperature measurement

Experiments on the effect of temperature were conducted using a waterbath incubated within an LMS cooled incubator. Temperatures within each waterbath were monitored continuously with a Comark data logging thermometer using a type T thermocouple inserted into a Hungate tube containing 10 ml of water. The thermocouples were checked against a calibrated mecury in glass thermometer BS 593 C 105C. Tubes were

incubated in the waterbath, which acted as insulation against temperature fluctuations within the incubator.

Isolation of *C. butyricum* from food and environmental samples

Preliminary experiments with soil samples spiked with *C. butyricum* showed that the method which gave the best results was selective enrichment with Modified Bhat and Barker (1947) medium (MBB) medium followed by plating out onto iron sulphite agar (ISA) (Annex 2). This method was therefore used to isolate strains from foods. Fresh vegetables were purchased from a local farmers' market. Food samples (pasteurised milk, pate, sieved tomato) were purchased from a local supermarket.

Five gram samples of food were stomached with 50 ml MBB medium for 10 min. Five samples of 1 ml were inoculated into 9 ml MBB and incubated anaerobically at 37°C for 5 d. After 5 d the cultures were diluted 1:10 in fresh MBB and incubated for a further 5 d. After a second subculture into MBB and incubation, the culture was streaked onto plates of ISA which were incubated anaerobically at 30°C for 48 h. Catalase negative colonies having typical appearance of butyric clostridia were streaked onto RCM. Colonies were picked from RCM and stored in cryovials at -70°C.

Growth in food

Passata di Campagna thick country style sieved tomatoes were obtained from Mediterranean Growers Ltd. (Mill Hill, London, UK). Ardennes pate, chicken, bacon and red wine pate, duck and orange pate and Brussels pate were obtained from a local supermarket. Sieved tomatoes were transferred to Duran bottles (500ml) and the pH adjusted to 4.0, 4.2, 4.4, 4.6, 4.8 or 5.0 using 1M NaOH or 1M HCl as required. The initial pH of sieved tomatoes used in this study varied from pH 4.1 to pH 4.3. For some experiments 50 mM citric acid was added prior to the adjustment of pH. After pH adjustment, the bottles were sterilised by autoclaving. Autoclaved product was transferred aseptically to sterile universal bottles (20ml per bottle). Thirty universal bottles were prepared for each experimental strain at each pH. Twenty-four were inoculated with spores, the other six were used as controls for pH before and

after incubation. Tomato experiments were inoculated with either sterile physiological saline or approximately 10^5 heat-shocked spores from a mixture of non-toxigenic *Clostridium butyricum* strains (NCTC 6084, NCTC 7423, NCTC 8082, NCIMB 9575) or the toxigenic *C. butyricum* strain ATCC 43181 or the toxigenic *C. butyricum* strain ATCC 43755. Inoculated tubes were incubated at either 15°C or 30°C and viable counts were determined at intervals by plate counting.

Screening culture filtrates of *C. bifermentans* and *C. tertium* for toxic effects against Vero cell lines

Growth of test strains. All strains were grown in 25 ml Brain Heart Infusion broth (Oxoid, Basingstoke, UK). Each bottle was inoculated with approximately 2.5 x 10^4 spores (equivalent to 1 x 10^3 spores/ml) from one of the test strains listed above and incubated at 30°C under anaerobic conditions. Growth was monitored by measuring the change in optical density at 540nm (OD_{540}) and by determining viable counts on Wilkins-Chalgren anaerobe agar (Oxoid) and incubated under anaerobic conditions at 35°C for 48 h.

Preparation of culture filtrates. Cells were separated from the culture supernatant by centrifugation at 15,000x*g* and 4°C for 10 min. The supernatant was collected and filtered aseptically though a 0.45 μm filter. All filtrates were stored at 4°C until required. For experiments at a fixed pH, each filtrate was adjusted to the required pH using 0.1 M NaOH or 0.1 M HCl. After the pH was adjusted, samples were sterilised by filtration though a 0.45μm filter.

Maintenance of Vero cell line. The Vero cell line used in this study was Vero ECACC 84113001, supplied by the European Collection of Cell Cultures (ECACC, Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG). Cells were defrosted at room temperature and suspended in 5 ml of Vero cell growth medium (VCGM). Suspended cells (1.5 ml) were inoculated into 25 cm^2 tissue culture flasks containing 5ml VCGM and incubated at 37°C

for up to 7 days in 5% CO_2. Flasks were examined daily for cell growth. Medium was replaced at 48h intervals and cells were harvested when the cells covered a minimum of 70% of the flask surface (70% confluence). The VCGM in flasks containing cells at a minimum of 70% confluence was removed by aspiration and the attached cells washed twice with phosphate buffered saline. Trypsin-EDTA buffer (0.25%, Sigma T4049, Sigma-Aldrich, Poole, Dorset, UK) was added (2 ml for 25 cm^2 flasks and 5 ml for 75 cm^2 flasks). Each flask was gently agitated by hand for 1 min and the trypsin-EDTA buffer removed by aspiration. The flask containing the tissue culture monolayer was incubated at 37°C for 2-3 min with cell detachment encouraged by tapping the side of the flask. Detachment was confirmed by observation with an inverted microscope. Detached cells were suspended in 5 ml of fresh VCGM and gently suspended using a pipette. Suspended cells were pelleted by centrifugation at 1,000 rpm for 5 min, the medium removed by aspiration and the cells resuspended. A 200µl aliquot of this suspension was then added to 15 ml VCGM in a 75 cm^2 tissue culture flask and incubated at 37°C and 5% CO_2.

Preparation of microwell plates. Once the VERO cells were suspended in 1ml of fresh medium, the number of cells within the suspension was determined using a coulter counter on 10µl of cell suspension in 10ml of Isoton® II diluent (Beckman Coulter, High Wycombe, Buckinghamshire, UK). Cell counts were averaged for 3 readings. Cells were diluted in VCGM to give a final volume of 20,000 cells per cm^2 within each 12-well microwell plate (76,000 cells per well, 912,000 cells per plate). Each well was inoculated with a total of 1.5 ml diluted cell suspension. Inoculated plates were incubated at 37°C in 5% CO_2 until the cell monolayer was confluent.

Visual determination of cytotoxicity. Each culture filtrate was diluted in series to 10^{-6} in VCGM. A 1.5ml aliquot of each dilution was inoculated into one of the wells of a 12-well microwell plate containing confluent growth Vero cells. Inoculated plates were incubated at 37°C and 5% CO_2. After 24 h, each well was examined for cytotoxic effects with an inverted microscope. The

highest concentration at which only minimal cell damage occurred was recorded and this limit used to determine the range of concentrations of culture filtrate to use in the neutral red cytotoxicity assay.

Neutral red cytotoxicity assay. Filtrates were diluted fourfold in VCGM to give a percentage filtrate content of 50, 25, 12.5 or 6.25%. Each dilution was added to a separate well of a 12-well microwell plate containing confluent growth Vero cells to a final volume of 1.5ml per well. Plates were incubated at 37°C and 5% CO_2 for 24 or 48 h. After incubation, the media/filtrate mix was removed by aspiration and replaced with neutral red working solution filtered though a 0.20 μm filter. Neutral red working solution was a solution of 60 μg/ml cell-culture tested neutral red (Fisher Scientific Limited, Loughborough, Leicestershire, UK) in VCGM. The solution was prepared 24 h prior to use and filtered immediately before use. Cells were incubated with the neutral red working solution for 3 h. Following incubation, the neutral red working solution was removed by aspiration. The cell monolayers in each well were washed with a 0.5% formaldehyde/1% calcium chloride solution and the dye from each monolayer extracted into 1.5 ml of an acetic acid, ethanol and water mix (1:49:50, *v/v/v*) over 60 min. Neutral red uptake by Vero cells was estimated by measuring the dye extracted into the acetic acid/ethanol/water mix at 540nm using a Biolise microwell plate reader (BMG LabTechnologies, Champigny-sur-Marne, France). Toxicity was defined as the reduction in neutral red uptake by Vero cells incubated with filtrate compared to the uptake by Vero cells incubated with the same concentration of sterile filtered growth medium.

Methods for sequencing 16S rRNA of bacterial strains

DNA extraction from cell suspensions. DNA was extracted using a modified version of the method of Lawson (1989). A 500μl aliquot of each cell suspension prepared from lawn cultures (section 1.2) was incubated with 5μl lysozyme (10mg/ml) at 37°C for 20 minutes to lyse the cells. After this period, 8μl of proteinase K (10mg/ml) and 8μl of RNase (10mg/ml) were added. The suspension was mixed by inversion and incubated in a waterbath at 65°C for

1 hour. The increased temperature inhibited non-specific nucleases that would otherwise degrade the DNA. Sodium dodecyl sulphate (SDS [10%, 120µl]) was then added and the mixture incubated at 65°C for a further 10 minutes. The mixture was then removed from the waterbath and allowed to cool. Once the mixture was cooled, 640µl of phenol/chloroform mix (an equal volume to the volume present within the tube) was added and mixed by inversion until emulsified. The mix was centrifuged at 5,000rpm for 10 minutes. This separated the mixture into three layers, a lower solvent layer, an upper aqueous layer containing DNA and a layer of protein/cell debris separating the other two layers. The upper layer was removed to a clean Eppendorf tube using a wide bore blue tip. When the initial sample contained a large amount of protein, not all of the protein was separated by a single phenol/chloroform extraction. In these cases, TES was added to the upper layer until the solution cleared and the phenol extraction step was repeated to remove the excess protein. The DNA solution was mixed gently with 2.5 volumes of 100% ice cold (-20°C) ethanol (*e.g.* if there was 200µl of solution, 500µl of ethanol would be added) to precipitate the DNA and the mixture centrifuged on high speed for 5 minutes. The fluid was removed and the resultant pellet of DNA was allowed to air-dry for 20 minutes. Once dry, the DNA pellet was resuspended in 50-500µl of TE buffer (10mM Tris, 1mM EDTA pH 8.0). A 5µl aliquot was taken from this suspension and run against a λ standard (1µg) by gel electrophoresis to give a visual estimate of DNA concentration. A 0.8% agarose gel prepared with 30ml TAE (4.84g Tris-base, 1.09g glacial acetic acid and 0.292g EDTA in 1L of distilled water) buffer and 0.5% ethidium bromide (EtBr) was used for this purpose, run at 50mV in TAE buffer. Extracted DNA was then stored at -20°C until required.

PCR amplification of extracted DNA PCR amplifications were conducted using the following mix of reagents in a 0.5ml Eppendorf tube:

10X NH_4 reaction buffer (10µl), dNTP (10µl), primer 1 (1µl), primer 2 (1µl)
template DNA (2-10µl), sterile distilled water (67-75µl, 77µl for control)

The majority of amplifications were conducted on 2μl of DNA suspension. All PCR amplifications were conducted in a Biometra Thermoblock. The primers used were all commercially available products specific to a section of the 16S rRNA gene sequence. The forward primers (primer 1) used were pA, KK and Ari T. The reverse primer (primer 2) used was 3. Reagents were mixed by centrifugation for 30 seconds at high speed and the DNA within the mixture denatured to single strands by heating to 94°C for 3 minutes in a Biometra Thermoblock. The mixture was then placed on ice to prevent re-annealing. Taq polymerase (2μl) was added to the mixture, which was subsequently mixed by centrifugation at high speed for 30 seconds. The mixture was then overlaid with three drops of mineral oil and subjected to 25 PCR amplification cycles of 94°C for 1 minute, 55°C for 1.5 minutes and 72°C for 1.5 minutes with a final treatment of 72°C for ten minutes at the end of the last cycle. The lower layer of fluid within the Eppendorf (underneath the mineral oil) was removed to a fresh tube and 2μl assayed by gel electrophoresis using the parameters described for running DNA against a standard.

Purification of PCR amplification products. DNA amplified by the polymerase chain reaction was cleaned using a QIAgen PCR clean-up kit. PB buffer (500μl) was added to PCR product (100μl) and inverted to mix. This mixture was transferred to a QIAgen spin column over a 2ml collection tube and centrifuged for 2 minutes at 13,000rpm. The flow-through was discarded and 750μl of PE buffer added to the spin column. The column was then spun twice at 13,000 rpm for 1 minute, discarding the flow-through after each spin. The spin columns were then transferred to fresh 1.5ml Eppendorf tubes (with lids cut off to allow centrifugation). EB (elution buffer) buffer (45μl) was added to each column and the column spun at 13,000 rpm for 1 minute. The spin column was then discarded and the Eppendorf (now containing the purified PCR product) labelled and stored at -20°C.

Sequencing. Sequencing reactions were performed by Dr. Paul Lawson and were run on a Perkin Elmer ABI Prism 377 automatic sequencer. DNA

sequence was analysed using a combination of the Genetic Computer Group (University of Wisconsin) and Lasergene DNAStar Computer Packages.

