Manufacture of *Clostridioides difficile* spores for experimental infection of human volunteers

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Abstract

Background: Infections with *C. difficile* remain a significant global healthcare problem for which novel (preventive) treatment strategies are urgently needed. Controlled human infection models (CHIMs) can contribute to a better understanding of the disease and accelerate (novel) medicinal product development.

Methods: A robust production process for *C. difficile* spores for experimental human administration, was implemented. Direct-release capsules containing viable *C. difficile* spores were formulated. The manufacturing process aligns with good manufacturing practices (GMP) guidelines, including quality controls and release by a qualified person (QP).

Results: Selected non-toxigenic and toxigenic *C. difficile* strains were used to create a master cell bank and working cell bank from which multiple batches of purified active substance were produced. Substance was then used to manufacture the final active product. The active product and its intermediate products passed all quality controls for identity, potency, purity and safety, and were individually released by the QP. Stability of active substance and product is confirmed up to 12 months.

Conclusion: Although there are no clear standardized international guidelines for challenge material production, we demonstrate that it is feasible to produce *C. difficile* human challenge material for small scale application in CHIM trials. This application of GMP principles to an unconventional production process of a bacterial spore-forming anaerobic challenge agent is an example for the production of challenge material as described in the auxiliary medicinal product guidelines of EMA and FDA.

Keywords: controlled human infection model, CHIM, *Clostridioides difficile*, *C. difficile*, live biotherapeutic product, challenge material, auxiliary medicinal products, GMP, capsule

41 **Background**

42 *Clostridioides difficile* is the leading cause of healthcare-associated diarrhoea, causing substantial
43 morbidity and mortality. Globally it is responsible for an estimated 15,600 deaths and 284,000
44 disability adjusted life years (DALYs), representing the primary cause of diarrhoea-related mortality
45 in high-income countries (1). Concerningly, the incidence of community-acquired *C. difficile*
46 infections (CDI) (defined as disease in patients not hospitalized during the 12 weeks prior to
47 diagnosis) is on the rise, almost doubling the past years (2). As such CDI causes a substantial clinical,
48 social and economic burden (3).

49 An acute episode of CDI is treated with antimicrobial therapy, but alternative therapies are
50 urgently needed because of high relapse rates (approximately 20% after treatment of an initial
51 episode and up to 60% after multiple recurrences) and a rise in antimicrobial resistance (4).
52 Microbial restoration therapies, such as faecal microbiota transplantation (FMT) seem to deliver on
53 the promise of high level sustained prevention of recurrent CDI (5-7). However, the complex
54 interactions between *C. difficile* and the human microbiome remains largely poorly understood,
55 hampering the translation of FMT into a medicinal product. Additionally, animal models cannot fully
56 replicate the human microbiome and its interactions, making preclinical down selection and
57 optimization for novel medicinal products complex. Innovations to accelerate such processes are
58 thus needed.

59 Controlled human infection models (CHIMs), in which human volunteers are deliberately
60 inoculated with an infectious organism (so-called "challenge agent") under controlled conditions, are
61 a valuable tool to rapidly evaluate medicinal products (e.g. vaccines) for efficacy in early phase
62 clinical development (8, 9). CHIMs have been developed for a wide variety of pathogens (including
63 parasites, viruses and bacteria) over the past 200 years and CHIM experiments have even led to the
64 registration of a novel cholera vaccine (10). The development of a *C. difficile* CHIM, in which healthy
65 adult volunteers are experimentally exposed to *C. difficile* spores in a controlled clinical
66 environment, would provide the opportunity to identify microbiota and immunological targets for

67 novel (preventive) medicinal products, and ultimately test the preliminary efficacy of such products
68 (e.g. vaccines or microbial restoration therapies).
69 The development of a *C. difficile* CHIM starts with the production of *C. difficile* challenge
70 material. To ensure safety of CHIM participants a challenge agent which complies with regulatory
71 requirements for human use must be available. Within the EU, challenge agents are classified as
72 auxiliary medicinal products (AxMPs) by the European Medicines Agency (EMA), defined as a
73 medicinal product used for the needs of a clinical trial as described in the protocol, and should be
74 manufactured according to Good Manufacturing Practice (GMP) equivalent standards. GMP
75 guidelines describe the minimum requirements which medicinal products must meet in their
76 manufacturing process to ensure that the medicinal product is 1) of consistent high quality 2)
77 appropriate for their intended use and 3) meets the requirements of the marketing or clinical trial
78 authorization (11).
79 Aligning the manufacturing process of *C. difficile* challenge material with GMP guidelines,
80 presents several key challenges, particularly in meeting the quality attributes of identity, purity,
81 potency and safety, as outlined in the technical white paper on the quality considerations for
82 challenge agent production for use in CHIM studies (9). Here, we addressed these challenges and
83 describe a robust manufacturing process for challenge material derived from cultures of *C. difficile*,
84 performed in an academic laboratory by trained personnel with an appropriate quality control
85 strategy. The described process for producing encapsulated viable *C. difficile* spores serves as an
86 example of the production of auxiliary medicinal products under the European Medicines Agency
87 (EMA) or U.S. Food and Drug Administration (FDA) regulations.
88

Methods

Manufacturing a *C. difficile* challenge product

Production was performed at a dedicated laboratory for *C. difficile* production of the Experimental Bacteriology research group of the Leiden University Center for Infectious Diseases in the Leiden University Medical Center (LUMC) under the quality management system (QMS) of LUMC's department of Clinical Pharmacy and Toxicology (KFT) and Center for Cell and Gene Therapy (CCG). The production process of *C. difficile* spores has been established using known and established methods and protocols at the LUMC (12).

