



## Anaerobes in animal disease

# A comparative study on the use of selective media for the enumeration of *Clostridium perfringens* in poultry faeces

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## ABSTRACT

Isolation and fast detection of *Clostridium perfringens* is essential in veterinary medical diagnostics and veterinary research, as it allows to recommend suitable treatment options after antimicrobial resistance determination, and is essential to study pathogenesis. In this study four selective media were tested for the enumeration of, and selectivity towards *C. perfringens* in faecal samples from poultry. The routinely used Columbia agar with 5% sheep (CBA), Shahidi-Ferguson-perfringens agar (SFP), tryptose sulphite cycloserine agar (TSC), and a novel chromogenic medium, CHROMagar™ *C. perfringens* (CHCP), were tested. Overall, no difference in *C. perfringens* recovery could be observed between the selective media. The limit of quantification was  $10^3$  CFU/mL for all agars. CHCP showed the highest specificity, especially when low *C. perfringens* loads were present in the faeces, with TSC being the second most specific selective medium. Both CBA and SFP allowed considerable growth of other faecal microbiota and were not specific for *C. perfringens*. On CHCP, differentiation of *C. perfringens* from other faecal bacteria was straightforward due to the appearance of *C. perfringens* as orange colonies, with other bacteria being absent or appearing as blue/green colonies. On TSC, *C. perfringens* appeared as black colonies, but longer incubation periods were sometimes needed for the black colour to develop. Therefore, CHCP can be recommended when timely and easy identification and enumeration of *C. perfringens* from complex samples, such as faeces, is needed.

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## 1. Introduction

In diagnostic bacteriology laboratories, a new generation of molecular tools has become available that partly replace traditional methods [1,2]. Despite these advances, isolation of bacterial strains is still indispensable for multiple purposes. First, isolation of causative bacterial strains is often needed in routine diagnostics for antimicrobial susceptibility testing and thus recommending treatment options for medical doctors and veterinarians. Secondly, bacterial isolates are essential to establish links between bacteria and diseases, as isolates are needed to perform infection trials and fulfil Koch's postulates, to identify genomic elements that are crucial for disease, and to develop control methods, including

antigen selection for vaccine development [3–6].

In veterinary medical diagnostics, one of the bacteria that is often encountered in intestinal and faecal samples is *Clostridium perfringens*. This is a Gram positive, non-motile, spore-forming, anaerobic rod-shaped bacterium that is commonly found in water, soil and the intestinal tract of healthy animals [7]. However, it is also one of the most common pathogens, causing a variety of important human and animal diseases, ranging from histotoxic to enteric infections [8,9]. In poultry, *C. perfringens* causes necrotic enteritis (NE), which is one of the most important enteric diseases of broilers. NE can also occur in layers and replacement pullets [10]. Furthermore, *C. perfringens* is associated with focal duodenal necrosis in layers [11]. In order to isolate this bacterium from the faeces or intestinal digesta, a medium is needed that suppresses outgrowth of concomitant microbiota but permits growth of *C. perfringens* as easily differentiated colonies. Several selective media are being used for isolating *C. perfringens* from complex samples, but none of them seems to be ideal, because of either partial inhibition of *C. perfringens* or permission of growth of too

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many other bacterial species, hampering isolation and quantification of *C. perfringens* [12]. In order to allow growth of *C. perfringens* in a readily recognizable form and thus improve the ease of identification of its colonies, various supplements are used. Most common is the use of sheep blood, egg yolk or sulphite in the agar plates. When grown on agar containing sheep blood, the haemolytic activity of *C. perfringens* causes a typical narrow zone of complete haemolysis inside a larger zone of partial haemolysis [13]. However, growth of other haemolytic bacteria (amongst others *Bacillus* sp., *Enterococcus* sp., some *E. coli* strains and *Clostridium bifermentans*) can impede the correct identification of *C. perfringens* on blood agar plates [14–17]. Supplementation of agar plates with egg yolk results in opaque zones around the *C. perfringens* colonies, which is due to its lecithinase activity [13]. However, *Clostridium sordelli* and *C. bifermentans* produce enzymes that are closely related to *C. perfringens* alpha toxin (lecithinase), and can therefore lead to false positives [18]. In addition to sheep blood and egg yolk, also sulphite is commonly used to aid in the isolation of *C. perfringens*. Sulphite has the additional advantage that it is inhibitory to many bacteria, while *C. perfringens* is not inhibited by this substance but reduces the sulphite to hydrogen sulphide, which leads to precipitation of iron sulphide, resulting in black colonies [19–21].