Screening *C. butyricum* isolates for BoNT toxin genes

All *Clostridium* strains were cultured in 20 ml of pre-reduced Peptone Yeast Glucose Starch (PYGS) medium and incubated under anaerobic conditions at 37°C for 24 to 48 h. The method for screening for the type E BoNT gene was as described by Lindstrom *et al.* (2001) with minor modifications

Template preparation. Harvested cells from 1 ml of each clostridial culture were washed with 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA) and centrifuged for 5 min. at 10.000 × *g*. The 1h pre-incubation stage as suggested by Lindstrom *et al.* (2001) was omitted. The pellet was resuspended in 1ml distilled water (GIBCO, UK). All suspensions were heated at 99 °C for 10 min to release the bacterial DNA and were centrifuged for 5 min at 10,000× *g*, and 1 µl of each supernatant was used as template in the PCR mixture.

Primers. One primer pairs with each being specific for *C. botulinum* type E was used (Lindstrom *et al.*, 2001) (Table 1).

Table 1. Primers for PCR detection of *C. botulinum /C. butyricum* type E

Type	Primer	Sequence (5'-3')
Ef	CBMLE1	CCA AGA TTT TCA TCC GCC TA
Er	CBMLE2	GCT ATT GAT CCA AAA CGG TGA

PCR. PCR was performed with 25 µl [a] of reaction mixture containing 1 µl [a] of template and 1 µl of each primer (Sigma-Genosys Ltd., Cambridgeshire, United Kingdom) and Titanium™ Taq PCR Kit # 639210/11 (BD Biosciences, Oxford, UK) including the following reagents [b] (Table 2)

Table 2. PCR reagents and their concentrations		
Reagent	**Ingredients/Concentration**	**Volume**
Titanium 10x PCR buffer	400 mM Tricine-KOH (pH 8.0 at 25°C)	100µl
	160 mM KCl	
	35 mM MgCl2	
	37.5 mg/ml BSA	
Titanium 50x NTP mixture	10 mM each of (dATP, dCTP, dGTP, and dTTP)	20µl
Titanium 50x PCR *Taq*	50 % Glycerol	20µl
	20 mM Tris-HCl (pH 8.0)	
	100 mM KCl	
	0.1 mM EDTA (pH 8.0)	
	0.25 % Tween-20	
	0.25 % Nonidet P-40	
Primer E1 [c]	0.3 µM	10µl
Primer E2 [c]	0.3 µM	10µl
Nuclease-free water		440µl

[a] In most cases 15 µl reaction mix and 10 µl of template was enough
[b] All reagents were stored at -20°C until use
[c] The concentration of forward and reverse primers were 30µM each

The amplification was carried out using a PTC-200 Peltier Thermocycler (MJ Research Inc., Watertown, Mass.). After initial denaturation at 95°C for 1 min. each PCR cycle consisted of denaturation at 95°C for 30 s, annealing at 60°C for 25 s, and extension at 72°C for 1 min 25 s and was repeated 27 times. Final extension was at 72°C for 3 min. The amplified PCR products were visualized in 0.8 or 2% agarose gels (Sigma, Pool, UK) stained with ethidium bromide. Bromophenol blue (0.25%) in Glycerol (30%) was used as loading buffer and 100 bp and/or 1kb DNA ladder (Promega, Madison, USA) were used as molecular weight markers to indicate the sizes of the amplification products. An aliquot (3 μl) of each amplification reaction mixed with 2 µl of loading buffer and was analysed on 0.8 or 2% w/v agarose gels cast and run in TAE buffer which contained 0.04 M Tris base, 0.04 M acetate, 0.01 M EDTA (Amresco, Ohio, USA). Gels were stained with ethidium bromide and photographed using transmitted U.V. light.

Development of a gene probe method to distinguish *C. butyricum* from *C. beijerinckii*.

Template preparation. Cells were harvested from freshly grown culture (24-48 h) in PYGS broth at 10,000 × *g* for 10 min and resuspended in 500 µl TES buffer (0.05 M Tris, 0.05 M NaCl, 0.005 M EDTA, pH 8.0). 5 µl lysozyme (10 mg/ml) was added, mixed and incubated in water bath at 37 °C for 30 min. Then 8 µl each of "Proteinase K" and RNAse (10 mg/ml) was added, mixed and incubated in water bath for 1 h at 65 °C, followed by the addition of 120 µl 10% SDS and immediate return to the 65 °C water bath for a further 10 min. The tubes were left to cool and then an equal volume of phenol / chloroform was added and mixed until emulsion formed. The mixture was centrifuged at 5000 × *g* for 10 min. The top layer was carefully taken off into a clean 1.5 ml Eppendorf tube. To this DNA solution, 2.5 volumes of 100 % ethanol (-20 °C) was added and gently mixed. The mix was centrifuged at 10,000 × *g* for 5 min and the top layer was removed. The tube content was air dried and resuspended in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA extract kept at -20 °C until use. Table 3 summarizes the primers used in this study.

Table 3. Primers for PCR detection of *C. butyricum* /*C. beijerinckii* by nested PCR

Type	Primer	Sequence (5'-3')
(S)*	P1	GCGGCGTGCCTAATACATGC
(A)*	P2	GGGTTGCGCTCGTTGCGGGA
(S)	P5	GGAATCTTCCACAATGGGCG
(A)	Pbe, *C. beijerinckii*	CTTCCCCGATTAAGGGTAATTCAG
(A)	Pbu, *C. butyricum*	GTGGCTTGCTCCATTACAGAGTAA

* S, sense; A, antisense

Nested PCR. Nested PCR was performed by first amplifying a part of the 16S rRNA gene (nucleotides 41 to 1114) with primers P1 and P2 (Initial PCR), using a PTC-200 Peltier Thermocycler (MJ Research Inc., Watertown, Mass.). The reactions were carried out in sterile 0.2-ml tubes which contained 45 µl of the following reagents and 5 µl of DNA template (Table 4).

Table 4. PCR reagents and their concentrations used for nested PCR

Reagent	Ingredients/Concentration	Volume
10 × PCR buffer	100 mM Tris-HCl, pH :8.3 500 mM KCl 15mM MgCl2 0.01% Gelatin	5 µl
dNTPs	dTTP, dCTP, dGTP, dATP (0.2 mM each)	5 µl
Taq DNA Polymerase	5 U	0.2 µl
Primer 1	P1 or P5, 15ng/ µl	1 µl
Primer 2	P2 or Pbu/Pbe, 15ng/ µl	1 µl
Nuclease-free water		32.8 µl

After being heated to 95 °C for 3 min, to eliminate all protease activity, amplification was done in 45 cycles of melting DNA at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and elongation at 72 °C for 2.5 min. The resulting PCR product was diluted 10-100 fold to decrease the remaining concentrations of primers P1 and P2. Then 5 µl of this dilution was used as the template for the second PCR amplification (Specific PCR) with one of the specific primers (Pbu/Pbe) and P5. In this second step, the amplification was done in 25 cycles with the same condition as above. The amplified PCR products were visualized in 0.8 agarose gels (Sigma, Pool, UK) stained with ethidium bromide. Bromophenol blue (0.25%) in Glycerol (30%) was used as loading buffer. 3 µl of reacted samples were mixed with 2 µl of loading buffer and agarose gel electrophoresis was carried out in TAE buffer which contained 0.04 M Tris base, 0.04 M acetate, 0.01 M EDTA (Amresco, Ohio, USA).

RESULTS

Objective 1. To establish the proportion of *Clostridium butyricum* strains isolated from natural sources that carry the botulinum neurotoxin gene.

Task 01/01. Assemble a collection of strains of *Clostridium butyricum*, *C. bifermentans* and *C. tertium*.

The following strains expressing botulinal neurotoxin were assembled:

Table 5. Strains expressing botulinal neurotoxin

Strain designation	Botulinal toxin type
C. barati ATCC 43756	F
C. butyricum ATCC 43181	E
C. butyricum ATCC 43755	E
C. botulinum NCTC 7272	A
C. botulinum NCTC 7273	B
C. botulinum NCTC 8265	D
C. botulinum NCTC 8266	E
C. botulinum NCTC 10281	F

In addition, the following strains not expressing botulinal neurotoxin were assembled

Table 6. Other *Clostridium* species used in experiments

Strain designation	Number of strains*
C. barati	6
C. butyricum	4
C. bifermentans	6
C. tertium	5
C. beijerinckii	1
C. acetobutylicum	1
C. tyrobutyricum	1
Isolates from food believed to be either *C. butyricum* or *C. beijerinckii*	6

*A full list of strains and their origins is given in Annex 1.

A strain of *C. barati* from the Food Microbial Sciences Unit (FMSU) collection originally designated as being toxigenic, *C. barati* ATCC 43756, was shown by partial sequencing of the γ-section of the 16S ribosomal RNA to be *C. botulinum* type B. This strain was not used further and a replacement strain (also *C. barati* ATCC 43756) was obtained from CAMR Porton. Other strains shown by 16S sequencing to be misidentified were: *C. barati* NCIMB 10652 (*C. novyi*), *C. butyricum* NCTC 7423 (*C. beijerinckii/roseum*) *C. butyricum* NCIMB 9380 (*putrificum/sporogenes*), *C. tertium* NCIMB 9363 (*C. sartagoformum*). *Clostridium bifermentans* strains NCTC 6800 and NCTC 10696 proved to be *C. sordellii*.

The cultures provided by Unilever PLC (Colworth strains 3669-3675) had been isolated from chocolate custard and/or processing machinery and were believed to be either *C. butyricum* or *C. beijerinckii*. It is very difficult to distinguish between these species using phenotypic tests. Sequencing of a 1000 base-pair sequence the 16S RNA showed all strains to have identical sequences, which were 99.7% homologous to the reference strain of *C. beijerinckii* (Annex 3).

Task 01/02. Isolate *C. butyricum* strains from natural sources (soil, food raw materials etc) using elective enrichment methodology.

A number of different methods have been published for isolating *C. butyricum and* other butyric anaerobes from soil, milk and silage. However, the specificity of these methods for distinguishing between different species of the Genus *Clostridium* has not been reported. This series of experiments was designed to identify the methods most suitable for the isolation of *C. butyricum* from environmental samples based on the frequency with which *C. butyricum* and other clostridial strains could be recovered from spiked soil samples.

The liquid enrichment media that were used were (a) the medium of Bhat and Barker (1947)(BB) which depends for its selective effect on the ability of organisms to ferment a mixture of lactate and acetate; (b) modified Bhat and Barker (MBB) which is BB with the addition of trimethoprim, cycloserine and polymyxin B; (c) Butyricum Isolation Medium (BIM, Popoff 1984) which contains no nitrogen source and depends on the ability of *C.*

butyricum to fix atmospheric nitrogen with glucose as carbon and energy source as well as its resistance to trimethoprim, cycloserine and polymyxin B; and (d) Butyricum Isolation medium with lactate and acetate in place of glucose (BIMLA). Soil samples (1g) were inoculated with approximately 100 spores of one of the following: *C .butyricum* (5 strains), *C. barati, C. beijerinckii, C. bifermentans, C. tertium C. tyrobutyricum, C. acetobutylicum, C. botulinum types* A, B, C, D, E, F. Inoculated soil was added to four different enrichment broths (BB, MBB, BIM or BIMLA medium, Annex 2) and incubated under anaerobic conditions for five days. After five days, 1 ml was taken from each slurry, diluted in 9 ml of fresh medium and incubated for a further five days. A further dilution and incubation was carried out after ten days, giving a total incubation period of fifteen days. After incubation, aliquots (100 µl) were plated out onto BIM, ISA, RCM, SCA, TS or TSC agar and incubated under anaerobic conditions for 48 h. Colony isolates were examined for cell morphology and biochemical profile using API 20A (BioMerieux) to confirm the identity of isolates. The results are summarised in Table 7.

Table 7. Recovery of *Clostridium butyricum* and other clostridia from soil using combinations of selective enrichment and plating media							
		Plating medium					
Selective Enrichment Medium		**BIM**	**ISA**	**RCM**	**SCA**	**TS**	**TSC**
Bhat and Barker medium	Recovery of *C. butyricum* (%)	8	58	67	58	67	92
	Recovery of other clostridia (%)	0	37	13	0	13	89
Modified Bhat and Barker medium	Recovery of *C. butyricum* (%)	0	92	67	50	67	75
	Recovery of other clostridia (%)	0	19	13	0	13	39
Butyricum isolation medium	Recovery of *C. butyricum* (%)	0	8	58	37	58	58
	Recovery of other clostridia (%)	0	0	50	0	37	50
Lactate and acetate-modified butyricum isolation medium	Recovery of *C. butyricum* (%)	0	67	0	0	0	8
	Recovery of other clostridia (%)	0	53	0	0	0	0

*Plating media: BIM, Butyricum Isolation Medium; ISA, Iron Sulphate Agar; RCM, Reinforced Clostridial Medium; SCA, Sulphite Cycloserine Azide agar; TS, Perfringens base minus cycloserine, TSC, Tryptose Sulphite Cycloserine.

