# Recovery of *Clostridioides difficile* in different enrichment culture media


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## Introduction and Objective

*Clostridioides difficile* (formerly *Clostridium difficile*) [1] is a spore-forming human pathogen that is the main cause of antibiotic- and hospital-associated diarrhoea [2]. In the past decade, there has been a dramatic change in the epidemiology *of C. difficile* infection (CDI), with the emergence and spread of new and hypervirulent strains and an increase in the incidence of community-acquired CDI, particularly in populations previously not considered at high risk [3]. Given the absence of clear explanations for so rapidly increased rates of CDI in recent years, a common vehicle of *C. difficile* spores dissemination such as the food products may not be excluded. To diagnose infection and contamination by *C. difficile*, enrichment in an appropriate culture medium is frequently required. Since there is still no standard methodology for isolation and/or enumeration of *C. difficile* in foods, different researchers have used different successfully developed methodologies applied for stool samples [4,5]. However, differences in methodologies compromise the comparison of data reported in different studies.

This study aimed to evaluate non-selective and selective enrichment culture media for the efficient recovery of *C. difficile* strains for further application to foods.

## Methods

### *Clostridioides difficile* strains and preparation of inoculum

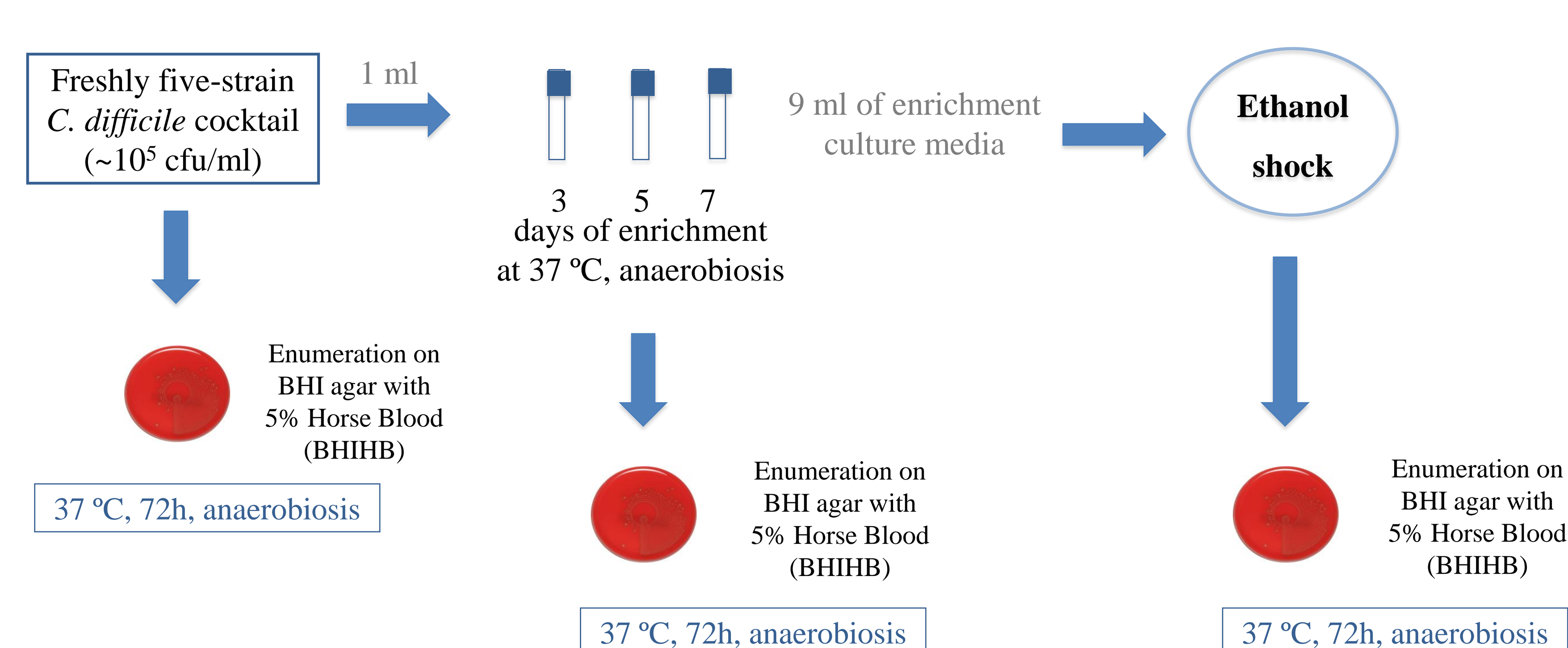
Five strains were used in this study: four gently provided by Hospital de S. Marcos, Braga (*C. difficile* H63866, *C. difficile* I805452, *C. difficile* I805937 and *C. difficile* V315638) and one from German collection of microorganisms and cell cultures (*C. difficile* DSMZ 1296). Prior to inoculation, each *C. difficile* strain was separately cultivated in Brain Heart Infusion broth (Biokar diagnostics, Beauvais, France) at 37 ºC for 24 h under anaerobic conditions. A freshly five-strain *C. difficile* cocktail was prepared by mixing an equal volume of each *C. difficile* culture, diluted with ¼ strength Ringer's solution to a final level of ~$10^5$ CFU/ml and plated on BHI agar plates with 5% (v/v) of horse blood defibrinated (BHIHB; Oxoid, Hampshire, United Kingdom) for enumeration and immediately used to inoculate 10 different enrichment media (Table 1).