Recently, a novel chromogenic agar has been developed for selective isolation of *C. perfringens* (CHROMagar™ *C. perfringens*, CHCP) but this agar has not yet been benchmarked in scientific literature. The aim of the current study was to compare this novel chromogenic agar to three commonly used agars (CBA, SFP and TSC), to determine the recovery rate of and specificity towards *C. perfringens*, as well as the ease of differentiation between *C. perfringens* and contaminating bacteria present in complex samples.

## 2. Material and methods

### 2.1. Media preparation

Columbia blood agar (CBA) was prepared using Columbia blood agar base (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood, 12 mg/L kanamycin sulphate and 30 000 IU/L polymyxin B sulphate. Shahadi-Ferguson Perfringens (SFP) agar and tryptose sulphite cycloserine (TSC) agar were prepared using Perfringens Agar Base (Sigma-Aldrich) supplemented with either 5% sterile egg yolk emulsion (Oxoid, Basingstoke, UK), 12 mg/L kanamycin sulphate and 30 000 IU/L polymyxin B sulphate for SFP or 400 mg/L D-cycloserine for TSC. Sodium metabisulphite and ferric ammonium citrate are present in both media. CHROMagar™ *C. perfringens* (CHCP) was prepared according to the manufacturer's instructions. Briefly, CHCP base was supplemented with CHCP supplement 1 to a final concentration of 2000 mg/L, as well as CHCP supplement 2 to a final concentration of 120 mg/L. Sterile supplements were added after sterilization of the respective media, after which they were dispersed in 120 × 120 mm petri dishes, air dried at room temperature and stored at 4 °C for maximum 30 days.

### 2.2. Bacterial strains and culture conditions

A collection of 44 bacterial strains was used to assess the growth and colony appearance on CHCP (Table S1). This included 28 *C. perfringens* strains isolated from 7 different animal species, four *Clostridioides difficile* strains, one *Bacillus subtilis*, one *Bacillus amyloliquefaciens*, one *Enterococcus gallinarum*, one *Enterococcus faecalis*, four chicken isolates of *Escherichia coli*, one *Lactobacillus crispatus*, one *Lactobacillus vaginalis* and one *Lactobacillus salivarius* strain. Bacteria were routinely grown on Columbia agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated sheep blood

and anaerobically incubated, except the *Bacillus*, *Lactobacillus* and *E. coli* strains that were both aerobically and anaerobically incubated. To assess the selectivity of CHCP towards *C. perfringens*, all strains listed in Table S1 were plated on CHCP and incubated overnight at 37 °C under anaerobic conditions.

From this collection, two pathogenic *C. perfringens* type G strains isolated from necrotic enteritis lesions (CP56 and EHE-NE18) and two commensal *C. perfringens* type A strains isolated from healthy broilers (CP10 and CP20) were grown overnight in brain heart infusion broth (BHI; Sigma-Aldrich, Steinheim, Germany) at 37 °C under anaerobic conditions. From each overnight culture, bacterial suspensions were prepared in sterile phosphate-buffered saline (PBS) to obtain 10<sup>7</sup>, 10<sup>5</sup>, 10<sup>3</sup> or 10<sup>1</sup> CFU/mL for use in the *C. perfringens* isolation experiments using spiked chicken faeces. The number of *C. perfringens* in the final suspensions was determined by plating 10-fold serial dilutions and colony counting.