Clostridium butyricum strains were isolated most consistently using the combination of either BB medium and TSC agar (Table 7) or MBB medium and ISA agar with 92% isolation in both cases. Isolation from BIM medium onto ISA, RCM, TS and TSC agars was successful in only 50% of samples. Isolation using BIMLA medium was not successful. Isolation using the

BB/TSC and the MBB/ISA combinations was not specific for *C. butyricum.* Samples spiked with other clostridia were also recovered by some combinations.

Analysis of isolates by API20A and cell morphology showed all *C. butyricum* isolates to be subterminal spore-forming rods with a biochemical profile consistent with *C. butyricum* or *C. beijerinckii* (97.8% confidence). Isolates from samples spiked with other clostridial strains did not yield a recognisable API profile, although cell morphology matched those of the species used to spike the samples. In further work to isolate *C. butyricum* from food and environmental samples the MBB medium and ISA agar were used.

A range of samples (978) of fresh vegetables and food samples were examined for the presence of *C. butyricum* using this method. Of 978 samples tested 302 (31%) yielded presumptive *C. butyricum* isolates (Table 8). The food samples tested were mainly fresh vegetables but also included milk, cream, yoghurt and pâté. The highest percentage of positives came from soil, potato skins, swede skin, yoghurt and cream. No positive isolates were obtained from pate, garlic or spring greens. *Clostridium butyricum* and other butyric clostridia have been commonly isolated from silage, milk and cheese and also from other environments such as poultry processing plants and canned vegetables (Gibson, 1965, Bergère and Hermier, 1965, Gibbs, 1971, de Jong, 1998). A subsample of 93 isolates was examined for the presence of gene encoding the Type E botulinum neurotoxin (Task 01/03).

Table 8. Samples processed and isolates obtained

Material	Source	Number of samples	Number of positive samples	Percentage recovery
Arica Tomatoes	Isle of Wight tomatoes	25	4	16
Beefsteak Tomato	Isle of Wight tomatoes	25	4	16
Brussels Sprouts	Tilehurst allotments	8	4	50
Campari Tomatoes	Isle of Wight tomatoes	25	4	16
Carrot Flesh	Manor Farm	25	8	32
Carrot Skin	Manor Farm	25	10	40
Cauliflower	Manor Farm	25	16	64
Celeriac Flesh	Manor Farm	25	5	20
Celeriac Skin	Manor Farm	25	8	32
Coriander	Manor Farm	25	10	40
Cream	Prosperous Farms	7	7	100
Cucumber Flesh	Manor Farm	25	1	4
Cucumber Skin	Manor Farm	25	7	28
Eggs (shell and flesh)	Reading Farmer's Market	25	8	32
Garlic	Manor Farm	25	0	0
Kale	Manor Farm	25	8	32
Leeks	Manor Farm	25	2	8
Lettuce	Manor Farm	25	4	16
Milk - Pasteurised	Prosperous Farms	8	5	62
Mushrooms - Button	Reading Farmer's Market	8	4	50
Mushrooms - Chestnut	Reading Farmer's Market	8	2	25
Mushrooms - Cremini	Reading Farmer's Market	8	5	62
Mushrooms - Flat	Reading Farmer's Market	8	6	75
Pak Choi	Manor Farm	25	3	12
Parsley	Manor Farm	25	3	12
Parsnip Flesh	Manor Farm	25	8	32
Parsnip Skin	Manor Farm	25	8	32
Pate – Ardennes	Asda	25	0	0
Pate – Brussels	Asda	25	0	0
Potato Flesh	Tilehurst allotments	30	14	47
Potato Skin	Tilehurst allotments	30	30	100
Rocket	Manor Farm	25	3	12
Savoy Cabbage	Manor Farm	25	16	64
Soil	Lower Earley garden soil	10	16	100
Spinach	Manor Farm	25	5	20
Spring Greens	Manor Farm	25	0	0
Swede Flesh	Manor Farm	25	9	36
Swede Skin	Manor Farm	21	25	84
Sweet Potato Flesh	Manor Farm	25	8	32
Sweet Potato Skin	Manor Farm	25	9	36
Tomatoes – Sieved	Mill Hill, London, UK	25	0	0
Turnip Flesh	Manor Farm	25	4	16
Turnip Skin	Manor Farm	25	2	8
White Onion	Manor Farm	25	1	4
Yoghurt	Prosperous Farms	7	6	86
Total		**978**	**302**	**31**

Task 01/03. Screen *C. butyricum* isolates for toxin genes

Development and testing of PCR method.

Two *C. butyricum* type E strains and one strain of *C. botulinum* type E were included in the study. Also four non-toxigenic *C. butyricum* strains were used as negative controls. Three DNA extracts of *C. botulinum* type E were also provided by IFR, Norwich and used as positive controls (x 7, x 8, ABEF Table 9).

Table 9. *Clostridium* strains used in this study

Species	Strain/code	Toxin/Type	Culture collection/supplier
C. botulinum	8266	Type E	NCTC
C. butyricum	43755	Type E	ATCC
C. butyricum	43181	Type E	ATCC
C. butyricum	7423	Type strain	NCTC
C. butyricum	9575		NCIMB
C. butyricum	8082		NCIMB
C. butyricum	6084		NCTC
C. botulinum [a]	× 7	Type E	Beluga = BL81/026
C. botulinum [a]	× 8	Type E	Colworth 96 = BL81/027
C. botulinum [a]	ABEF	Type A, B, E, F	

[a] Positive DNA controls (Provided by the Institute of Food Research, Norwich)

Considerable time and effort was initially spent in testing various PCR protocols for detecting the toxin genes of *C. botulinum*, without success. A large number of factors were tested to discover the cause of the problem. The primers and methods described by Szabo *et al.* (1993), Campbell *et al.* (1993), Franciosa *et al.* (1994), Fach *et al.* (1995) and Sciacchitano and Hirshfield (1996) were all tried together with a range of DNA extraction methods and a range of concentrations of reagents for each method. It was eventually discovered that, irrespective of the exact protocol, good results were only obtained using the highest quality Taq polymerase (`Titanium' BD Biosciences, Oxford, UK).

A typical agarose gel of PCR product when the template contained a mixture of four (ABEF) toxin producing *C. botulinum* strains is shown below (Fig. 1).

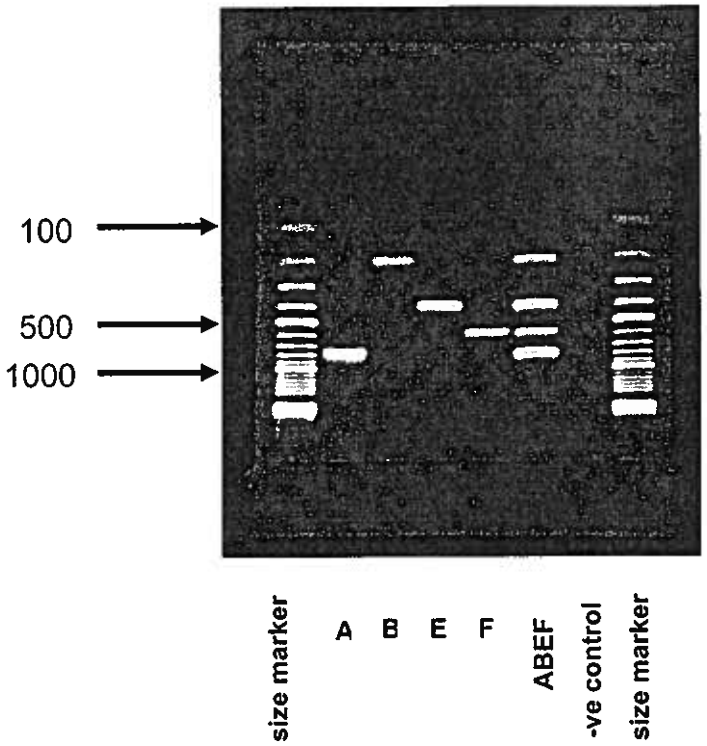


Fig 1 Agarose gel of the botulinum toxin gene multiplex PCR (courtesy G. Wyatt, IFR, Norwich)

The DNA from both toxigenic *C. butyricum* strains (43755, 43181) amplified as expected when the diluted (1:10) or original DNA extracts were used. However, when 1µl of DNA template was mixed with 25 µl PCR mix reagents, the quality of bands improved (Fig. 2).

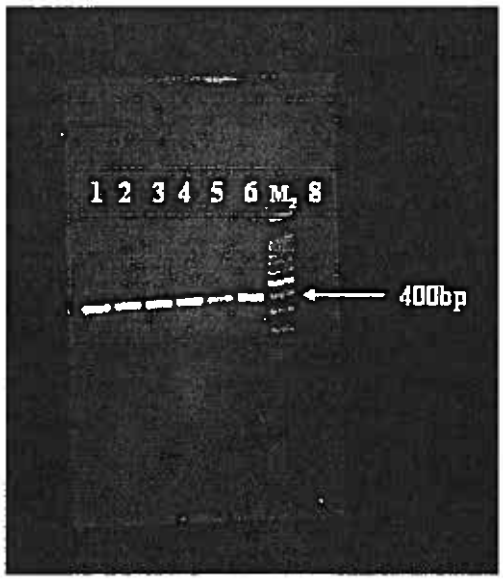


Fig 2. Agarose gel of PCR products generated from template DNAs[a] from toxigenic clostridia. Lane 1(*C. butyricum* 43181), Lane 2(*C. butyricum* 43181 diluted 1:10), Lane 3(*C. butyricum* 43755), Lane 4(*C. butyricum* 43755 diluted 1:10), Lane 5(*C. botulinum* type E, control ×7), Lane 6(*C. botulinum* type E, control ×8), M_2 (100 bp DNA ladder).

[a] 1 µl template (original or diluted 1:10) + 25 µl PCR reagents mix.

Screening isolates obtained from food and environmental samples.
Based on these results a random selection of 93 strains selected from those isolated from food and environment (as described earlier) were screened for the type E toxin gene. No positive samples were found. A typical agarose gel of PCR products of these isolates is shown in Fig 3. As can be seen, some PCR products produced a weak band at the gel injection point. These bands disappeared when PCR of such isolates was repeated using 1 µl DNA template mixed with 25 µl PCR mix reagents.

Fig 3. A typical agarose gel of PCR products generated from template DNAs[a] of isolated *C .butyricum* strains from food and environment. Lane 1-12 Food & environment isolates, Lane 13 (*C. butyricum* type E, 43181), Lane 14 (*C. butyricum* type E, 43755), Lane 15 (*C. botulinum* type E, ×8)

[a]10 µl template + 15 µl PCR reagents mix

None of the 93 presumptive *C. butyricum* isolates yielded a positive result when screened for the type E toxin gene.

Task 01/04. Develop and test a gene-probe method for identifying *C. butyricum* that is able to distinguish it from *C. beijerinckii*.

The following strains were used in this part of the work: *Clostridium butyricum* non-toxigenic strains (NCTC 7423, NCTC 6084, NCIMB 8082, NCIMB 9575) and toxigenic strains (ATCC 43181, ATCC 43755); The following *C. beijerinckii* strains from Unilever C3669, C3670, C3671, C3672, C3673, C3674, C3675; *Clostridium bifermentans* NCTC 13019, NCTC 506, NCTC 1341; *C. barati* NCTC 10986, ATCC 27638; *C. tertium* NCIMB 10697; *C. sartagoformum* NCIMB 9363

The differences between the 16S rRNA target gene sequences of *C. butyricum* and *C. beijerinckii* are quite small, so a nested PCR approach was used, based on the method described by Klijn *et al.* (1995). In the first step of the nested PCR, part of the 16S rRNA gene, corresponding to nucleotides 40 to 1114 on the *E. coli* nomenclature, was amplified with primers P1 and P2. The success of this stage was checked by running the PCR products on an agarose gel. If the first step was successful a second amplification was performed using a diluted product of the first step with a specific primer for *C. beijerinckii* (Pbe) or *C. butyricum* (Pbu) and primer P5 specific for part of the conserved region.

As shown in Fig 4 all strains of *C. butyricum* and *C. beijerinckii* were amplified well in the first PCR (IPCR), except *C. butyricum* 43755, which produced a very weak band.

