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Investigating the effect of supplementation on Clostridium difficile spore recovery in two solid agars

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Abstract

Background: A variety of supplemented solid media are used within Clostridium difficile research to optimally recover spores. Our study sought to investigate different media and additives, providing a method of optimised C. difficile spore recovery. Additionally, due to the results observed in the initial experiments, the inhibitory effects of three amino acids (glycine, L-histidine & L-phenylalanine) on C. difficile spore outgrowth were investigated.

Methods: Spores of five C. difficile strains (PCR ribotypes 001,015,020,027,078) were recovered on two commonly used solid media (BHI & CCEY) supplemented with various concentrations of germinants (taurocholate,glycine & lysozyme). Agar-incorporation minimum inhibitory concentration (MIC) testing was carried out for glycine and taurocholate on vegetative cells and spores of all five strains. Additionally a BHI broth microassay method was utilised to test the growth of C. difficile in the presence of increasing concentrations (0,1,2,3,4%) of three amino acids (glycine,L-histidine,L-phenyalanine).

Results: CCEY agar alone and BHI supplemented with taurocholate (0.1/1%) provided optimal recovery for C. difficile spores. Glycine was inhibitory to spore recovery at higher concentrations, although these varied between the two media used. In agar-incorporated MIC testing, glycine concentrations higher than 2% (20 g/L) were inhibitory to both C. difficile spore and vegetative cell growth versus the control (mean absorbance = 0.33 ± 0.02 vs 0.12 ± 0.01)($P < 0.001$). This indicates a potential mechanism whereby glycine interferes with vegetative cell growth. Further microbroth testing provided evidence of inhibition by two amino acids other than glycine, L-histidine and L-phenylalanine.

Conclusions: We provide two media for optimal recovery of C. difficile spores (CCEY alone and BHI supplemented with 0.1/1% taurocholate). CCEY is preferred for isolation from faecal samples. For pure cultures, either CCEY or supplemented BHI agar are appropriate. The inhibitory nature of three amino acids (glycine,L-histidine,L-phenylalanine) to C. difficile vegetative cell proliferation is also highlighted.

Keywords: lysozyme, germination, spores, MIC, culture media, taurocholate

1.1 Introduction

Clostridium difficile is a Gram-positive anaerobe responsible for C. difficile infection (CDI). CDI can vary in severity; mild diarrhoea is the most common presentation but some patients may develop pseudomembranous colitis (1) and subsequent toxic megacolon, a surgical emergency with a high mortality rate (~30-80%) (2). Although cases of CDI have declined considerably in the UK, from 55,498 cases in 2007/2008 to 13,361 in 2013/2014 (3), it remains an infection of concern and the subject of considerable research efforts.

Different solid media are used for C. difficile recovery according to particular requirements. However the design of all C. difficile isolation media must optimise vegetative outgrowth from C. difficile spores while providing a selective environment to suppress other bacteria present in the sample .CCEY (Cycloserine-cefoxitin egg yolk) agar is a selective medium previously shown to be the most sensitive and cost-efficient medium for isolating C. difficile from stool samples when compared to Cycloserine-cefoxitin fructose agar (CCFA), ChromID C. difficile and tryptone soy agar (TSA) with 5% sheep's blood (4). CCEYL (cycloserine-cefoxitin and 5% lysozyme) is used by the C. difficile Ribotyping Network (CDRN) for the isolation of C. difficile from faecal samples (5) on the basis of evidence suggesting the increased recovery of environmental spores treated with lysozyme (6). CCEYL is suitable for faecal C. difficile isolation due to the antibacterial actions against the normal microflora of cycloserine-cefoxitine. Brain Heart Infusion (BHI) agar with incorporation of taurocholate (a primary bile acid) and glycine in combination has been widely used to recover spores from pure culture . BHI is not suitable for the isolation of C. difficile from faecal samples; it is non-selective media allowing the growth of multiple organisms (7). The effect of bile acids on C. difficile spore

germination has been recognised since 1983, and these interactions are still the focus of much research (8-12). Subsequently, Sorg et al have further investigated glycine and taurocholate as stimulatory cogerminants (11) and have recorded the inhibitory nature of secondary bile acids on C. difficile spore germination (13, 14). More recently, Buffie et al (2015) have demonstrated the protective effect of C. scindens against C. difficile (15), due to the conversion of primary to secondary bile acids by 7α-hydroxylation. The inhibitory nature of secondary bile acids has subsequently been supported by in vitro work (16).