### 2.3. Evaluation of the selective media for isolation and quantification of *C. perfringens* from chicken faeces

Four selective media (CBA, TSC, SFP and CHCP) were compared for their ability to isolate and quantify a known concentration of *C. perfringens* present in chicken faeces. Therefore, faecal samples were collected from healthy chickens housed at the Faculty of Veterinary Medicine, Ghent University, Belgium. To account for differences in microbial composition between individual birds, the experiment was performed on six different days, each time using a fresh faecal sample from a healthy chicken (one chicken per day, six different birds in total). Furthermore, to account for differences in the ability of *C. perfringens* strains to outgrow the residing microbiota, each faecal sample was spiked with four different *C. perfringens* strains (CP10, CP20, CP56 or EHE-NE18). More specifically, each faecal sample was collected in a sterile tube and subsequently divided into 200 mg aliquots (17 aliquots per sample), which were spiked with respectively 1800 µL of the *C. perfringens* strain dilutions expected to be 10<sup>7</sup>, 10<sup>5</sup>, 10<sup>3</sup> or 10<sup>1</sup> CFU/mL, as described above. As a negative control, faeces were spiked with sterile PBS only, to determine the level of *C. perfringens* present in the collected faeces. In two faecal samples, no *C. perfringens* could be detected in the unspiked aliquots, whereas in the other four faecal samples, some *C. perfringens* colonies could be observed (2–12 counted colonies in 6 × 20 µL droplets, not expected to have major influence on the results of the spiking experiments). After homogenization of the *C. perfringens*-spiked faecal aliquots by vortex mixing, *C. perfringens* was enumerated by plating 10-fold serial dilutions and colony counting. Therefore, 10-fold dilutions of the faeces were prepared by diluting 20 µL of each sample with 180 µL PBS. For each dilution, six droplets of 20 µL were pipetted on the different selective media. The plates were air-dried and incubated overnight at 37 °C under anaerobic conditions. Plates containing between 30 and 300 colonies in 6 × 20 µL droplets were counted and numbers of CFU/g faeces were calculated. All plates with droplets consisting of more than 300 colonies were determined as uncountable. For each growth medium the recovery of *C. perfringens* from the spiked faeces was calculated relative to the growth of the starting inoculum on the same growth medium. Furthermore, to assess the specificity of the four tested media, both *C. perfringens* colonies as well as the total amount of colonies (contaminant microbiota at the countable dilution (30–300 colonies)) were recorded.

### 2.4. Identification of bacterial isolates using MALDI-TOF MS

From each of the selective media, at least 4 different colonies visually identified as *C. perfringens*, as well as a selection of

contaminant colonies that were visually different from *C. perfringens* were randomly picked and identified with Matrix Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) using the direct transfer method and  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) as a matrix, according to the manufacturer's guidelines (Bruker MALDI Biotyper MSP, Bruker Daltonics GmbH, Bremen, Germany) as described before [17]. This technique is based on detection of bacterial proteins and their resulting protein mass spectrum. The spectra were obtained and analysed with the MBT Compass software version 4.1 (Bruker Daltonik), and compared with a database of 6120 mean spectra projections.

### 2.5. Statistical analysis

All analyses were performed in the R statistical environment (R Core Team 2017, version 3.6.0). For each selective medium, the influence of *C. perfringens* inoculum level in the chicken faeces on both the *C. perfringens* recovery rate and *C. perfringens* specificity (i.e. the growth of concomitant microflora at the countable dilution (dilution used to enumerate *C. perfringens*, containing 30-300 *C. perfringens* colonies)) was determined. Furthermore, at each inoculum level, the difference between the selective media on both *C. perfringens* recovery and specificity was assessed. For all analyses a linear mixed-effect model (LMM) was used (LME, lme4 package [22]), with *P*-values computed via the Satterthwaite approximation (lmerTest package [23]). Post-hoc analysis was performed using the emmeans package, with Tukey adjustment for multiple comparisons. The model was run separately for *C. perfringens* recovery and *C. perfringens* specificity. In each model *C. perfringens* strain ID and faecal sample ID were included as random terms to account for possible non-independence of *C. perfringens* strain and faecal microbial background. The significance level was set at *P* = 0.05.

## 3. Results

### 3.1. Selectivity of CHROMagar™ *C. perfringens* for pure cultures

All tested *C. perfringens* strains, independent of their origin, were able to grow on the CHCP plates and appeared as orange colonies (Table S1). The selectivity of the CHCP plates was evaluated based on their ability to inhibit the growth of pure cultures of a selection of other bacteria commonly found in poultry faeces. None of the tested *Bacillus*, *Lactobacillus* and *E. coli* strains were able to grow on CHCP, both under aerobic and anaerobic conditions. The *C. difficile* and *Enterococcus* strains grew on CHCP but were easily distinguishable from *C. perfringens* by the blue to green colony colour. No false positives or negatives could be observed in this experiment.