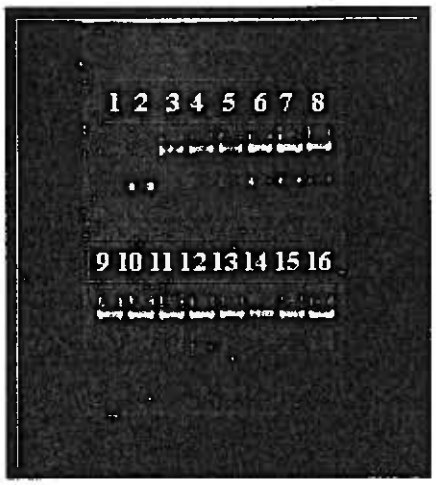


Fig 4. Agarose gel of products of first PCR (IPCR) generated from template DNAs[a] from *C. butyricum* and *C. beijerinckii*. Lane 1 (*C. butyricum* 43775), Lane 2 (Control), Lane 3 (*C. butyricum* 43181), Lane 4 (*C. butyricum* 8082), Lane 5 (*C. butyricum* 9575), Lane 6 (*C. butyricum* 7423), Lane 7 (*C. beijerinckii* 3669), Lane 8 (*C. beijerinckii* 3670), Lane 9 (*C. beijerinckii* 3671), Lane 10 (*C. beijerinckii* 3672), Lane 11 (*C. beijerinckii* 3673), Lane 12 (*C. beijerinckii* 3674), Lane 13 (*C. beijerinckii* 3675), Lane 14 (*C. beijerinckii* 9362), Lane 15 (*C. beijerinckii* 3673 repeat), Lane 16 (*C. beijerinckii* 9362 repeat)

[a]5 µl template + 45 µl PCR reagents mix

In the second PCR (SPCR) specific primers for either *C. butyricum* (Pbu) or *C. beijerinckii* (Pbe) were used against the respective amplification products shown in Fig 4. As shown in Fig 5. *C. beijerinckii* 3672, 3673 and 3675 were amplified but *C. beijerinckii* 3669, 3670 3671 were not amplified and *C. beijerinckii* 9362 gave a weak band. In contrast all *C. butyricum* strains, even *C. butyricum* 43755 that was not amplified well in the first step, produced a clear band when the agarose gel was run.

Fig 5. Agarose gel of Specific PCR products (SPCR) generated from template DNAs[a] from *C. butyricum* and *C. beijerinckii*. Lane 1 (*C. butyricum* 43775), Lane 2 (Control), Lane 3 (*C. butyricum* 43181), Lane 4 (*C. butyricum* 8082), Lane 5 (*C. butyricum* 9575), Lane 6 (*C. butyricum* 7423), Lane 7 (*C. beijerinckii* 3669), Lane 8 (*C. beijerinckii* 3670), Lane 9 (*C. beijerinckii* 3671), Lane 10 (*C. beijerinckii* 3672), Lane 11 (*C. beijerinckii* 3673), Lane 12 (*C. beijerinckii* 3674), Lane 13 (*C. beijerinckii* 3675), Lane 14 (*C. beijerinckii* 9362)
Specific Nested PCR-Pure strains (Pbu +Pbe)
[a]5 µl template (1:10) + 45 µl PCR reagents mix

In order to check the specificity of the specific primers a PCR was set up and the primers were swapped (Pbu for *C. beijerinckii* and Pbe for *C. butyricum* strains), except in the case of *C. butyricum* 43181 and *C. beijerinckii* 9362 which were used as positive controls. Fig. 6 demonstrates a good specificity of the primers for all tested strains except for *C. beijerinckii* 3671, where the band indicates that the strain is most probably mislabelled.

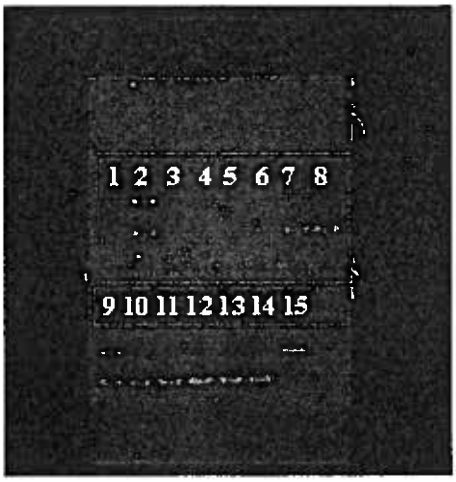


Fig 6. Agarose gel of PCR products with swapped specific primers except for positive controls Lane 1 (*C. butyricum* 43775), Lane 2 (*C. butyricum* 43181 +ve Control), Lane 3 (*C. butyricum* 43181), Lane 4 (*C. butyricum* 8082), Lane 5 (*C. butyricum* 9575), Lane 6 (*C. butyricum* 7423), Lane 7 (*C. beijerinckii* 3669), Lane 8 (*C. beijerinckii* 3670), Lane 9 (*C. beijerinckii* 3671), Lane 10 (*C. beijerinckii* 3672), Lane 11 (*C. beijerinckii* 3673), Lane 12 (*C. beijerinckii* 3674), Lane 13 (*C. beijerinckii* 3675), Lane 14 (*C. beijerinckii* 9362), Lane 15 (*C. beijerinckii* 9362 +ve control)

[a]5 µl template (1:10) + 45 µl PCR reagents mix

The specificity of *C. butyricum* primer (Pbu) was further examined with other species of clostridia including, *C. bifermentans*, *C. barati, C. tertium* and *C. sartagoformum.* All strains were amplified in the initial PCR (Fig 7). In the specific PCR (Fig 8) all three strains of *C. bifermentans*; 13019, 506 and 1341 and *C. barati* 10986 produced rather good bands with Pbu primers. No bands were produced with *C. tertium or C. barati.* Thus although the Pbu primer showed some discriminatory power it was not sufficiently specific for the unequivocal identification of *C. butyricum.*

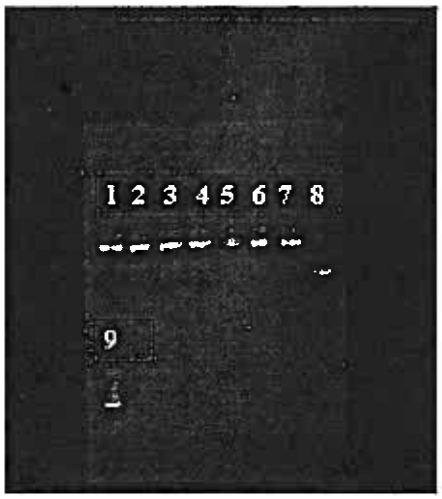


Fig 7. Agarose gel of Initial PCR products generated from template DNAs[a] from *C. bifermentans, C. barati* and *C. tertium*. Lane 1(*C. bifermentans* 13019), 2(*C. bifermentans* 506), 3(*C. bifermentans* 1341), 4(*C. barati* 10986), 5(*C. barati* 27638), 6(*C. tertium* 10697), 7(*C. C. satagoformum*), 8(control), 9(*C. barati* 27638 repeat)

[a]5 µl template + 45 µl PCR reagents mix

Fig 8. Agarose gel of Specific PCR products generated from template DNAs[a] from *C. bifermentans, C. barati* and *C. tertium*. Lane 1(*C. bifermentans* 13019), Lane 2(C. *bifermentans* 506), Lane 3(*C. bifermentans* 1341), Lane 4(*C. barati* 10986), Lane 5(*C. barati* 27638), Lane 6(*C. Tertium* 10697), Lane 7(*C. Tertium* 9363), Lane 8(*C. Butyricum* 43181), Lane 9(*C. butyricum* 8082), Lane 10 (*C. butyricum* 7423)

Specific Nested PCR-Pure strains (Pbu)

[a]5 µl template (1:10) + 45 µl PCR reagents mix

Objective 2. To determine whether *C. tertium* and *C. bifermentans* produce toxins.

Task 02/01. Screen culture filtrates of *C. bifermentans* and *C. tertium* for toxic effects against cultured cell lines.

The strains used for screening were: *E. coli* O157 strain 30-2C4 (positive control), *E. coli* strain K-12 (negative control) *C. bifermentans* strains NCTC 13019, NCTC 506, NCTC 1340, NCTC 1341, NCTC 6929 and NCIMB 6801, *C. tertium* strains NCTC 541, NCIMB 10697, NCIMB 701802, NCIMB 701803 and NCIMB 701804. They were grown in Brain Heart Infusion broth and supernatants were prepared from stationary phase cultures after one to eight days incubation. The toxicity of culture supernatants was assessed by microscopic examination of Vero cells for morphological changes (Fig 9) or by inhibition of neutral red uptake (see Materials and Methods).

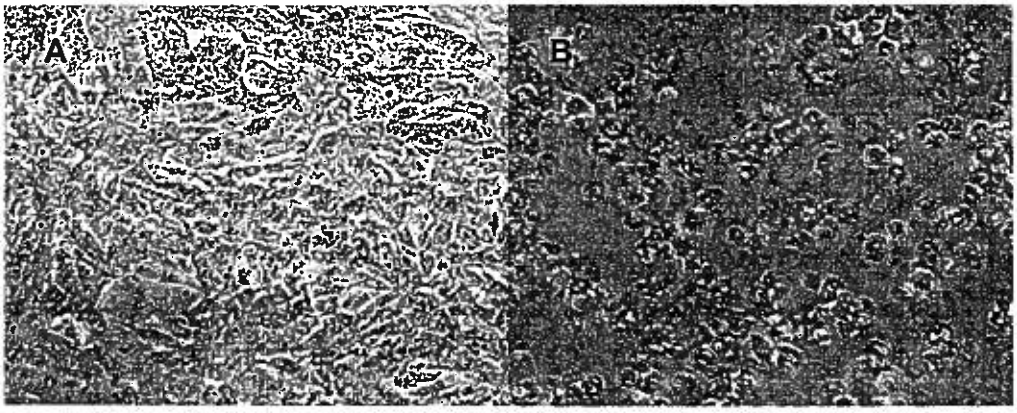


Fig 9. Vero cell monolayers after 48 h incubation with a 50% dilution of culture supernatant from non-cytotoxic *C. bifermentans* NCTC 6801 (a) and cytotoxic *C. bifermentans* NCTC 13019 (b).

Weak toxicity was detected in supernatants of three out of five *C. tertium* strains and five out of six *C. bifermentans* strains but, in most cases, it could only be demonstrated by incubating Vero cells for 48h rather than 24h and was quickly lost on moderate dilution. Moreover the toxic effect sometimes only appeared after several days' incubation of the culture.

Preliminary tests using visual estimation of cytotoxicity showed that a cytotoxic effect was apparent for filtrate from strain *C. tertium* NCIMB 10697 at a concentration of 10%, but not at a concentration of 1%. Filtrates from other clostridial strains did not exhibit a cytotoxic effect at 10% concentration. Concentrations of 50%, 25%, 12.5% and 6.25% were used for further screening.

Using the neutral red assay, a toxic effect against Vero cell lines was observed in culture supernatants from five out of six *C. bifermentans* strains tested (Table 10) and from three out of five *C. tertium* strains tested (Table 11). The effects were generally seen only in 50% concentrations of filtrate although toxicity of *C. bifermentans* NCTC 13019 could be detected at 12.5% and that of *C. tertium* NCIMB 10697 could be detected at 6.25%. The toxicity was retained when culture filtrates were adjusted to pH 7.0. Culture supernatants from *C. bifermentans* strain NCIMB 6801 and *C. tertium* strains NCIMB 701803 and 701804 were not cytotoxic under any of the study conditions.

Table 10. Cytotoxicity* of culture filtrates from strains of *C. bifermentans* after 48 hours incubation with Vero cells

Strain	Days of growth	Lowest toxic filtrate concentration (%)	Toxicity (%)	Significance (P)
NCTC 506	8	50	49	0.011
NCTC 1340	1	25	11	0.011
	8	50	31	0.001
NCTC 1341	8	50	33	0.002
NCTC 6929	3	50	49	0.009
	8	50	35	0.0043
NCTC 13019	1	12.5	25	0.037
	8	50	36	0.0007

*Inhibition of Neutral Red uptake

Table 11. Cytotoxicity* of culture filtrates from strains of *C. tertium* after 48 h incubation with Vero cells				
Strain	**Days of growth**	**Lowest toxic filtrate concentration (%)**	**Toxicity (%)**	**Significance (P)**
NCTC 541	8	50	48	0.012
NCIMB 10697	1	6.25	28	0.007
	3	50	64	0.0057
	8	50	55	0.0011
NCIMB 701802	3	50	72	0.0002
	8	50	60	0.000045

*Inhibition of Neutral Red uptake

To test the heat resistance of the toxic effect, filtrates were autoclaved before being screened against Vero cells. All toxic filtrates retained activity and in some cases showed a slightly increased effect. Significantly increased cytotoxicity was observed in filtrates from *C. bifermentans* strains NCTC 13019, NCTC 1340 and NCTC 6929 and *C. tertium* strains NCIMB 10697, NCIMB 701802 (Table 12). There was no significant increase or a reduction in the other strains. The toxicity of supernatants from *E. coli* O157 was eliminated by heat treatment.

There is thus a toxic effect associated with culture supernatants from some strains of *C. bifermentans* and *C. tertium* but it is very weak and lost when diluted 2 - 10 fold, in contrast to the toxicity of *E. coli* which was detectable after 1000-fold dilution (data not shown). The heat stability of the toxic effect suggests it is not due to a protein toxin.