In summary, this study seeks to optimise the recovery of C. difficile spores on solid media.

1.2 Methods

1.2.1 Spore Production

Spores of five PCR ribotypes (RT 001,015,020, 027 & 078) of C. difficile were prepared as previously described (17). Spore stocks were enumerated by serial dilution in phosphate-buffered saline (PBS) in a 96-well plate and growth of 20µl aliquots of each dilution on to CCEYL agar.

All experiments were carried out in triplicate unless otherwise stated. All spores were fresh (<30 days old) unless otherwise stated. In all experiments agar plates were incubated anaerobically at 37℃ and counts of colony forming units (CFU) were carried out at 48 hours post-inoculation. Spore suspensions were vortexed vigorously and homogenised for 20 seconds prior to use.

1.2.2 Recovery of C. difficile spores on solid media

A range of C. difficile test recovery agars were prepared. BHI and CCEY were used as the agar bases and prepared according to the manufacturer's instructions with

89 additions as detailed in table 1. Taurocholate and glycine additions were made prior
90 to autoclaving, but lysozyme was added subsequently.

91 Spore suspensions of the five C. difficile PCR ribotypes were serially diluted (10-fold)
92 in PBS to 10^{-9} and 20µl of each dilution were spread on to a range of solid agar
93 plates (Table 1).

94

Media	Additional Additives
BHI / CCEY	Nil
BHI / CCEY	5% lysozyme
BHI / CCEY	0.1% taurocholate
BHI / CCEY	1% taurocholate
BHI / CCEY	0.1% taurocholate, 0.4% glycine
BHI / CCEY	1% taurocholate, 4% glycine
BHI / CCEY	1% taurocholate, 4% glycine

95 **Table 1. Solid agar plates utilised in C. difficile spore recovery experiments.**
96 **Media types and additives are shown.**
97

98 ### ~~1.2.3~~2.2.3 Minimum inhibitory concentration (MIC) testing

99 Glycine and taurocholate were tested both independently and in combination (4:1
100 ratio glycine:taurocholate) using an agar-incorporation minimum-inhibitory
101 concentration method, as previously described (18). Briefly, test compounds were
102 weighed out in doubling concentrations and added to individual aliquots of Wilkins-
103 Chalgren anaerobe agar or CCEY agar. CCEY agar was supplemented with 2%
104 lysed, defibrinated horse blood. Both spore and vegetative (1:10 dilution of 24 hour
105 Schaedler's broth culture) populations of the five C. difficile strains were inoculated
106 (~10^4 cells) on to glycine/taurocholate incorporated agar. Inhibition of growth was
107 assessed after anaerobic incubation at 37℃ for 48 hours, where the lo west

concentration at which visible C. difficile growth was inhibited was recorded as the MIC.

1.2.4 Broth Microassay of C. difficile inhibition by L-amino acids

BHI broths with increasing concentrations (1, 2, 3, 4%) glycine, L-histidine or L-phenylalanine) were prepared and 180ul aliquots distributed into a 96-well plate. Twenty microlitres of spore suspension (~ $5x10^5$ CFU/ml concentration) were aliquoted in to each well at time zero. Five strains of different ribotypes were utilised (001, 015, 020, 027, 078). Plates were incubated anaerobically at 37℃ for 48 hours. At 0, 24 and 48 hours absorbance readings at 595nm were determined (Tecan Infinite 200 Pro reader).Reads were carried out at 25℃ under 1atm of pressure. Negative controls were prepared for each concentration, and the absorbance for the blanks was subtracted from the absorbance of the inoculated wells to determine an accurate absorbance reading based on growth alone. All wells were prepared in triplicate.

1.2.5 Data analysis

Statistical analysis was carried out on IBM SPSS Statistics 22. Data normality was assessed using histograms and Kolmogorov-Smirnov tests. Levene's test was used for calculating homogeneity of variance. In both experiments, the variance between groups was significantly different ($P< 0.001$), hence Welch's ANOVA was utilised. Group means were compared using Welch's ANOVA with Games-Howell multiple comparisons. $\bar{x}$ represents the mean spore recovery of all five ribotypes used. All means are reported with standard error of the mean (SEM). P values <0.05 were considered significant, <0.01 highly significant and $P< 0.001$extremely highly significant.