### 3.2. Identification of *C. perfringens* colonies from faecal samples on all tested media

Reliable and preferably easy identification of *C. perfringens* in samples with a complex microbial composition is important to avoid unnecessary additional purification and identification steps. Therefore, the visual appearance of *C. perfringens*, as well as the concomitant microbiota on the different plates was assessed. On CBA, *C. perfringens* can be recognized due the typical double haemolytic zone around the colonies. However, also growth of non-haemolytic colonies was observed, which were identified as *Enterococcus faecium* and *Enterococcus hirae*, whereas haemolytic colonies were confirmed to be *C. perfringens* (Fig. 1(e) and (f)). On both TSC and SFP, *C. perfringens* colonies appear black due to reduction of the sulphite incorporated in the medium. On TSC,

white colonies with black spots were confirmed to be *C. perfringens*, whereas growth of other faecal microbiota could be observed as translucent or white colonies, which both failed to be identified via MALDI-TOF, since the organism did not seem to be included in the database (Fig. 1(c) and (d)). However, it was observed that after an additional 24 h of incubation, more colonies developed black spots, which were confirmed to be *C. perfringens* as well. On SFP, *C. perfringens* was identified as white colonies with black spot, surrounded by an opaque halo which is due to its lecithinase activity on the egg yolk incorporated in the medium. Other faecal microbiota was present as white colonies without an opaque zone, some of which were identified as *Enterococcus faecalis* (Fig. 1(a) and (b)). On CHCP, *C. perfringens* produced orange colonies (Fig. 1(g) and (h)). On some plates, blue/green colonies could be observed which were identified as *Clostridium disporicum* (Fig. 1(i)).

### 3.3. *C. perfringens* recovery rates from faecal samples are similar on CBA, TSC, SFP and CHCP

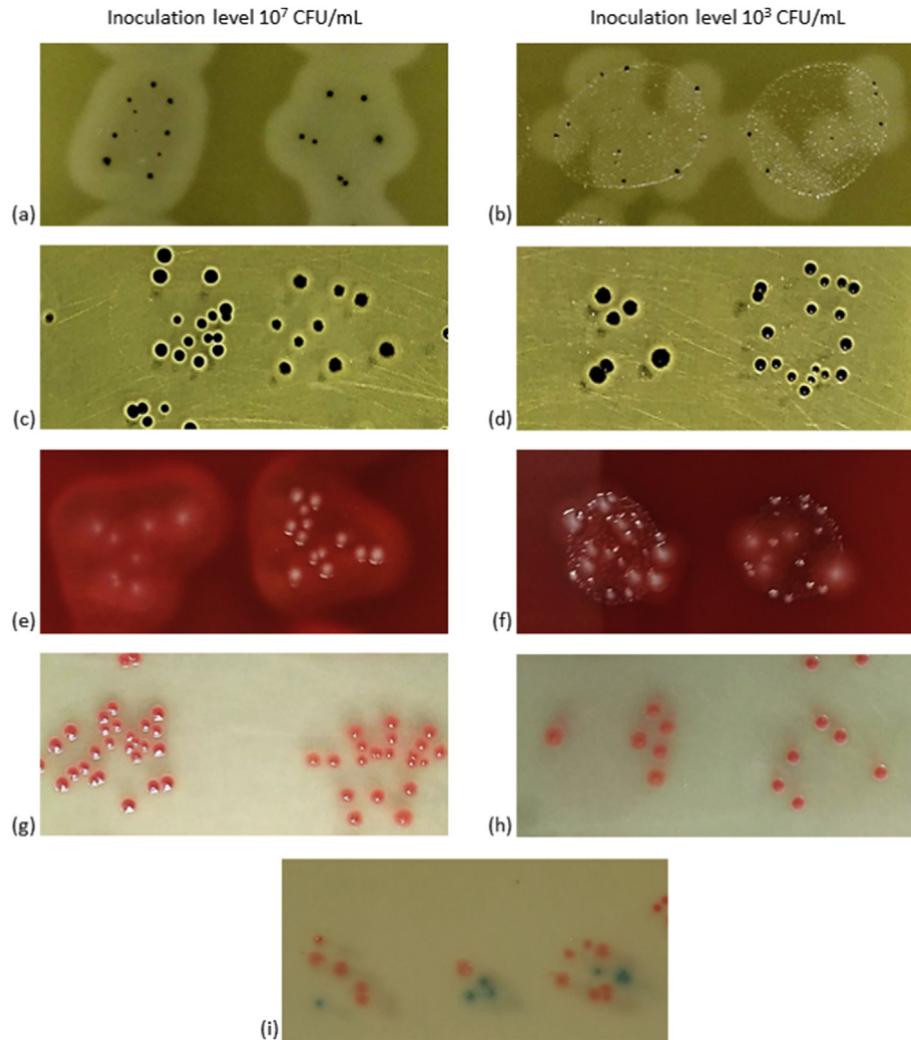
To determine the limit of detection and limit of quantification of *C. perfringens* from samples containing complex microbial mixtures, fresh chicken faeces were spiked with known concentrations of *C. perfringens*, ranging from  $10^7$  to  $10^1$  CFU/mL, after which the recovery rate was determined on each selective medium (Fig. 2, Table S2). Although *C. perfringens* could be detected on all selective media, for all tested inoculum levels, reliable quantification was only possible at an inoculum level of at least  $10^3$  CFU/mL. At the lowest tested inoculum level ( $10^1$  CFU/mL) high variability of recovery was observed for all four tested selective media, with recovery levels ranging from 0% (false negatives) to 270% in one replicate. Furthermore, the recovery rate from samples containing low *C. perfringens* load ( $10^1$  CFU/mL inoculum) was clearly different from all other inoculum levels for all tested selective media (*P*-values ranging from *P* = 0.16 to *P* = 0.0092, Table 1). However, at inoculation levels where *C. perfringens* could be accurately enumerated (above the limit of reliable quantification;  $\geq 10^3$  CFU/mL) no difference in *C. perfringens* recovery between the different inoculum levels could be observed, on all selective media (Table 1).