Table 12. Effect of autoclaving on toxicity of culture filtrates* against cultured Vero cells		
	% toxicity before autoclaving	% toxicity after autoclaving
E. coli O157	67	0.0
E. coli K-12	0.0	0.0
C. bifermentans NCTC 1340	31	51
C. bifermentans NCTC 1341	33	12
C. bifermentans NCTC 6929	35	72
C. bifermentans NCTC 13019	36	69
C. tertium NCTC 541	48	0.0
C. tertium NCIMB 10697	55	86
C. tertium NCIMB 701802	60	89

*Culture filtrates from 8-day old clostridial cultures were examined for toxicity before and after autoclaving. The filtrates were diluted 1:1 with Vero Cell growth medium and added to microwell plates containing confluent growth of Verocells. Toxicity was assessed by the neutral red assay after 48h incubation (see Materials and Methods).

Objective 3. To define the conditions of temperature, pH and solute concentration that will prevent growth of *Clostridium butyricum, C. tertium, C. bifermentans* and *C. barati.*

Tasks 03.01- 03.04. Define the growth limits for *C. butyricum, C. tertium, C. bifermentans* and *C. barati* in broth under anaerobic conditions.

Minimum pH for growth. Growth limits were determined in PYGS broth incubated anaerobically at 30°C for up to 42d. Initially, three replicate tubes were inoculated for each pH and, if growth was observed within a few days, no further replicates were done at that pH value. If no growth was observed within 42 d, or if growth only occurred after more prolonged incubation, the experiment was repeated up to a further two to five times so that a total of 6 - 15 tubes were examined. The values shown in the Tables represent the lowest pH values at which growth was observed.

The pH of the broth was adjusted in two ways. In the first method, acid was added to PYGS broth until the target value was achieved; in the second method the salt of the acid was added to a concentration of 50 mM, and the pH was then adjusted with HCl. It was not considered practical to compare acids at the same concentration of undissociated acid because the pK_a value for the dissociation of the last carboxyl group of citric acid is 3.14, which would mean adding unrealistically high concentrations of total acid to achieve reasonable levels of undissociated acid at relevant pH values (between 4 and 5). The limiting pH values are shown below.

Table 13. Limiting pH values for growth of *Clostridium butyricum*			
Acidulant	**Non-toxigenic *C. butyricum***	***C. butyricum* ATCC 43181**	***C. butyricum* ATCC 43755**
HCl	4.1	4.2	4.2
Citric	4.7	4.7	4.7
Lactic	4.7	4.8	4.8
Acetic	4.8	4.8	4.8
50 mM citric	5.0	5.1	5.0
50 mM Lactic	4.6	4.4	4.4
50 mM Acetic	4.7	4.8	4.8

Acidulant	***C. bifermentans***	***C. tertium***	***C. barati* (cocktail of strains)**	***C. barati* NCTC 43756**
HCl	4.1	4.2	3.7	4.0
Citric	4.7	4.6	4.4	4.4
Lactic	4.5	4.4	5.1	4.8
Acetic	4.9	4.8	4.4	4.3
50 mM Citric	4.9	4.9	4.8	4.2
50 mM Lactic	4.4	4.4	4.7	4.6
50 mM Acetic	4.9	4.9	4.8	4.8

The minimum pH for growth in PYGS broth acidified with HCl alone was 4.1 for the non-toxigenic strains of *C. butyricum* and 4.2 for the two toxigenic strains. Similar limiting pH values were observed for *C. bifermentans* and *C. barati*. The pH limits were lower for *C. barati* especially the non-toxigenic strains which grew down to pH 3.7. Although growth at this low pH has been reported previously (de Jong, 1989), the trial was repeated, measuring viable counts weekly for six weeks. On this occasion the lowest pH permitting growth of the non-toxigenic strains was pH 4.4 when an approximately tenfold increase in number occurred, but no growth of the non-toxigenic strain was detected at pH 4.4 or pH 5.0.

The organic acids were more inhibitory to growth as expected, and growth was inhibited at higher pH values between 4.4. and 5.1 . In general the toxigenic strains of both *C. butyricum* and *C. barati* were inhibited at similar or somewhat higher values than the non-toxigenic ones except the two toxigenic strains of *C. butyricum* grew to a slightly lower pH in 50mM lactic acid than the non-toxigenic strains and the toxigenic strain of *C. barati* grew to a lower pH in 50mM citric acid than the non-toxigenic strains. Growth at below pH 4.5, the limit for growth of proteolytic strains of *C. botulinum* occurred in all strains in media acidified with HCl, and in some cases in media acidified with organics acids.

Minimum water activities and temperatures. The lowest water activities and temperatures permitting growth are shown below. The measured water activity values were rounded up or down to two decimal places.

The mixture of non-toxigenic strains of *C. butyricum* grew at a lower water activity (0.95) than the toxigenic strains (0.96) in both NaCl and sucrose (Table 14). Similarly the minimum temperature at which growth was recorded was lower in the non-toxigenic strains (8°C) than in the toxigenic ones (10 - 11°C).

Table 14. Limiting water activities and temperatures for growth of *Clostridium butyricum*

Limiting factor	Non-toxigenic C. butyricum	*C. butyricum* ATCC 43181	*C. butyricum* ATCC 43755
a_w (NaCl)	0.95	0.96	0.96
a_w (Sucrose)	0.95	0.96	0.96
Temperature	6.8 > 7.9	9.3 – 10.2	10.3 – 11.4

With the other clostridial strains tested, sodium chloride was more inhibitory than sucrose (Table 15). Growth occurred down to a water activity of 0.95 in sucrose and 0.96 in NaCl except in the toxigenic strain of *C. barati* where growth in NaCl was only possible down to a water activity of 0.97. The minimum temperature allowing growth was approximately 8°C for all species.

It is worth noting that growth did not occur at or below 0.94 the limiting water activity for growth of proteolytic strains of *C. botulinum.*

Table 15. Limiting water activities and temperatures for growth of *C. bifermentans, C. tertium and C. barati*

Solute	*C. bifermentans*	*C. tertium*	*C. barati (cocktail of strains)*	*C. barati* NCTC 43756
a_w (NaCl)	0.96	0.96	0.96	0.97
a_w (Sucrose)	0.95	0.95	0.95	0.95
Temperature	6.8 > 7.9	7.4 > 7.9	6.8 > 7.9	6.4 > 7.9

Task 03/05. Examine growth behaviour in food substrates.

Samples of sieved tomato were adjusted to different pH values by addition of hydrochloric acid and triplicate tubes containing the tomato were inoculated with a either a mixture of non-toxigenic strains of *C. butyricum* or with one or other of the toxigenic strains. The tubes were incubated at 15°C or 30°C. for 42 days. A parallel set of triplicate tubes was set up containing additional citric acid (50mM). Different patterns of growth of *C. butyricum* in sieved tomato were observed including (i) an increase in number, (ii) a decline in number or (iii) an initial decline followed by an increase. The complete experiment was done twice. The results for each condition are average values of counts from six replicate tubes from two experiments. The results are presented as the increase in log count (if any) relative to the lowest count observed during incubation for 42d. The conditions in which counts increased by more than a factor of 10 above the inoculum level in at least one of the two trials are indicated in the Tables.

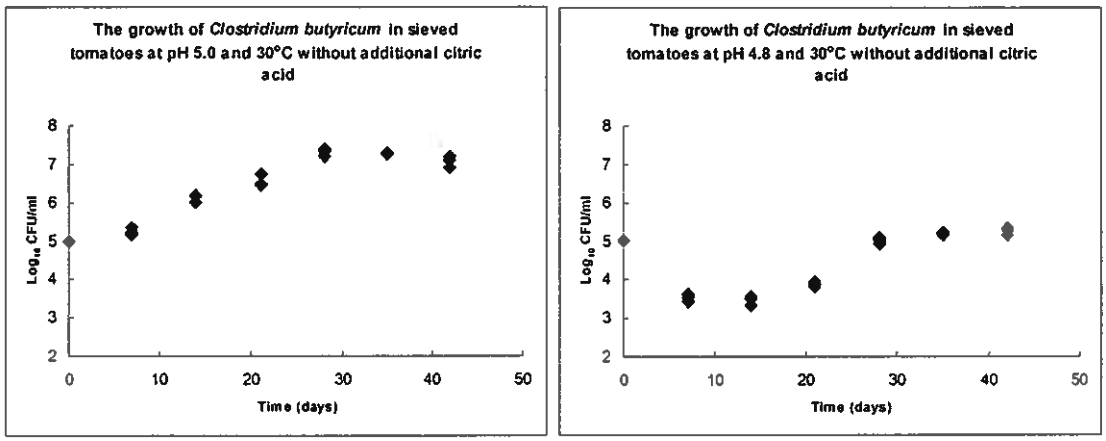


Fig 10. Examples of growth behaviour of C. *butyricum* in tomato

Table 16. Growth of *Clostridium butyricum* strains in sieved tomatoes at 30°C

	Log increase in cell numbers (CFU/ml)		
pH	**Non-toxigenic *C. butyricum***	***C. butyricum* ATCC 43181**	***C. butyricum* ATCC 43755**
4	1.18 ± 0.23	1.04 ± 0.33	1.88 ± 0.29
4.2	0.89 ± 0.30	1.17 ± 0.18	1.20 ± 0.40
4.4	0.59 ± 0.18	1.84 ± 0.22	0.69 ± 0.26
4.6	1.45 ± 0.10	1.06 ± 0.24	0.55 ± 0.26
4.8	1.48 ± 0.21	1.00 ± 0.26	0.35 ± 0.12
5	1.91 ± 0.07 ‡	0.80 ± 0.42	0.60 ± 0.18

*The increase in growth is reported relative to the lowest viable count as the baseline.

‡ Count increased by more than tenfold above inoculum level

Table 17. Growth of *Clostridium butyricum* strains in sieved tomatoes containing 50mM additional citric acid at 30°C.			
	Log increase in cell numbers (CFU/ml)		
pH	Non-toxigenic *C. butyricum*	*C. butyricum* ATCC 43181	*C. butyricum* ATCC 43755
4	ND	ND	ND
4.2	0.37 ± 0.63	0.25 ± 1.33	0.48 ± 0.76
4.4	0.26 ± 0.15	0.47 ± 0.51	0.16 ± 1.08
4.6	0.37 ± 0.46	0.16 ± 1.08	0.00 ± 0.00
4.8	0.72 ± 0.36	0.06 ± 0.15	1.40 ± 0.38 ‡
5	2.73 ± 0.45 ‡	0.14 ± 0.45	1.69 ± 0.35 ‡

‡ Count increased by more than tenfold above inoculum level

Table 18. Growth of *Clostridium butyricum* strains in sieved tomatoes at 15°C			
	Log increase in cell numbers (CFU/ml)		
pH	Non-toxigenic *C. butyricum*	*C. butyricum* ATCC 43181	*C. butyricum* ATCC 43755
4.0	0.18 ± 0.27	0.00 ± 0.00	ND
4.2	0.00 ± 0.00	ND	ND
4.4	1.40 ± 0.14	ND	ND
4.6	0.14 ± 0.11	0.24 ± 0.41	0.13 ± 0.27
4.8	0.87 ± 0.41	0.04 ± 0.36	0.00 ± 0.00
5.0	1.85 ± 0.26 ‡	0.20 ± 0.29	0.00 ± 0.00

‡ Count increased by more than tenfold above inoculum level

The sieved tomato did not support extensive growth of *C. butyricum* under the conditions of the experiment. The average log increases shown in the tables are generally rather small and in many cases occurred after an initial decrease of 1 - 2 logs. An increase in number of non-toxigenic *C. butyricum* strains above the inoculated level occurred at pH 4.8. - 5.0 at both 15 and 30°C. No increase above the inoculum level was detected in toxigenic strain

ATCC 43181 whilst an increase was detected in strain ATCC 43755 but only at 30°C. The additions of 50 mM citrate did not appear to affect the growth of *C. butyricum* in tomato.

Table 19. Growth of *Clostridium butyricum* strains in sieved tomatoes with 50mM added citric acid at 15°C

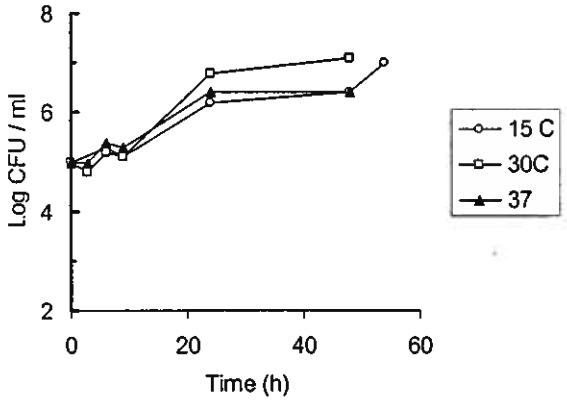
	Log increase in cell numbers (CFU/ml) *		
pH	Non-toxigenic *C. butyricum*	*C. butyricum* ATCC 43181	*C. butyricum* ATCC 43755
4	ND	0.00 ± 0.00	ND
4.2	0.24 ± 0.27	0.23 ± 0.21	0.68 ± 0.14
4.4	0.00 ± 0.00	0.64 ± 0.20	0.37 ± 0.46
4.6	0.38 ± 0.10	0.00 ± 0.00	0.72 ± 0.39
4.8	0.96 ± 0.22 ‡	0.62 ± 0.25	0.65 ± 0.31
5	1.99 ± 0.07 ‡	0.42 ± 0.20	0.37 ± 0.37

‡ Count increased by more than tenfold above inoculum level

Growth in chocolate custard

The strains provided by Unilever PLC had originally been isolated from chocolate custard. These were identified in this project as *C. beijerinckii*. However the concern is that if strains of the physiologically similar *C. butyricum* found their way into such products they might grow and, in the case of toxigenic strains, produce toxin. The toxigenic strain ATCC 43755 was therefore inoculated in to freshly boiled and cooled chocolate custard purchased at a local supermarket (pH 7.0, a_w 0.99) and examined for growth at 15, 30 and 37°C. Growth occurred within 24 h at all three temperatures and was accompanied by extensive gas production particularly at 37°C.