**Table 1.** Enrichment culture media used in this study

| | Enrichment culture media |
|---|---|
| **1** | Base medium + *C. difficile* moxalactam norfloxacin (CDMN) |
| **2** | Base medium + *C. difficile* moxalactam norfloxacin + Sodium taurocholate (ST) |
| **3** | Brain heart infusion (Biokar diagnosis) + *C. difficile* moxalactam norfloxacin + Sodium taurocholate |
| **4** | Base medium + *C. difficile* selective supplement (SS) |
| **5** | Base medium + *C. difficile* selective supplement + Sodium taurocholate |
| **6** | Brain heart infusion + *C. difficile* selective supplement + Sodium taurocholate |
| **7** | Mannitol broth + *C. difficile* selective supplement + Sodium taurocholate |
| **8** | Brain heart infusion + Sodium taurocholate |
| **9** | Cooked meat (Lab M, Bury, UK) + Sodium taurocholate |
| **10** | Brain heart infusion + Yeast extract (YE) + Cysteine hydrochloride (CH) + Sodium taurocholate |

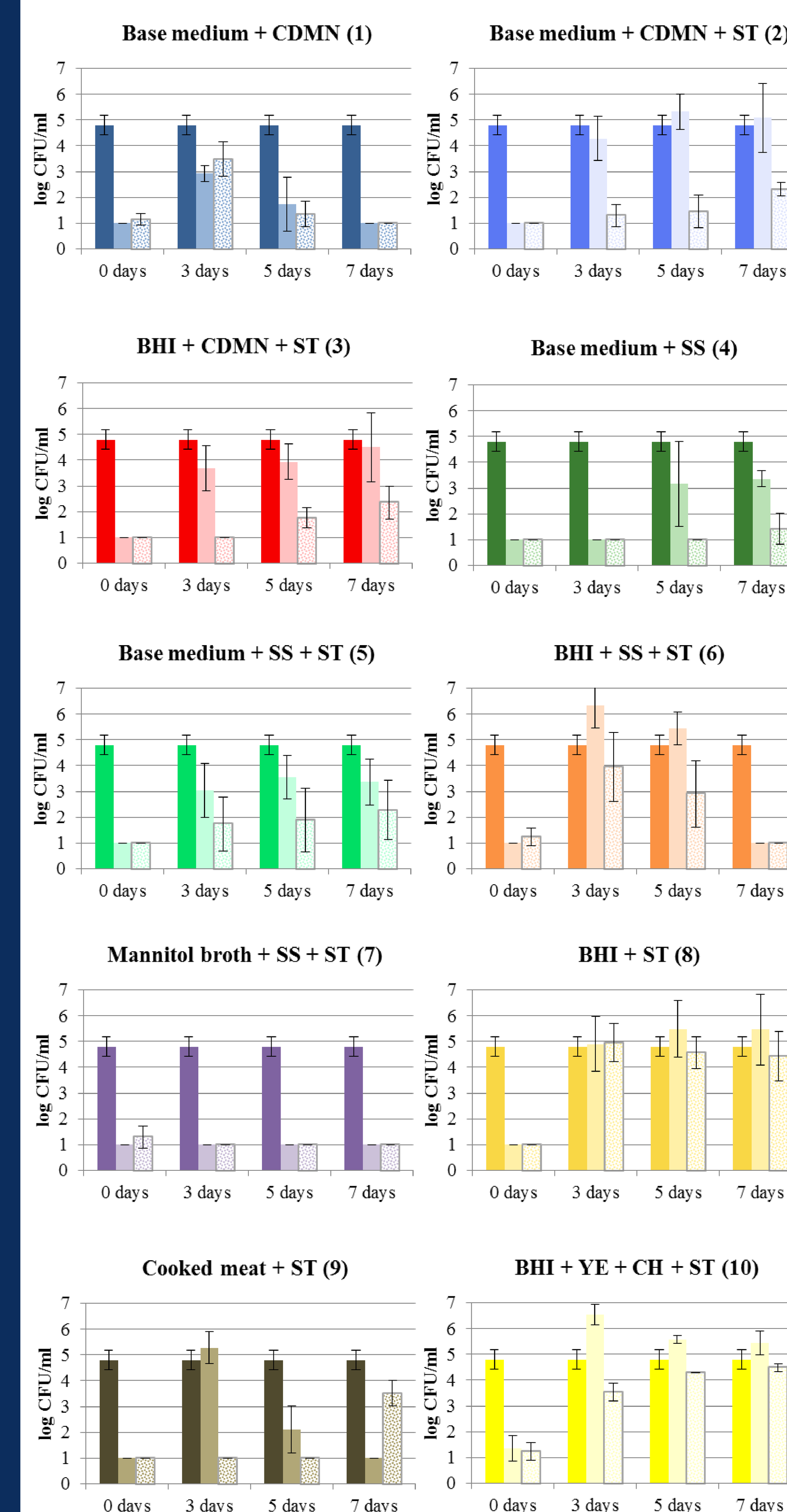
Base medium: proteose peptone (40 g/l), fructose (6 g/l), disodium hydrogen phosphate (5 g/l), sodium chloride (2 g/l), potassium dihydrogen phosphate (1 g/l) and magnesium sulfate (0.1 g/l). Mannitol broth: peptone (3 g/l), yeast extract (5 g/l) and mannitol (25 g/l). Solutions of sodium taurocholate (**ST**, ThermoFisher, Kandel, Germany) and cysteine hydrochloride (AppliChem, Darmstadt, Germany) were prepared, filtered and added to a final concentration of 0.1% (v/v) and 0.05% (v/v), respectively, in each corresponding culture media. *Clostridium difficile* moxalactam norfloxacin selective supplement solution (**CDMN**, Oxoid) was added in each culture media in order to obtain a final concentration of 0.032 mg/ml moxalactam, 0.012 mg/ml norfloxacin and 0.05 mg/ml cysteine hydrochloride. *Clostridium difficile-selective* supplement solution (**SS**, Oxoid) was added in each culture media in order to obtain a final concentration of 0.25 mg/ml D-cycloserine and 0.008 mg/ml cefoxitin.