Furthermore, no significant differences in *C. perfringens* recovery were detected between the media at the inoculum levels of  $10^7$ ,  $10^3$  and  $10^1$  CFU/mL, whereas on CHCP recovery was significantly lower at the inoculum level of  $10^5$  CFU/mL as compared to SFP (*P* = 0.0132, Tables 1 and 2).

### 3.4. CHCP shows significantly higher specificity for isolation of *C. perfringens* from faecal samples compared to the other media

For accurate enumeration of *C. perfringens* from samples with mixed faecal microbiota, not only the recovery rate but also the specificity of the selective media is important. In order to objectively assess the specificity of the four tested selective media, the number of *C. perfringens* counts relative to the total colony count (including contaminant microflora) was recorded (Fig. 3).

At highest inoculation levels ( $10^7$  CFU/mL) *C. perfringens* outgrew other contaminants on all tested media, with highest specificity observed on CHCP and TSC, which performed equally well (*P* = 0.83) and significantly better than CBA and SFP (Table 2). At lower *C. perfringens* inoculation levels, increased growth of contaminants was observed on CBA, TSC and SFP, but not on CHCP, which showed significantly higher specificity for *C. perfringens* (still 95.05% specificity at the lowest *C. perfringens* inoculation level ( $10^1$  CFU/mL, Fig. 3(d)). TSC has good specificity at both  $10^7$  and  $10^5$  CFU/mL inoculum (respectively 95.05% and 85.55% *C. perfringens* specificity), but growth of contaminants significantly increased when lower *C. perfringens* levels were present in the faeces (*P*  $\leq$  0.0001, Table 1,



**Fig. 1.** Visual representation of *C. perfringens* colonies as well as concomitant bacteria from fresh faecal samples spiked with  $10^7$  (left column),  $10^3$  CFU/mL (right column) *C. perfringens* on four different selective media. Representative pictures were taken from the dilution used to enumerate *C. perfringens* recovery and specificity (dilution  $10^{-5}$  for  $10^7$  CFU/mL inoculation level or dilution of  $10^{-1}$  for an inoculation level of  $10^3$  CFU/mL). *C. perfringens* can be identified as white colonies with black spot, surrounded by an opaque zone on SFP (a, b) or black colonies without halo on TSC (c, d). On CBA (e, f), *C. perfringens* is identified as haemolytic colonies, whereas on CHCP, *C. perfringens* is typically orange (g, h), with growth of other faecal bacteria as blue/green colonies (i). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3(b)). No difference in specificity towards *C. perfringens* could be observed between CBA and SFP medium, with acceptable background growth only when high *C. perfringens* load was present in the faeces ( $>80\%$  background at  $10^5$  CFU/mL inoculum) (Fig. 3(a) and (c)).