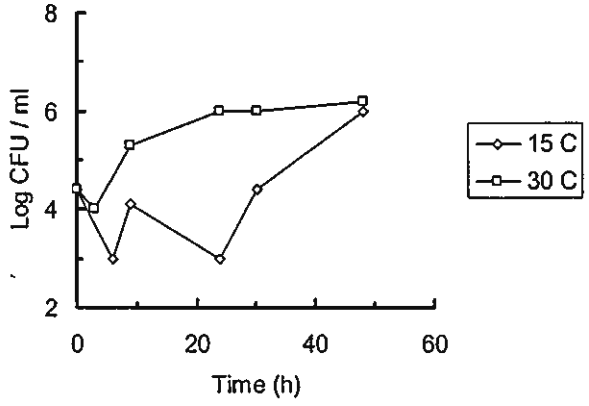
Fig 11. Growth of a toxigenic strain of *Clostridium butyricum* in chocolate custard



Growth in creamed rice

Growth occurred in creamed rice (pH 6.7, a_w 0.99) incubated at 30°C and was accompanied by gas production and disruption of rice structure in the tubes. Growth also occurred at 15°C after an initial decrease in numbers.

Fig 12. Growth of a toxigenic strain of *C. butyricum* in creamed rice



Growth in Pate

In preliminary experiments a range of pate products (Ardennes, chicken, bacon and red wine, duck and orange, Brussels) were inoculated with approximately 10^5 spores or vegetative cells of mixed strains of *C. bifermentans* or *C. tertium* and incubated at 8°C, 15°C, 22°C or 30°C. At weekly intervals, samples were removed, heated at 65°C for 10 min and viable counts determined on differential clostridial agar incubated

anaerobically. Representative results are shown below. Variable amounts of growth were observed, but since the pate was not sterile the dominant flora did not correspond to the inoculated organism. Most isolates from samples incubated at abuse temperature were identified as *C. sporogenes* by API. Further experiments were conducted with pate that had been sterilised by irradiation.

Table 20. Growth of *Clostridium* species in Pate incubated at 15°C

		Maximum log increase in CFU				
Inoculum	**Organism**	**Ardennes**	**Brussels**	**Chicken, bacon red wine**	**Chicken and mushroom**	**Duck and orange**
Vegetative	*C. bifermentans*	2.3	2.8	1.2	3.5	2.1
Vegetative	*C. tertium*	2.1	3.5	3.3	3.6	5.1
Spores	*C. bifermentans*	0.0	0.0	0.0	1.0	0.8
Spores	*C. tertium*	1.9	0.0	0.8	2.4	2.3

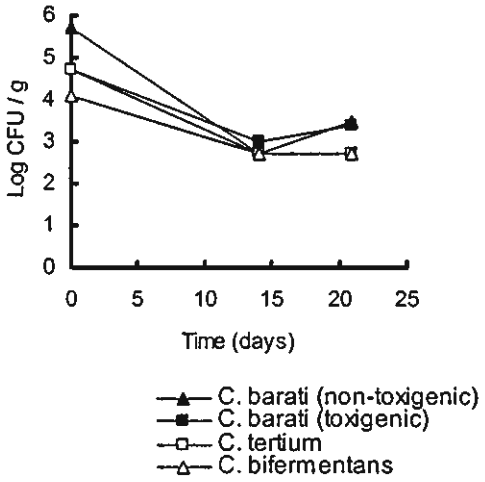
Table 21. Growth of *Clostridium* species in Pate incubated at 22°C

		Maximum log increase in CFU				
Inoculum	**organism**	**Ardennes**	**Brussels**	**Chicken, bacon red wine**	**Chicken and mushroom**	**Duck and orange**
Vegetative	*C. bifermentans*	2.4	2.1	1.8	2.9	4.7
Vegetative	*C. tertium*	3.2	0.3	2.5	2.7	1.6
Spores	*C. bifermentans*	1.6	0.9	0.0	0.9	2.5

Table 22. Biochemical characterisation of dominant species isolated from pate incubated at 30°C		
Pate type	**Organism inoculated**	**Identity**
Ardennes	Control	No identification
Ardennes	*C. bifermentans*	*C. sporogenes*
Ardennes	*C. tertium*	*C. sporogenes*
Brussels	Control	*C. sporogenes*
Brussels	*C. bifermentans*	No identification
Brussels	*C. tertium*	*C. sporogenes*
Chicken, bacon, red wine	Control	*C. sporogenes*
Chicken, bacon, red wine	*C. bifermentans*	*C. sporogenes*
Chicken, bacon, red wine	*C. tertium*	*C. botulinum*
Duck and orange	Control	*C sporogenes*
Duck and orange	*C. bifermentans*	*C. histolyticum* 53.4%, *Clostridium* spp. 43.5%
Duck and orange	*C. tertium*	*C. sporogenes*

Growth of *C. barati*, *C. tertium* or *C. bifermentans* did not occur within 21 days at 20°C, rather a decline in viable numbers was observed. When the pate

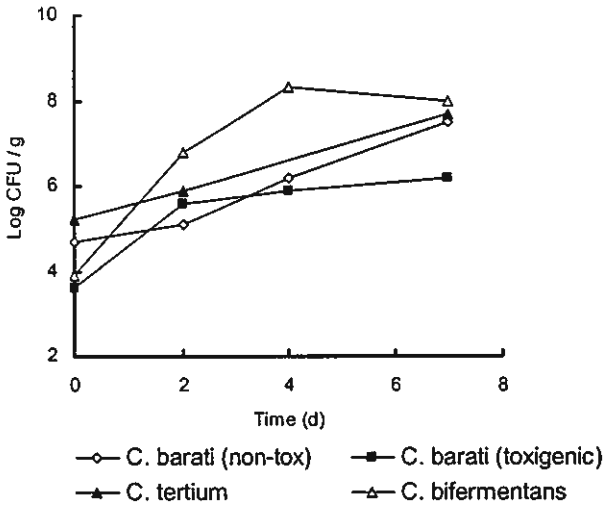
Fig 13. Growth of clostridia in pate at 20° C



was incubated at the more severe abuse temperature of 30°C an increase in

count was observed and this was accompanied by obvious gas production and splitting of the pate within its pack.

Fig 14. Growth of clostridia in pate at 30° C



DISCUSSION

Establishing the proportion of *Clostridium butyricum* strains isolated from natural sources that carry the botulinum neurotoxin gene.

Despite the wide range of selective media available for isolating and enumerating specific groups of organisms from food and environmental samples, there are relatively few reliable selective media available for clostridia – exceptions being the media developed for *C. perfringens* and *C. difficile*. Some effort has been devoted to devising media for the butyric clostridia because of their role in spoilage of canned acid foods and cheese, but no medium has been extensively tested and accepted for routine use. In this work 24 combinations of different enrichment broths and plating media were tested for their ability to recover *C. butyricum* and other clostridia. The method which gave the best results was selective enrichment with Modified Bhat and Barker (MBB) medium followed by plating out onto iron sulphite agar (ISA). MBB depends on the ability of butyric clostridia to grow on a relatively minimal medium with lactate and acetate as a source of carbon and energy. In addition, selective antibiotics are present in the medium to favour growth of *C. butyricum* specifically. This method was therefore used to isolate strains from foods. Just under 1000 food samples were tested, mainly fresh vegetables but also including milk, cream, yoghurt and pâté. The highest percentage of positives came from soil, potato skins, swede skin, yoghurt and cream. No positive isolates were obtained from pate, garlic or spring greens. A subset of 93 of the positive samples was tested for the presence of the gene encoding type E botulinal toxin but no toxin positive samples were detected. If the true incidence of toxin positive samples was 1%, there would be a 95% chance of detecting a positive by examining 93 samples. Since there were no positives we may assume the real incidence of toxin positive samples is less than 1%. Unfortunately considerable time was spent solving the initial problems with the PCR method and this restricted the amount of time available for testing more food samples.

Development of gene-probe method for identifying *C. butyricum* that is able to distinguish it from *C. beijerinckii*.

The availability of a specific confirmatory test for *Clostridium butyricum* would be very helpful in food sampling or in assessing the risk of the organism being present in the event of process failures. Unfortunately other butyric clostridia particularly *C. beijerinckii* that have not been associated with toxin formation, are physiologically very similar and difficult to differentiate from *C. butyricum* on the basis of phenotypic tests. The two species can be readily differentiated by sequence analysis of the variable regions of the 16S rRNA genes, as shown in this work. Gene probe tests are therefore obvious candidates for a diagnostic test but, because there are relatively few differences between the sequences of *C. butyricum* and *C. beijerinckii* rRNA genes, this is not straightforward. Some progress was made with the nested PCR approach but the primers use in the second amplification proved to be insufficiently specific for *C. butyricum*. Further work is needed to develop a gene-based test.

Determining whether *C. tertium* and *C. bifermentans* produce toxins.

The clostridial species *Clostridium tertium* and *Clostridium bifermentans* sometimes occur in food in high numbers. *Clostridium tertium* has been isolated from processed and white-brined cheeses (Hood and Smith, 1951; Bintsis and Papademas, 2002), pasteurised milk (Goudkov and Sharpe, 1965) and chocolate custard. *Clostridium bifermentans* has been isolated from cheese (Goudkov and Sharpe, 1965), poultry (Gibbs, 1971), cooked meats (Kokubo *et al.*, 1986) and bean sprouts (de Jong, 1989). Both clostridial species are mildly pathogenic and can act as opportunistic pathogens (e.g. Kolander *et al.*, 1989; Rehany *et al.*, 1994; Gosbell *et al.*, 1996; Miller *et al.*, 2001). However these organisms have not been associated with foodborne illness and no guidelines exist for acceptable numbers of these species in food. This study was conducted to establish the toxigenicity of strains of C. tertium and *C. bifermentans* against Vero kidney tissue cells *in vitro*.

Some weak toxicity against Vero cells was detected in some strains of both *C. bifermentans* and *C. tertium* but in most cases this was lost when the culture supernatant was diluted more than twofold. Several bacterial toxins affect cultured cell lines including the *C. perfringens* enterotoxin and the *B.*

cereus diarrhoeal toxin that cause cytopathic changes in Vero and CaCo2 cells and the *B cereus* emetic toxin causes vacuolation in cultured Hep-2 cells (Labbé 2000). The toxic effect from clostridial culture supernatants was heat stable which argues against its being a protein. It is more likely to be a small molecule such as a peptide. The peptide toxins produced by *Bacillus cereus* or *Staphylococcus aureus* for example are both heat stable. The nature of the toxic effect from the clostridial cultures is unknown but because of its weakness it could be a `non-specific' effect caused by cell component. For example lipoteichoic acid, found in the Gram-positive cell wall is not normally regarded as a toxin but under some circumstances can activate the cascade of steps that lead to toxic shock (Salyers and Whitt, 2002).

If we assume that the levels of toxin needed to produce an effect in Vero cells also caused illness in humans when ingested, then the implication is that very high concentrations of clostridial cells would be needed before food became toxic. If this is so, then food would be obviously spoiled and unpalatable before that stage had been reached. It is possible that more potent toxic effects would be detected using a different assay system but, from evidence obtained here it seems unlikely that growth of *C. bifermentans* and *C. tertium* constitutes an important risk factor in foodborne illness.