1.3 Results

1.3.1 Recovery of C. difficile spores on solid media

Considerable variation in spore recovery was observed between the solid media used (Fig 1). In the absence of any additives, spore recovery was on average 1log CFU/mL greater for CCEY vs BHI (range = 0.1 – 2.4log_{10}CFU/ml) and was significantly different for all but the 078 strain ($P > 0.05$). Greatest spore recovery was observed for CCEY ($\overline{x}$ = 8.2 ± 0.03log_{10}CFU/ml) and taurocholate supplemented BHI ($\overline{x}$ = 8.3 ± 0.06log_{10}CFU/ml). The addition of lysozyme to either media appeared to have no substantial effect on the recovery of spores (CCEY $\overline{x}$ = 8.2 ± 0.03 vs 8.3 ± 0.04log_{10}CFU/ml, BHI $\overline{x}$ = 7.3 ± 0.18log_{10}CFU/ml vs 7.1 ± 0.20log_{10}CFU/ml) ($P > 0.05$). Spore recovery increased on average by 1logCFU/mL when BHI was supplemented with 0.1 or 1% taurocholate (range = 0 – 2.7log_{10}CFU/mL), but no equivalent increase was observed in CCEY. Strain dependent differences were also evident in regards to this increase, with the 001 strain showing a considerable increase in recovery in response to taurocholate (5.8 ± 0.13log_{10}CFU/ml vs 8.5 ± 0.08log_{10}CFU/ml) ($P<0.0001$), in contrast to the negligible difference in the 078 (8.0 ± 0.08log_{10}CFU/ml vs 8.0 ± 0.12log_{10}CFU/ml) ($P>0.05$). When glycine was incorporated into media alongside taurocholate, it had no substantial effect on spore recovery at 0.4 or 0.8% in BHI. Whereas recovery decreased by ~3log and ~6log_{10}CFU/mL in CCEY at the same concentrations. The 027 strain appeared to be recovered to a greater extent at these concentrations than the other strains. At the inclusion of the 4% glycine concentration, no spore recovery was seen on plates of either medium