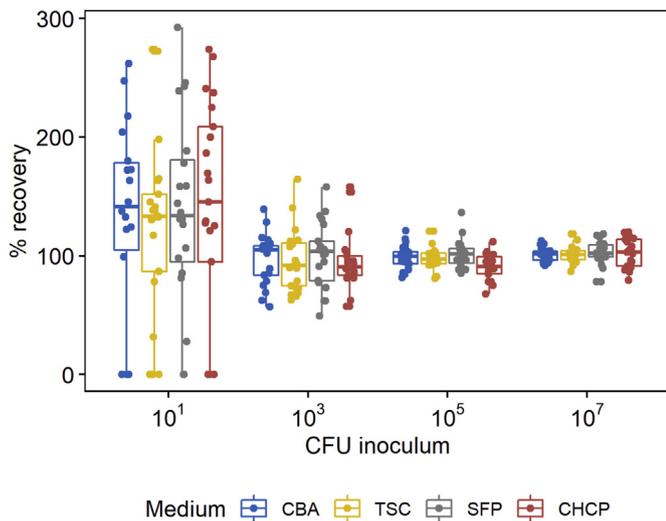
#### 4. Discussion

Isolation and fast detection of *C. perfringens* is essential in veterinary medical diagnostics and veterinary research, as it allows to choose antimicrobial therapies, either or not after antimicrobial resistance determination. The isolation of *C. perfringens* from complex microbiota as a pure culture can, however, be challenging, as to date, no selective culture medium is described that allows maximal recovery of *C. perfringens* while preventing all growth of other bacterial types present in the sample.

In this study, the use of a novel chromogenic selective medium (CHROMagar™ *C. perfringens*) was described. As the inclusion of antibiotics in the selective media used to date can also hamper the ability of certain *C. perfringens* strains to grow on the plates [12], the recovery of pure cultures of *C. perfringens* on CHCP was assessed. All

tested *C. perfringens* strains were easily recovered from the plates, indicating that the supplements used to suppress the growth of other bacteria had no influence on the viability of *C. perfringens*. Furthermore, all 28 *C. perfringens* strains appeared as orange colonies, while all the strains that were not *C. perfringens*, appeared as blue/green colonies. In fact, all *Bacillus*, *Lactobacillus* and *E. coli* strains that have the ability to grow on CBA, did not grow on CHCP plates at all, which underlines the selectivity of this novel medium.

In a second part of the study, CHCP was compared to three traditionally used selective media (CBA, TSC and SFP) in its ability to isolate and enumerate a known concentration of *C. perfringens* cells from spiked chicken faeces. Overall, no difference in *C. perfringens* recovery could be observed between the different media. However, at the inoculation level of  $10^5$  CFU/mL, CHCP had significantly lower recovery than SFP. Nevertheless, this difference in recovery was not observed when both higher or lower *C. perfringens* levels were present in the faeces, suggesting that all media performed equally well. This is in accordance with a study conducted by Harmon et al. [24], where similar *C. perfringens* recovery of pure cultures of *C. perfringens* on SFP and TSC was observed. However, some of the



**Fig. 2. Overall recovery percentage, based on four different *C. perfringens* strains from spiked, fresh chicken faeces inoculated at different levels and incubated on CBA, TSC, SFP and CHCP.** Fresh chicken faeces were spiked with *C. perfringens* concentrations ranging from  $10^7$  to  $10^1$  CFU/mL, followed by determination of the recovery rate on the selective media CBA (blue), TSC (yellow), SFP (grey) and CHCP (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

*C. perfringens* strains tested were more sensitive to D-cycloserine, resulting in lower recovery of those specific strains [24]. Although *C. perfringens* could be recovered from spiked faeces at all inoculum levels, large variability was observed at low *C. perfringens* loads,

resulting in a limit of quantification of  $10^3$  CFU/mL for all selective media. Detection at inoculum levels as low as  $10^1$  CFU/mL is in general difficult due to contaminants in faeces that outgrow *C. perfringens* more easily.