### Recovery of *C. difficile* in each enrichment media




## Results

The graphs in the following figure represent the results obtained after the incubation of a *C. difficile* cocktail in 10 different enrichment culture media. Each graph represents a culture medium, with log CFU/ml obtained after counting with and without prior ethanolic shock.




- For day 0, contrary to what happens with most aerobic bacteria, immediately after placing *C. difficile* cocktail in each enrichment media, no *C. difficile* cells were recovered. This may be due to the fact that the cells were fragile and did not have time to recover in an enriched culture media.
- Comparing enrichment media 1 and 2, which consist of the same base culture medium differing only in the presence of sodium taurocholate (to increase germination), it is possible to verify that the presence of sodium taurocholate allowed an increase in the *C. difficile* recovery over time. In contrast, there was a marked decrease in the number of cells when recovery was done in the absence of sodium taurocholate. For the enrichment medium 3, BHI with sodium taurocholate, the same trend was observed over time as in the culture base medium with sodium taurocholate, although the number of recovered cells had been lower.
- Regarding the results obtained for the enrichment media 4, 5 and 6, which were similar to the enrichment media 1, 2 and 3, respectively, but with the selective agents cycloserine and cefoxitin, the BHI medium (6) seems to allow a better recovery in comparison to the culture base medium, at least until the 5th day of incubation.
- The enrichment medium 7 was the only culture medium for which no recovery of *C. difficile* occurred after each day of incubation.
- The recommended enrichment medium for the cultivation of *Clostridium* species, cooked meat medium (medium 9), only allowed a good recovery after 3 days of incubation. After that period, the number of recovered cells gradually deacreased until they were not detected.
- The enrichment medium 8, also BHI with sodium taurocholate, but without selective agents, also allowed a good recovery. Somehow, this was an expected result, since on the one hand sodium taurocholate increase germination and for another, the absence of selective agents allowed better recovery *C. difficile* cells. Finally, the enrichment medium 10, with the same composition but with cysteine hydrochloride (reduce the redox potential and protect cells against oxidative stress) and also with yeast extract (growth promoter), allowed a greater recovery after 3 days, but overall the results were very similar to those obtained for the enrichment medium 8.
- Ethanol shock is often used when detecting *C. difficile* in food. It is a recommended technique, because it eliminates the contaminating microbiota, stimulating the germination of *C. difficile* spores. However, there seems to be no direct relationship with the use of ethanolic shock and the increase in the number of *C. difficile*. With the exception of the enrichment medium 9, where there was an increase in the number of *C. difficile* at the 7th day of incubation, no differences were found for any of the other enrichment media.

**Figure 1.** Growth of *Clostridioides difficile* in each enrichment media immediately inoculated with a five-strain cocktail of *C. difficile* (0 days) and after 3, 5 and 7 days of anaerobic incubation at 37 ºC (initial inoculum ( ); enriched *C. difficile* culture ( ) and enriched *C. difficile* culture prior ethanolic shock ( )). Detection limit of the enumeration technique was 1 log CFU/ml and the counts of the initial inoculum were shown in all graphs and for each day of incubation to facilitate comparison of the results.

## Conclusion and Relevance

Since *C. difficile* is a human pathogen and as several studies have reported its presence in foods, more studies are necessary in order to define an appropriate methodology which could, ideally, become standardized.

With these preliminary results, the next step will be the simulation of real scenarios, evaluating the enrichment culture media which allow the best recovery of *C. difficile* from different artificially contaminated food matrices.

## References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