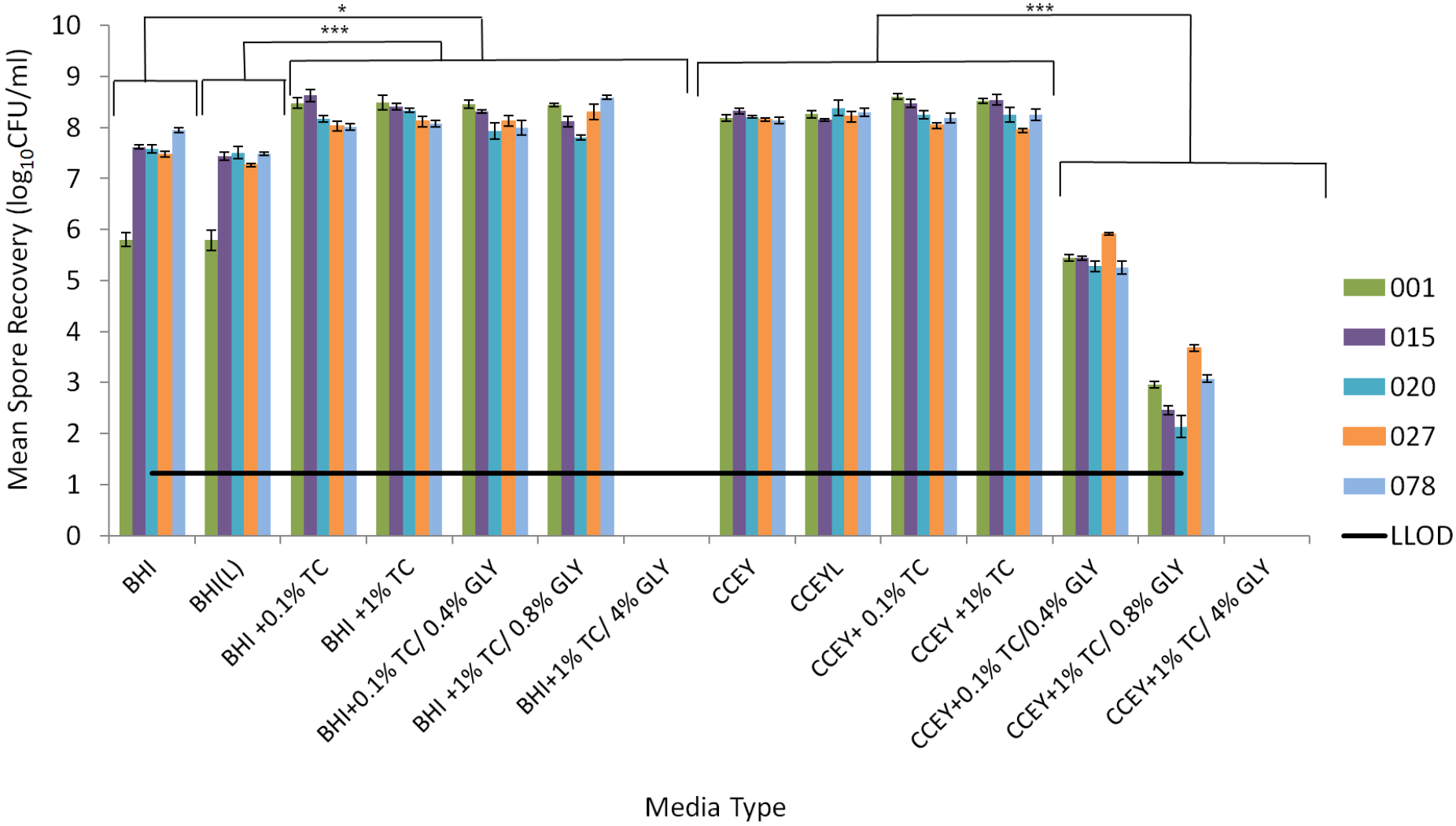

Figure 1. Mean (± SE) spore recovery (log_{10}CFU/ml) of spores of five C. difficile strains inoculated on to a variety of solid agars. Spores were serially diluted in PBS to 10^{-9} and each dilution spread on to agar plates in triplicate. Plates were read after 48h of anaerobic incubation. TC= taurocholate, GLY= glycine, L = lysozyme, LLOD = lower limit of detection

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1.3.2 MIC testing

The minimum inhibitory concentration of glycine for all five strains was 20g/L (2.0%) (Figure 2). The MIC was equivalent for both spore and vegetative C. difficile populations (only spore data shown).The inhibition was observed in glycine alone and in combination with taurocholate. No inhibition was seen in the presence of the highest concentration of taurocholate (10g/L) (data not shown).

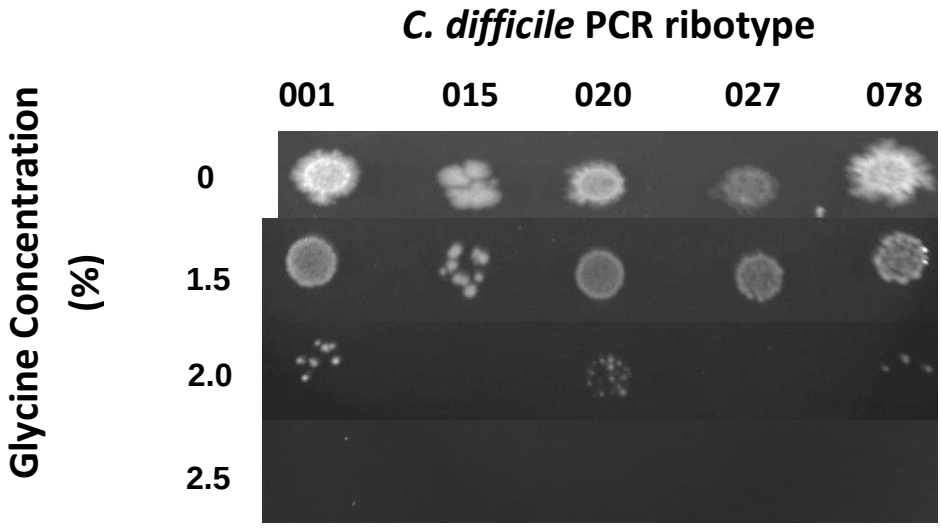

Figure 2. MIC testing of spores of five C. difficile strains against increasing concentrations of glycine (0, 1.5, 2.0 & 2.5%). Growth was substantially inhibited in all strains at a concentration of 2.0%.