In a third part of the study, the ease of visual identification of *C. perfringens* on the plates when other bacterial types were present, was assessed. This includes both the specificity of the selective media for *C. perfringens* as well as the ease of identification of *C. perfringens* on the plates when other bacterial types were present as well. The specificity of the selective media for *C. perfringens* was assessed by enumerating both the *C. perfringens* growth as well as the growth of accompanying microbiota. CHCP showed the overall best specificity towards *C. perfringens*, and clearly outperformed other selective media when only low *C. perfringens* numbers were present in the faeces. Indeed, even at the lowest inoculum level ( $10^1$  CFU/mL), less than 5% growth of non-*C. perfringens* colonies was observed on CHCP, whereas the non-*C. perfringens* fraction accounted for more than 80% of the colonies on all other tested agars. Furthermore, a clear colour difference between orange *C. perfringens* colonies and blue/green non-*C. perfringens* colonies was observed, which facilitates correct identification. At the highest tested *C. perfringens* load ( $10^7$  CFU/mL), TSC and CHCP were equally specific for *C. perfringens*. However, when inoculated with lower *C. perfringens* concentrations, increasingly more growth of other bacteria was observed on TSC. *C. perfringens* could easily be identified on TSC due to the black colony-appearance. However, 48h of incubation might be needed for all *C. perfringens* colonies to turn black, a phenomenon that has been observed before [25], which does not make it a suitable medium if the goal is fast identification and distinction of *C. perfringens* from other strains. CBA and SFP were not specific for *C. perfringens* as both showed a large number

**Table 1**

*P*-values for the comparison of different inoculum levels for *C. perfringens* recovery from faeces (CP recovery) and *C. perfringens* counts relative to total colony count (CP specificity). *P*-values were assessed for each selective medium.

CFU inoculum	CBA		TSC		SFP		CHCP	
	CP recovery <sup>a</sup>	CP specificity <sup>b</sup>	CP recovery	CP specificity	CP recovery	CP specificity	CP recovery	CP specificity
$10^1$ – $10^3$	<b>0.0192*</b>	0.0975	0.0628	0.9645	<b>0.0109*</b>	0.4913	<b>0.0233*</b>	0.2233
$10^1$ – $10^5$	<b>0.0272*</b>	<b>0.0088**</b>	0.0865	<b>0.0001***</b>	<b>0.0123*</b>	0.9857	<b>0.0092**</b>	0.1705
$10^1$ – $10^7$	<b>0.0411*</b>	<b>&lt;.0001***</b>	0.1558	<b>&lt;.0001***</b>	<b>0.0172*</b>	<b>&lt;.0001***</b>	0.0711	0.1379
$10^3$ – $10^5$	0.9991	0.6988	0.9991	<b>&lt;.0001***</b>	1	0.7001	0.9698	0.9959
$10^3$ – $10^7$	0.9904	<b>&lt;.0001***</b>	0.9762	<b>&lt;.0001***</b>	0.9985	<b>&lt;.0001***</b>	0.9709	0.9908
$10^5$ – $10^7$	0.9983	<b>&lt;.0001***</b>	0.9927	0.753	0.9994	<b>&lt;.0001***</b>	0.8194	0.9999

<sup>a</sup> *P*-values of the comparison of *C. perfringens* recovery rates from chicken faeces between different inoculum levels.

<sup>b</sup> *P*-values of the comparison of the difference of *C. perfringens* counts relative to total colony count between different inoculum levels (Colonies were counted at the same dilution that was used to enumerate *C. perfringens* (30–300 colonies in  $6 \times 20 \mu\text{l}$  droplets)).

\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

**Table 2**

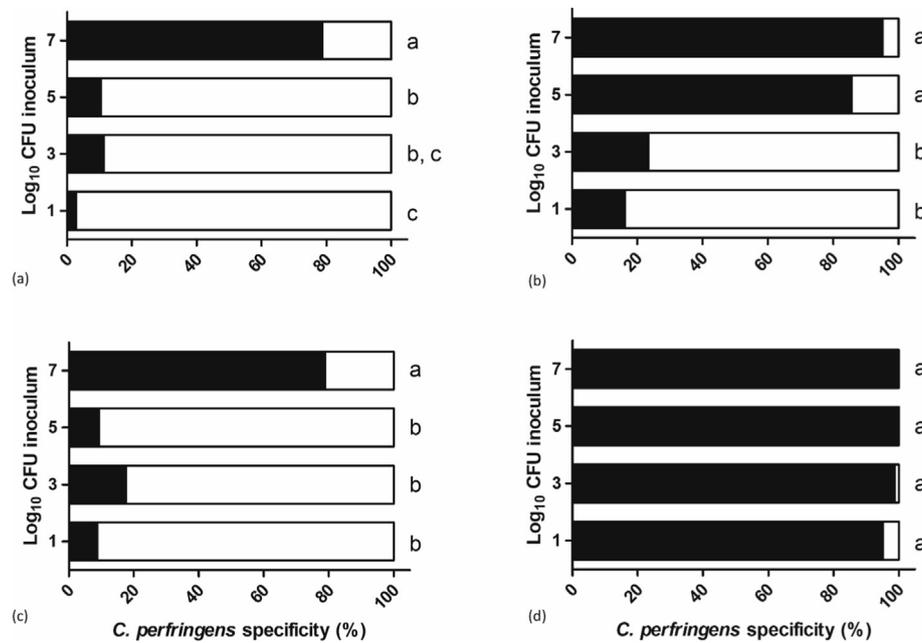
*P*-values for the comparison of different media for *C. perfringens* recovery from faeces (CP recovery) and *C. perfringens* counts relative to total colony count (CP specificity). *P*-values were assessed for each inoculum level.