Defining the conditions of temperature, pH and solute concentration that will prevent growth of *Clostridium butyricum, C. tertium, C. bifermentans* and *C. barati*

The limiting pH values for growth of *C. butyricum* obtained in this work are compared with data from previous studies in Annex 4. The minimum pH values permitting growth depended on the acidulant and strain. Organic acids were more effective at inhibiting growth than HCl as expected. In general the toxigenic strains of *C. butyricum* appear to be less tolerant of acid conditions than the non-toxigenic ones, i.e. they are inhibited at higher pH values, though this did not hold true for all the tested conditions. The lowest pH at which growth of non-toxigenic strains of *C. butyricum* has been observed is pH 4.0 (de Jong 1989, acidified bean sprouts). The lowest pH allowing growth in this work was 4.1 with broth acidified with HCl. Morton *et al.* (1970) observed growth of non-toxigenic *C. butyricum* in tomato juice at pH 4.2. The lowest pH

supporting growth of toxigenic strains of *C. butyricum* was 4.2 (this work, broth acidified with HCl) whereas the lowest pH values for growth of toxigenic strains of *C. butyricum* observed by Annibali *et al.* (2002) and Morton *et al* (1970) were pH 4.8 and 5.2 respectively. These differences in pH tolerance of toxigenic strains may be due to strain differences; the two toxigenic strains used in this work were ATCC 43181 and ATCC 43755, whilst Annibali *et al.* used a cocktail of srains from the Istituto Superiore di Sanita, Italy (CL20, CL21, CL86, CL109, CL145/1, CL146), and Morton *et al.* used two strains (5262 and 5520) obtained from the Centers for Disease Control, Atlanta, Georgia. The practical implications are that *C. butyricum* can grow at pH values below the cut-off point for growth of proteolytic stains of *C. botulinum* (pH 4.6) which is used as control measure in the food industry. The question of whether all non-toxgenic strains are less acid tolerant than non-toxigenic ones needs to be addressed by examining a wider range of available isolates. Unfortunately obtaining strains is now difficult because of security concerns.

The lowest pH values for growth of *C. bifermentans* and *C. tertium* in broth with HCl as acidulant were 4.1 and 4.2 respectively whilst in the presence of organic acids the minimum pH for both species was between 4.4 and 4.9. These two species are similar in their acid tolerance and both can grow below pH 4.6. Since these strains are not a severe hazard, this property is less significant than with *C. butyricum.*

The minimum pH for growth of *C. barati* observed in this work was 3.7 for non-toxigenic strains and 4.0 for the toxigenic strain. This low pH limit is unusual although a similar minimum pH of 3.7 has been reported previously (de Jong, 1989) for non-toxigenic strains isolated from acidified Mung Beans. However strains of *C. barati* isolated from dry Mung beans were unable to grow at 4.5 suggesting there may be large strain differences in acid tolerance within this species. The nature of acid tolerance in this organism requires more study but because there were long delays in acquiring the toxigenic strain we were unable to complete a more detailed and comprehensive study.

Growth of toxigenic and non-toxigenic strains of *C. butyricum* was inhibited at water activities of 0.95 and 0.96 respectively. Both these values are above the limiting value for proteolytic strains of *C. botulinum* (0.94) so water activities that prevent growth of *C. botulinum* will also ensure control of

toxigenic *C. butyricum*. The minimum growth temperatures of these strains are somewhat lower than those of proteolytic *C. botulinum* although the minimum for toxigenic strains (ca 10 -11°C) was somewhat higher than for non-toxigenic ones (7-8°C). Similarly growth of *C. bifermentans, C. tertium* and *C. barati* will be prevented by water activity conditions that prevent growth of proteolytic *C. botulinum* but again, the minimum growth temperatures for these organisms (7-8°C) are below the minimum for proteolytic *C. botulinum*.

Spores of butyric clostridia may be found in milk from time to time, hence it is important to ensure that spores are eliminated from dairy-derived foods that will support their growth. The *C. beijerinckii* strains used in this work were isolated from chocolate custard. *Clostridium butyricum* was able to grow in chocolate custard and also in creamed rice, emphasising the possible risk that could arise from process failures or post-process contamination in these types of foods.

CONCLUSIONS

The PCR method for screening *Clostridium* isolates for the presence of the type E toxin gene worked satisfactorily but was sensitive to the commercial source of the Taq polymerase. Screening of clostridial isolates from a range of foods failed to detect any type E toxin genes. The frequency of gene carriage in the bacterial population from which the isolates were obtained was estimated to be less than 1%.

Weak toxicity against Vero cells was detected in culture supernatants from three out of five *C. tertium* strains and five out of six *C. bifermentans* strains. Since prolonged incubation of the cell cultures was needed before an effect was detectable, and toxicity was lost on diluting the supernatant more than two-fold, it was concluded that growth of these organisms in food is unlikely to be a significant risk factor in food safety.

Growth of *C. butyricum, C. bifermentans, C tertium* and *C. barati* was prevented by reduced water activities that will prevent growth of proteolytic strains of *C. botulinum*. These organisms can grow at pH values below those that prevent growth of *C. botulinum*. Control of *C. butyricum* in the food

industry needs to allow for the greater pH tolerance of this species compared with proteolytic *C. botulinum.*

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ANNEX 1. List of bacterial strains

Genus	Species	Strain designation	Received from	Comments
Clostridium	*barati*	ATCC 43756	Ian Davison (CAMR)	Type F neurotoxin-producing strain (sequenced)
Clostridium	*barati*	NCTC 10986	NCTC	ATCC 27639 (NCTC listed as paraperfringens)
Clostridium	*barati*	NCTC 10987	NCTC	Originally deposited as *C. paraperfringens.* ATCC 27640.
Clostridium	*barati*	NCIMB 10652	NCIMB	ATCC 25753
Clostridium	*barati*	NCIMB 10675	NCIMB	ATCC 25782. DSM 1401.
Clostridium	*barati*	ATCC 27638	FMSU Collection	Originally deposited as C. paraperfringens.
Clostridium	*beijerinckii*	C3669	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	C3670	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	C3671	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	C3672	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	C3673	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	C3674	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	C3675	Unilever	API profile 47756423. Identitiy confirmed by 16s rRNA sequencing.
Clostridium	*beijerinckii*	NCIMB 9362	NCIMB	Type strain for *C. beijerinckii*
Clostridium	*bifermentans*	NCTC 13019	NCTC	Originally deposited as *Bacillus centrosporogenes.* ATCC 638. NCIMB 10716
Clostridium	*bifermentans*	NCTC 506	NCTC	Type strain for *C. bifermentans.* NCIMB 506.
Clostridium	*bifermentans*	NCTC 1341	NCTC	

Clostridium	*bifermentans*	NCTC 1340	NCTC	Carious tooth.
Clostridium	*bifermentans*	NCTC 6929	NCTC	Originally deposited as *C. sordellii*
Clostridium	*bifermentans*	NCIMB 6801	NCIMB	
Clostridium	*botulinum*	NCTC 7272	NCTC	Type strain for type A botulism
Clostridium	*botulinum*	NCTC 7273	NCTC	Type strain for type B botulism
Clostridium	*botulinum*	NCTC 8265	NCTC	Type strain for type D botulism
Clostridium	*botulinum*	NCTC 8266	NCTC	Type strain for type E botulism
Clostridium	*botulinum*	NCTC 10281	NCTC	Type strain for type F botulism
Clostridium	*butyricum*	NCTC 7423	NCTC	Type strain for *C. butyricum*. ATCC 19398.
Clostridium	*butyricum*	NCTC 6084	NCTC	Originally deposited as *Bacillus saccharobutyricus*. ATCC 6015.
Clostridium	*butyricum*	NCIMB 8082	NCIMB	ATCC 860
Clostridium	*butyricum*	NCIMB 9575	NCIMB	
Clostridium	*butyricum*	ATCC 43181	FMSU Collection	Type-E neurotoxin-producing strain. Isolated from infant botulism case Italy 1984.
Clostridium	*butyricum*	ATCC 43755	FMSU Collection	Type-E neurotoxin-producing strain. Isolated from infant botulism case Italy 1984.
Clostridium	*tertium*	NCTC 541	NCTC	Type strain for *C. tertium*. ATCC 19405
Clostridium	*tertium*	NCIMB 10697	NCIMB	ATCC 14573
Clostridium	*tertium*	NCIMB 701802	NCIMB	Isolated from processed cheese
Clostridium	*tertium*	NCIMB 701803	NCIMB	Isolated from cheese
Clostridium	*tertium*	NCIMB 701804	NCIMB	Isolated from milk
Clostridium	*tyrobutyricum*	NCIMB 10635	NCIMB	Type strain for *C. tyrobutyricum*
Clostridium	*acetobutylicum*	NCIMB 13357	NCIMB	Type strain for *C. acetobutylicum*

Clostridium	*tyrobutyricum*	NCIMB 10635	NCIMB	Type strain
Escherichia	*coli*	strain 30-2C4	M. Doyle	O157 Verotoxin-producing strain
Escherichia	*coli*	K-12	Laboratory collection	Standard laboratory strain

ANNEX 2. Media and diluents

A. General media used for growth limitation studies and spore production.

Media recipes for tasks 3.1 – 3.4

Peptone-Yeast-Glucose-Starch Medium (PYGS medium) Agar and Broth

Proteose petone (Oxoid L46)	5.0g/l
Yeast extract	10.0g/l
Meat extract powder (Oxoid Lab Lemco powder L29)	10.0g/l
Glucose	2.0g/l
Soluble starch	1.0g/l
Resazurin	0.001g/l
Salts solution A	40.0ml/l
Salts solution B	40.0ml/l
Silicon antifoaming agent (BDH) solution 20% v/v	0.25ml/l
Cysteine hydrochloride	0.2g/l.

Salts solution A: $CaCl_2.2H_2O$ 0.265g/l, $MgSO_4.7H_2O$ 0.48g/l and NaCl 2.0g/l
Salts solution B: KH_2PO_4 1.0g/l, $K_2HPO_4.3H_2O$ 1.3g/l, $NaHCO_3$ 10.0g/l.
Adjust pH to 7.0 with 1M HCl.

The medium was prepared and deoxygenated by heating to 121°C for 15 min. Deoxygenated medium was allowed to cool overnight in an anerobic cabinet and aliquots (9.5ml) transferred into Hungate tubes under anaerobic conditions. The tubes were then sterilised by autoclaving for 15 minutes at 121°C.

For PYGS agar, 15g/l bacteriological agar (Technical Number 3) was added to the medium after the pH had been adjusted and the agar was cooled and poured aerobically before being left to equilibrate in reduced conditions.

Growth and storage medium

Casitone	30.0g/l
Yeast extract	5.0g/l
K_2HPO_4	5.0g/l
Resazurin	0.001g/l
Glucose	4.0g/l
Cellobiose	1.0g/l
Maltose	1.0g/l
Soluble starch	1.0g/l
Cysteine hydrochloride	0.5g/l

Add all the ingredients except cysteine hydrochloride. Boil to dissolve, then adjust medium to pH 7.0. Add cysteine hydrochloride. Dispense into tubes (10ml). Sterilise by autoclaving at 121°C for 20 min. This medium was used for the storage of

vegetative cells in pure culture at 4°C. The inoculated tubes were incubated at 37°C for 24 - 48 h before transferring to storage at 4°C.

Differential clostridial agar (Blood agar with neomycin)

Blood agar base	40g
Defibrinated horse blood	7% (v/v)
Neomycin sulphate	75mg
Distilled water	1000 ml

The blood agar base was dissolved in distilled water by heating to 121°C for 15 min. Prepared agar was cooled to 50°C in a waterbath. Once cooled to 50°C, defibrinated horse blood and neomycin sulphate were added, mixed and the agar poured into Petri dishes aseptically.

Duncan and Strong sporulation medium

Proteose peptone	15.0g
Yeast extract	04.0g
Sodium thioglycollate	01.0g
Na2HPO4.7H2O	10.0g
Raffinose	04.0g
Distilled water	1000ml

Dissolve by autoclaving at 121°C for 15 min. Adjust pH to 7.8 + 0.1 using filter-sterilised 0.66M sodium carbonate. Filter-sterilise pH-adjusted medium.

Robertson's two-phase cooked meat medium

Bottom Layer

Cooked meat medium	200g
Glucose	1g
Bacteriological agar	45g
Distilled water	1000ml

Top Layer

Resazurin	1mg
Distilled water	1000ml

The lower layer was prepared within crimp-sealable vials and deoxygenated by boiling for 15 minutes. The vials were left to cool under anaerobic conditions overnight, after which they were crimp-sealed and sterilised by autoclaving for 15 min at 121°C. The upper layer of deoxygenated water was prepared by boiling water containing 0.001g/l resazurin for 15 min and allowing to cool under anaerobic conditions. The upper layer was then applied to the lower layer aseptically by

inoculating the water through the butyl rubber cap of each vial with a sterile disposable needle and syringe.

B. Media used in isolating clostridia from food and environmental samples.
The following media were used to test recovery of a range of *Clostridium* species after inoculation into sterile soil samples. (See Results Section, Task 01/02)

Bhat and Barker (BB) medium
(Bhat, J.V. and Barker, H.A. (1947) Journal of Bacteriology **54,** 381-391)
0.5 g K_2HPO_4, 2.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.02 g $FeSO_4.7H_2O$, 0.5 g yeast extract, 0.1 µg biotin, 100 µg *p*-aminobenzoic acid (PABA), 10.0 g lactic acid lithium salt, 8.0 g sodium acetate, 0.5 g sodium thioglycollate, 0.5 g cysteine hydrochloride and 1.0 mg resazurin.

Modified Bhat and Barker (MBB) medium: 0.5 g K_2HPO_4, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$, 0.02 g $FeSO_4.7H_2O$, 0.5 g yeast extract, 0.1 µg biotin, 100 µg *p*-aminobenzoic acid (PABA), 10.0 g lactic acid lithium salt, 8.0 g sodium acetate, 0.5 g sodium thioglycollate, 0.5 g cysteine hydrochloride, 16 mg trimethoprim, 10 mg cycloserine, 20 mg polymyxin B sulphate and 1.0 mg resazurin.