1.3.3 Broth Microassay of C. difficile inhibition by L-amino acids

Glycine, L-phenylalanine and L-histidine completely inhibited growth in all five C. difficile ribotypes at higher concentrations (3%) (Figure 3). Glycine and L-phenylalanine appeared to inhibit growth to a lesser extent at lower concentrations (1% and 2% respectively) compared to L-histidine. At 48 hours, absorbance began to decrease for all strains (data not shown), indicating transition from stationary phase growth to sporulation/death phase.

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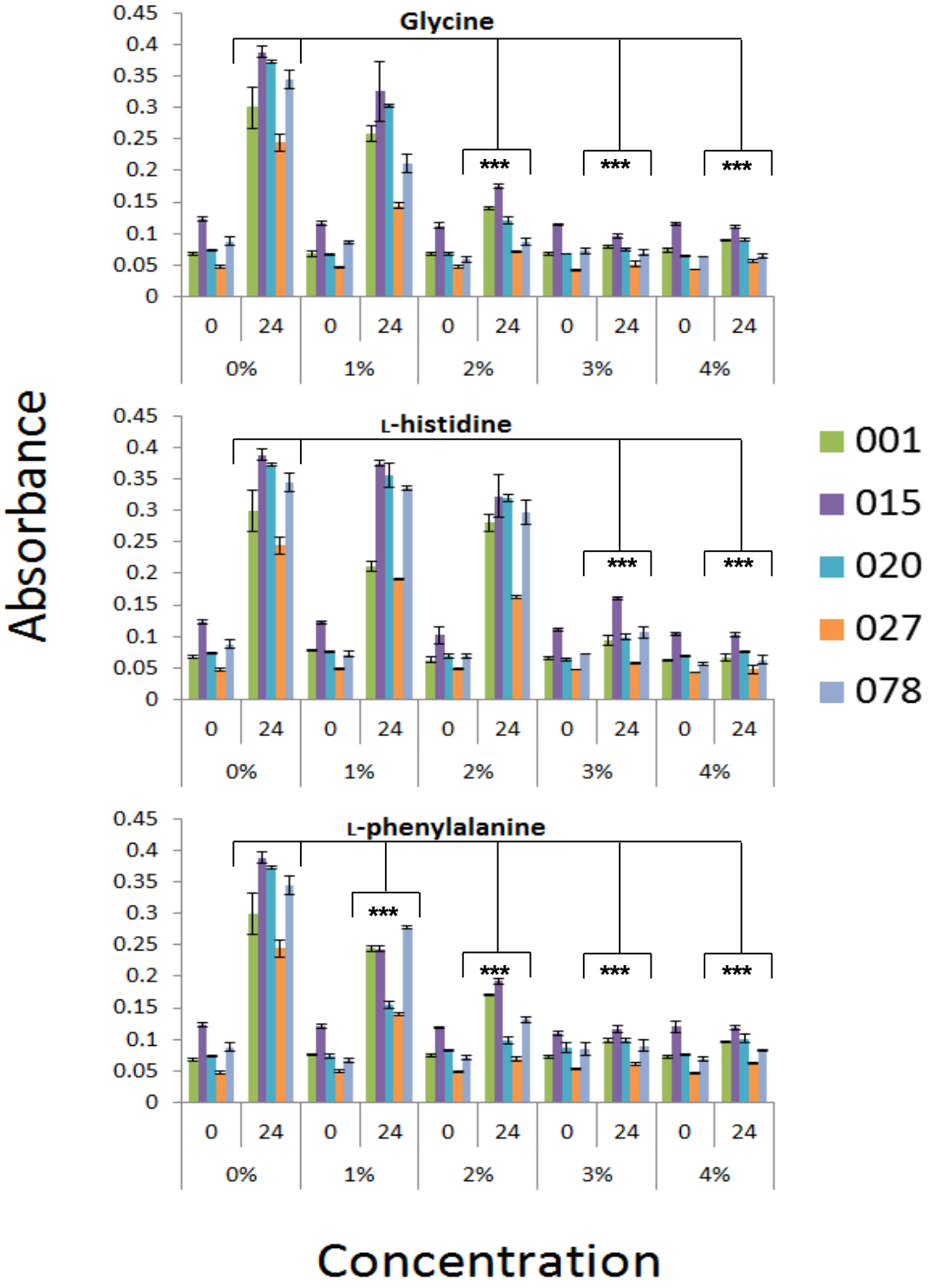

Figure 3. Mean (± SE) growth of five C. difficile strains in BHI with increasing concentrations (0, 1, 2, 3, & 4%) of 3 amino acids. Absorbance readings (595nm) shown are at 0 and 24 hours post-spore inoculation. At higher concentrations of amino acids, growth is inhibited.