Compared media	CFU inoculum							
	$10^7$		$10^5$		$10^3$		$10^1$	
	CP recovery <sup>a</sup>	CP specificity <sup>b</sup>	CP recovery	CP specificity	CP recovery	CP specificity	CP recovery	CP specificity
CBA - CHCP	0.9778	<b>0.0071**</b>	0.0771	<b>&lt;.0001***</b>	0.9746	<b>&lt;.0001***</b>	0.8986	<b>&lt;.0001***</b>
CBA - SFP	0.9262	0.9995	0.8917	0.6804	0.919	0.2789	0.9449	0.9776
CBA - TSC	0.9999	<b>0.0488*</b>	0.9651	<b>&lt;.0001***</b>	0.9914	<b>0.0083**</b>	0.9993	0.1092
CHCP - SFP	0.9965	<b>0.0093**</b>	<b>0.0132*</b>	<b>&lt;.0001***</b>	0.7142	<b>&lt;.0001***</b>	0.999	<b>&lt;.0001***</b>
CHCP - TSC	0.9678	0.8336	0.1982	<b>0.0246*</b>	0.9992	<b>&lt;.0001***</b>	0.9314	<b>0.0002***</b>
SFP - TSC	0.9068	0.062	0.6388	<b>&lt;.0001***</b>	0.7888	0.3656	0.9693	0.2245

<sup>a</sup> *P*-values of the comparison of *C. perfringens* recovery rates from chicken faeces between media.

<sup>b</sup> *P*-values of the comparison of the difference of *C. perfringens* counts relative to total colony count between media (Colonies were counted at the same dilution that was used to enumerate *C. perfringens* (30–300 colonies in  $6 \times 20 \mu\text{l}$  droplets)).

\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



**Fig. 3.** Specificity (%) of the different selective media for the recovery of *C. perfringens* from fresh chicken faeces spiked with known levels of *C. perfringens*. Fresh chicken faeces was spiked with  $10^7$ ,  $10^5$ ,  $10^3$  or  $10^1$  CFU/mL *C. perfringens* and plated on either (a) CBA, (b) TSC, (c) SFP or (d) CHCP. *C. perfringens* specificity was determined as the total number of *C. perfringens* versus the total number of bacterial colonies present at the countable dilution (i.e. containing 30–300 *C. perfringens* colonies). For each selective medium, inoculation levels not sharing the indicated letters show significantly different *C. perfringens* recovery ( $P < 0.05$ ).

of non-*C. perfringens* colonies. This is in line with previous reports, describing that SFP medium was not sufficiently selective to limit the growth of facultative anaerobes such as enterococci [26], which were also part of the contaminant microbiota on SFP in this work. Although not specific, the visual identification of *C. perfringens* on SFP is quite clear due to the opaque halo and black colour of colonies on the medium. On CBA, *C. perfringens* grows as haemolytic colonies, but, conclusive identification can be more challenging than on SFP. Indeed, it occurred that the haemolytic zone around one colony was so large that other, non-*C. perfringens* colonies fell into this zone and could therefore lead to false positives. Moreover, although not identified in this study, other haemolytic bacteria such as *Bacillus* sp., *Enterococcus* sp. and some *E. coli* strains could also give false positive results [14,15].

In conclusion, all tested selective media (CBA, TSC, SFP and the novel CHCP) had a limit of quantification at the inoculation level of  $10^3$  CFU/mL. However, CHCP outperformed the other media when it came to specificity, especially when low *C. perfringens* loads were present in the sample. Therefore, CHROMagar™ for *C. perfringens* can be recommended for easy and fast detection of *C. perfringens* in samples containing complex bacterial mixtures, such as faeces.

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### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.anaerobe.2020.102205>.

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