Butyricum Isolation (BIM) medium
(Popoff, M.R. (1984) *Journal of Clinical Microbiology* **20**, 417-420)
7.0 g K_2HPO_4, 500 µg biotin, 0.9 g NaCl, 0.02 g $CaCl_2$, 0.01 g $MgCl_2.6H_2O$, 0.01 g $MnCl_2.4H_2O$, 1.0 mg $CoCl_2.6H_2O$, 7.0 g KH_2PO_4, 0.05 g $Fe_2SO_4.3H_2O$, 0.25 g cysteine hydrochloride, 24 mg trimethoprim, 10 mg cycloserine, 20 mg polymyxin B sulphate, 1.0 mg resazurin and 10.0 g glucose.

Lactate and Acetate-modified Butyricum Isolation (BIMLA) medium: 7.0 g K_2HPO_4, 500 µg biotin, 0.9 g NaCl, 0.02 g $CaCl_2$, 0.01 g $MgCl_2.6H_2O$, 0.01 g $MnCl_2.4H_2O$, 1.0 mg $CoCl_2.6H_2O$, 7.0 g KH_2PO_4, 0.05 g $Fe_2SO_4.3H_2O$, 0.25 g cysteine hydrochloride, 16 mg trimethoprim, 10 mg cycloserine, 20 mg polymyxin B sulphate,1.0 mg resazurin, 10.0 g lactic acid, 8.0 g sodium acetate and 0.5 g sodium thioglycollate.

All media were adjusted to pH 7.7 after autoclaving and sterilised by filtration.

Materials were prepared in bulk, distributed to Duran bottles (500ml) and autoclaved at 121°C for 15 min. In the case of BIM and BIMLA medium, trimethoprim, cycloserine and polymyxin B sulphate were sterilised by filtration and added after autoclaving and cooling to room temperature.

BIM isolation agar:
BIM isolation medium plus 15.0 g/L agar. Prepare medium as described for BIM medium.

TS agar: This is perfringens agar base (Oxoid CM587) without the antibiotic cycloserine. Suspend 46 g of perfringens agar base (tryptose 15.0 g, soya peptone 5.0 g, 'Lab-Lemco' powder 5.0 g, yeast extract 5.0 g, sodium metabisulphite 1.0 g, ferric ammonium citrate 1.0 g and agar 14.0 g) in 1 L distilled water and sterilise by autoclaving.

TSC agar: Suspend 46 g of perfringens agar base (tryptose 15.0 g, soya peptone 5.0 g, 'Lab-Lemco' powder 5.0 g, yeast extract 5.0 g, sodium metabisulphite 1.0 g, ferric ammonium citrate 1.0 g and agar 14.0 g) in 1 L distilled water and sterilise by autoclaving. Cool to 50°C. Add 400 mg D-cycloserine (Oxoid supplement SR88) reconstituted in sterile distilled water and 50ml egg yolk emulsion. Overlay streaked plates with TS agar (above).

Iron sulphite agar: Suspend 23 g of iron sulphite agar (tryptone 10.0 g, sodium sulphite 0.5 g, iron (III) citrate 0.5g and agar 12.0 g) in 1 L distilled water and sterilise by autoclaving.

Sulphite-cycloserine-azide agar: Prepare TS agar as described above. Add 0.2 g/L sodium azide to 1 L of agar suspension and sterilise by autoclaving. Cool to 50°C. Add 400 mg D-cycloserine (Oxoid supplement SR88) reconstituted in sterile distilled water.

Reinforced Clostridial Medium: Suspend 52.5 g of reinforced clostridial agar (Yeast extract 3.0 g, 'Lab-Lemco' powder 10.0 g, peptone 10.0 g, glucose 5.0 g,

soluble starch 1.0 g, sodium chloride 5.0 g, sodium acetate 3.0 g, cysteine hydrochloride 0.5 g and agar 15.0 g) in 1 L of distilled water. Sterilise by autoclaving.

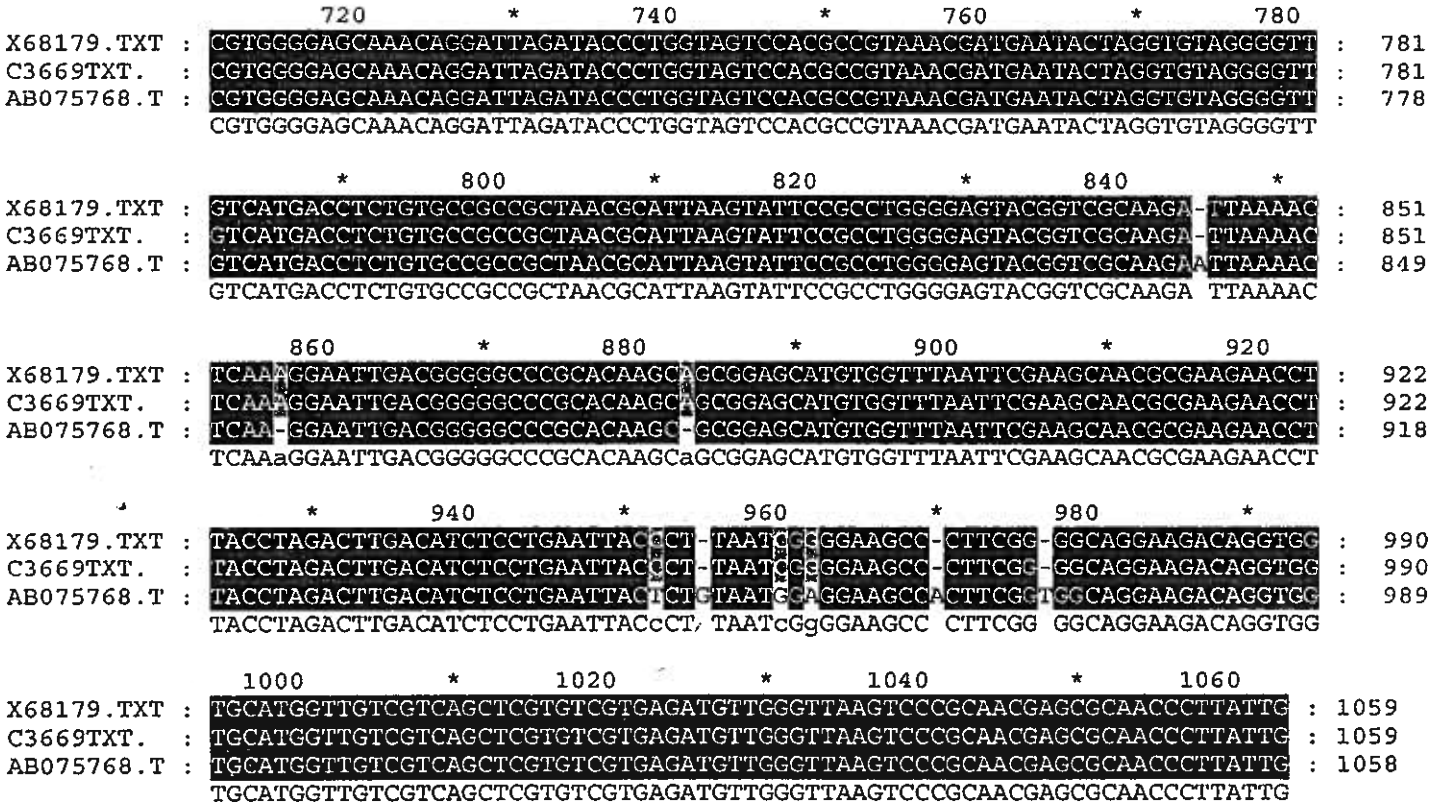
Brain Heart Infusion broth (Oxoid, Basingstoke, UK)
Wilkins-Chalgren anaerobe agar (Oxoid, Basingstoke, UK)

Ttryptone 10 g, gelatine peptone 10 g, yeast extract 5 g, glucose 1 g, sodium chloride 5 g, L-arginine 1 g, sodium pyruvate 1 g, menadione 0.5 mg, haemin 5 mg, agar 10g,

Vero cell growth medium (VCGM)
One litre of VCGM consisted of 880 ml Dulbecco's modified eagle medium (DMEM, Cambrex BE12-733F, Cambrex, Nottingham, UK), 100 ml foetal bovine serum (South African origin, Cambrex DE14-801F, Cambrex, Nottingham, UK), 10 ml penicillin-streptomycin (5,000 units/ml, Sigma P0781, Sigma-Aldrich, Poole, Dorset, UK) and 10 ml L-glutamine (200 mM, Cambrex, BE17605E. Cambrex, Nottingham, UK).

Phosphate buffered saline
(Cambrex 17-516F, Cambrex, Nottingham, UK)

Annex 3. Part of sequence from *C. beijerinckii* (top), isolate from Unilever (middle) and *C. butyricum* (bottom)



	Annex 4. Limiting pH values for growth of *Clostridium butyricum*								
Organism	**Toxigenicity**	**Growth medium**	**Incubation temp (C)**	**acidulant**	**pH allowing growth**	**pH preventing growth**	**Reference**	**Comments**	
C. butyricum	Non-toxigenic	broth				5.1	Bergere and Hermier (1970)	Spore outgrowth	
C. butyricum	Non-toxigenic	Dextrose tryptone broth		citric	4.4	4.2	Morton *et al.* (1970)		
C. butyricum (5 strains)	Non-toxigenic	Broth		HCl	4.1		This work		
C. butyricum (5 strains)	Non-toxigenic	Broth		acetic	4.8		This work		
C. butyricum (5 strains)	Non-toxigenic	Broth		acetic (50mM)	4.7		This work		
C. butyricum (5 strains)	Non-toxigenic	Broth		citric	4.7		This work		
C. butyricum (5 strains)	Non-toxigenic	Broth		citric (50mM)	5		This work		
C. butyricum (5 strains)	Non-toxigenic	Broth		lactic	4.7		This work		
C. butyricum (5 strains)	Non-toxigenic	Broth		lactic 50mM)	4.6		This work		
C. butyricum	Non-toxigenic	Acidified bean sprout brine		citric/lactic	4	3.7	de Jong (1989)		
C. butyricum	Non-toxigenic	Pear juice		citric	>4.8		Jakobsen and Jensen (1975)		
C. butyricum	Non-toxigenic	Tomato juice		HCl	4.2	4	Morton et al (1970)		
C. butyricum	Non-toxigenic	Tomato homogenate	30	Citric	5	4.4	This work	Our limiting pH in tomato is higher than Morton's	
C. butyricum	Non-toxigenic		15	Citric	5	4.8	This work		
C. butyricum	Toxigenic	TPYG broth		HCl	4.8	4.6	Annibali et al (2002)		
C. butyricum	Toxigenic	Dextrose tryptone broth		Citric	5.2	5	Morton et al. (1990)		
C. butyricum ATCC 43181	Toxigenic	Broth	30	HCl	4.2		This work	Lower pH limit than Morton	

C. butyricum ATCC 43181	Toxigenic	Broth	30	Acetic	4.8		This work		
C. butyricum ATCC 43181	Toxigenic	Broth	30	Acetic 50mM)	4.8		This work		
C. butyricum ATCC 43181	Toxigenic	Broth	30	Citric	4.6		This work		
C. butyricum ATCC 43181	Toxigenic	Broth	30	Citric (50mM)	5.1		This work		
C. butyricum ATCC 43181	Toxigenic	Broth	30	Lactic	4.6		This work		
C. butyricum ATCC 43181	Toxigenic	Broth	30	Lactic (50mM)	4.4		This work		
C. butyricum ATCC 43755	Toxigenic	Broth	30	HCl	4.2		This work		
C. butyricum ATCC 43755	Toxigenic	Broth	30	Acetic	4.8		This work		
C. butyricum ATCC 43755	Toxigenic	Broth	30	Acetic 50mM)	4.8		This work		
C. butyricum ATCC 43755	Toxigenic	Broth	30	Citric	4.7		This work		
C. butyricum ATCC 43755	Toxigenic	Broth	30	Citric (50mM)	5		This work		
C. butyricum ATCC 43755	Toxigenic	Broth	30	Lactic	4.7		This work		
C. butyricum ATCC 43765	Toxigenic	Broth	30	Lactic (50mM)	4.4		This work		
C. butyricum	Toxigenic	Tomato juice		HCl	5.2	4.8	Morton et al. (1990)		
C. butyricum ATCC 43181	Toxigenic	Tomato homogenate	30	Citric		5	This work		
C. butyricum ATCC 43181	Toxigenic	Tomato homogenate	15	Citric	5	4.8	This work		
C. butyricum ATCC 43755	Toxigenic	Tomato homogenate	30	Citric	5	4.4	This work		
C. butyricum ATCC 43755	Toxigenic	Tomato homogenate	15	Citric	4.6-4.8	5	This work		