1.4 Discussion

CCEY and taurocholate supplemented BHI are comparable for C. difficile recovery

We used an agar incorporation method to ascertain the optimal method for recovery of C. difficile spores. Two different solid media, CCEY and BHI were used, with various concentrations of additives (taurocholate, glycine and lysozyme). The response of the spores to the addition of the germinant taurocholate in our study was in accordance with the historical literature (9, 11). When taurocholate was added to BHI, spore recovery increased on average ~1$\log_{10}$CFU/mL. Lower concentrations of taurocholate were not tested but may be as effective as the 0.1%/1% used in the current study; 0.05% taurocholate has previously been investigated and found to be sufficient (19).

The addition of the cogerminant glycine at 0.4% did not increase the recovery of C. difficile spores further. However, BHI could provide a source of glycine without additional supplementation, as it is an undefined medium with unknown levels of amino acids. Therefore additional glycine supplementation may not be necessary. Taurocholate supplementation yielded no positive effect on spore recovery on CCEY. This is expected; spore germination may already be optimised in CCEY due to the presence of cholate, a stimulatory primary bile acid (10).

Most strikingly, when glycine supplementation was increased to a higher concentration (4%), spore recovery was completely inhibited to below the level of detection (1.2$\log_{10}$CFU/ml) on both CCEY and BHI. In CCEY, a linear concentration dependent reduction in spore recovery was observed in regard to glycine

supplementation. Even supplementation at the lowest concentration used in this study (0.4%) caused a ~3logCFU/mL drop in spore recovery on CCEY. This is in contrast to BHI, where the higher concentration of 0.8% glycine provided no impediment to recovery. BHI and CCEY are likely to contain differing levels of glycine; however, this cannot be verified as this is not detailed by the manufacturer. Finally, 5% lysozyme appeared to offer no additional benefit to spore recovery. Lysozyme has been historically been cited as a non- receptor based germinant (20, 21). Previously, research has shown increased recovery of lysozyme treated C. difficile spores from the environment (6).

The lack of effect seen in our work may reflect the different context in which lysozyme is used. In this study, lysozyme has been used to initiate germination in freshly produced non-stressed spores. Previous work has used lysozyme to recover spores from surfaces on hospital wards, where spore numbers are likely to be low. Environmental C. difficile spores are likely to be subjected to physical and chemical stresses such as detergents, UV light, desiccation and heat (6). This may potentially increase spore dormancy and reduce the rate of germination. It is possible the action of lysozyme increases germination of these ‘stressed’ spore populations, whereas the effect is unlikely to be observed in relatively newly produced spores. Previous work supports this hypothesis; lysozyme has been found to have no substantial effect on spore recovery when added to solid agar (19).

Our study demonstrates the comparability of some well used media in recovery C. difficile spores. CCEY or BHI supplemented with 0.1% taurocholate appear to be the most effective with the least amount of supplementation. BHI supplemented with 0.1% taurocholate is more cost effective (£0.15/plate) than CCEY (£0.25/plate); however it should noted these are estimated costs and there are a range of costs

dependent on supplier and supplement purity. BHI is also not appropriate for isolation of C. difficile from clinical specimens or mixed cultures due its non-selectivity(7).

Glycine and two L-amino acids appear to inhibit C. difficile vegetative growth

C. difficile was inhibited by increasing concentrations of glycine (2%) when grown on solid media. Both spores (data not shown) and vegetative cell inocula were used, and the effect observed was comparable in both cases. Both spore outgrowth and vegetative cell proliferation were inhibited in the presence of high concentrations of glycine (~2%) with ribotype variance likely due to natural variation. Differences in germination (22, 23), growth rates (24), sporulation (23, 25), and toxin production (26) between strains of different ribotypes have frequently been reported. Our observations indicate that growth resulting from both spore and vegetative inocula are inhibited by glycine suggesting a mechanism inhibiting the vegetative growth of C. difficile, rather than germination. This is not surprising; glycine has been identified as a cogerminant for C. difficile spores, with recent kinetic data suggesting a sequential binding of taurocholate and glycine to different receptors (27).

The inhibitory nature of glycine has been reported in other bacterial species (28). A recent study evaluating glycine as a replacement for sodium bicarbonate in dentistry found lower levels of bacterial colonisation on titanium appliances airbrushed with glycine (29). Synergism between glycine and penicillin has also been reported in H. pylori eradication (30). However, to the best of our knowledge, the glycine-mediated inhibition has not previously been reported C. difficile. It has been established that glycine could interfere with the formation of the bacterial cell wall, by replacing D-alanine residues in linking tetrapeptides and affecting downstream subunit cross-linkage of the developing peptidoglycan cell wall (31). D-amino acid enantiomers (D-

threonine, D-valine, D-leucine, D-methionine) have been found to have a similar effect on several species including E. coli (32, 33), following a similar mechanism to that proposed for glycine. Although D-amino acids are not ubiquitous in nature, they are increasingly being recognised as important in some bacterial regulatory processes, for instance in bacterial cell wall reorganisation in response to stress and biofilm formation (34). However, our study shows inhibition of C. difficile vegetative growth by not just glycine, but two L-amino acids; L- phenylalanine and L-histidine.

Given this discrepancy, it is unclear what the antibacterial mechanism of action is in C. difficile. It is possible that glycine may replace D-alanine in the bacterial cell wall, interfering with downstream cross-linkage of peptidoglycan by short-peptide chains. It seems unlikely that L-phenylalanine or L-histidine could do the same. However, Peltier et al. (2011)(35) recently described the unique nature of the C. difficile peptidoglycan cell wall in addition to the 4-> 3 cross links produced by D,D-transpeptidation in other bacteria, the peptidoglycan of C. difficile contains a predominance of 3->3 cross links catalysed by L,D-transpeptidation (35). In light of this amino acid substitutions may be possible.

In addition, recent evidence using Vibrio cholera and Bacillus subtilis has established that conversion of L-enantiomers to their D-isoforms could serve as a regulatory mechanism for cell wall remodelling and inhibiting growth in stationary phase cultures (36). Furthermore, the recent identification of an alanine-racemase in C. difficile that also accommodates serine as a substrate raises the possibility that other L-amino acids could be converted to their D-isoforms (37). Alternatively, a high concentration of D-amino acids could signal for vegetative cells to cease proliferation.

Although potential inhibitory mechanisms are described, it is possible that the effect observed is partly due to the hypertonicity of the solutions. This is likely to account for a degree of inhibition at higher concentrations of amino acids (3%+), as in the case of L-histidine. However, if the inhibition observed were solely due to the hypertonicity of the solutions, one would not necessarily expect differences in inhibition between the different amino acids utilised; inhibition was observed with 1% supplementation of L-phenylalanine In addition, the lowest concentration of glycine supplementation decreased spore recovery on CCEY solid agar. Hishinuma et al (1969) also found that the inhibition produced by 2% glycine was reversible upon addition of L-alanine. Considered together, these data suggest a unique mechanism of inhibition independent of solution hypertonicity (28).

The use of an undefined medium (BHI) is a limitation of the present study since the composition of amino acids is not known. Therefore, the concentrations stated in our broth microassay may not be accurate, and other amino acids may be present that could potentially influence C. difficile growth. The inhibitory concentrations obtained via agar-incorporated and broth microdilution methods were also different, however MIC discrepancies between broth microdilution and agar-incorporation methods have previously been reported (38). Despite these limitations, the results demonstrate a real antibacterial effect..

Previous research has highlighted that the majority of protein (90%) is enzymatically degraded and digested in the small intestines, with a small amount passing the terminal ileum (39). Converting previously reported values (40), free amino acids and peptides pass the terminal ileum at 0.2% and 0.7% respectively after ingestion of a 50g meal. These concentrations are below those utilised in the study, although ingestion of larger amounts of protein could increase free peptide concentrations to

therapeutic levels. The feasibility of utilising the described inhibitory effect in an in vivo setting is therefore unclear.

To summarise, we present data supporting the comparability of two commonly used media in optimising C. difficile recovery from prepared spore stocks. We also highlight the inhibitory nature of some amino acids, including glycine, on the vegetative growth of C. difficile. Future work should focus on the elucidation of a mechanism for this observed inhibition, and whether other amino acids have this inhibitory effect.

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