The manufacturing process was developed to be adaptable to diverse *C. difficile* strains, including production of non-toxigenic as well as toxigenic *C. difficile* strains. Here, the process to produce challenge material for the non-toxigenic *C. difficile* strain L-NTCD03 (PCR ribotype 416, ST39, clade 4, complete genome GenBank NZ_OX637968.1) and the toxigenic *C. difficile* strain L-TCD-01 (PCR ribotype 020, ST2, clade 1, complete genome GenBank NZ_OY731254.1) is described. Spores were produced in a dedicated anaerobic isolator cabinet (Whitley A135GMP, Don Whitley Scientific, Bingley, UK) in a restricted-access biosafety level 2 laboratory, using food-grade culture media (Soy Peptone A3X (SA3X), Organotechnie, La Courneuve, France) in batch fermentations in sterile single-use polyethylene terephthalate glycol-modified (PETG)-flasks (Sterile Disposable PETG Flask 2000 ml, Thermo Scientific, Rochester, US). Food-grade SA3X was selected as the production medium based on its absence of animal-derived components and its demonstrated capacity for robust growth and high spore yields (data not shown). Spores were purified using aerobic washing with phosphate-buffered saline (PBS) (Fresenius Kabi, Graz, Austria) supplemented with 0.1% Tween 80 (Polysorbatum 80, Duchefa, Haarlem, The Netherlands). Dose-adjusted aliquots (10^4 and 10^7 colony forming units (CFU) *C. difficile spores* in buffered 70/30% glycerol (Duchefa, Haarlem, The Netherlands) and PBS solution) were filled into liquid-filled direct-release capsules (Lonza Vcaps® Plus Capsules size 0, Lonza, Colmar, France) using an autoclavable capsule filling system (ProFiller1100, Torpac, Heerlen, The Netherlands). A detailed risk assessment of the *C. difficile*

115 manufacture process and impurities has been conducted. All raw materials used in the
116 manufacturing process are released for their use by a Quality Control (QC) officer. The qualification
117 of all compendial and non-compendial reagents are listed in **Supplementary Table 1**. Non-
118 compendial reagents are qualified based on vendor Certificate of Analysis (CoA) and meet pre-
119 defined acceptance criteria (**Supplementary Table 2 and 3**). The production process was formalized
120 in Standard Operating Procedures (SOPs), authorized by QC, QP and Head of Production.
121 Quality and in-process controls (QC and IPCs) are described further below and are based on
122 well-established protocols of the Dutch National Expertise Center for *C. difficile* infections (13-15) as
123 well as guidance documents of the European Centers for Disease Control and Prevention (12) and
124 the European Society for Clinical Microbiology and Infectious Diseases (16, 17). All IPCs and QCs
125 were validated or qualified for their intended use. Where possible, IPCs and QCs were performed
126 according to GMP requirements or, if not possible, under ISO15189 standards at the clinical
127 microbiology laboratory of the LUMC. If during culture steps a QC analysis was required, this was
128 performed in a Whitley A35 anaerobic cabinet (Don Whitley Scientific). All QC test results were
129 independently reviewed by a QC officer, and thereafter the master cell bank, working cell bank,
130 active substance and active product were released by a Qualified Person (QP). To verify the
131 robustness of the manufacturing process ≥3 preclinical test runs were performed per challenge
132 strain.
133 A product dossier was created according to EMA/CHMP/BWP/534898/2008 Rev. 2. for
134 pharmacological products. All raw materials, disposables and excipients were quarantined until
135 release was done by a QC officer.
136 The product was characterized as an auxiliary medicinal product (regulation EU No 536/2014
137 (CTR)). The principles of GMP as outlined in Regulation (EU) 536/2014 along with the white paper
138 "Considerations on the Principles of Development and Manufacturing Qualities of Challenge Agents
139 for Use in Human Infection Models" (9) were applied to the production process wherever possible
140 and audited as such.

141 **Results**

142 Master cell bank (MCB) and working cell bank (WCB)

143 The *C. difficile* challenge strains were selected based on considerations previously published
144 (18). The NTCD strain was isolated from faecal material of an asymptomatic nursing-home resident
145 and the TCD strain from faecal material of a symptomatic patient with uncomplicated CDI disease.
146 The selected isolated challenge strains were used to form a master cell bank (MCB), the starting
147 material for the production of the active product (**Figure 1**). MCB has been prepared under good
148 laboratory practice (GLP) conditions according to the SOPs of the Dutch National Expertise Center for
149 *C. difficile* infections, which is an ISO-15189 certified laboratory. In short, the MCB was created
150 through enrichment culture in CDEB MOD (*C. difficile* enrichment modified broth, Mediaproducts
151 BV, Groningen, The Netherlands), inoculated onto CLO plates (*Clostridioides difficile* selective agar
152 plates, bioMérieux, Marcy-l'Étoile, France), followed by re-culturing on a tryptic soy sheep blood
153 (TSS) plate (bioMérieux, Marcy-l'Étoile, France), after which a colony was transferred to glycerol for
154 storage at -80°C.

155 To produce the working cell bank (WCB) intermediate product (**Figure 1**), MCB was thawed
156 and plated out on a SA3X plate for expansion of *C. difficile*. After incubation of the plate, colonies
157 were picked and subsequently the WCB was stocked in a glycerol/SA3X solution and stored in a -
158 80°C freezer.

159 The MCB and WCB were released for further processing after undergoing successful quality
160 control testing for confirmation of identity, potency, purity and safety of the products and passing all
161 acceptance criteria (**Table 1**). NGS testing additionally showed stability of the challenge strain
162 genome, with no evidence of laboratory-driven adaptation of the original strain (data not shown).

163

164 Active substance (AS)

165 To produce purified spores in solution (**Figure 1**), which constitute the active substance (AS), the
166 WCB was recovered on a SA3X agar plate and re-streaked on a fresh SA3X plate to increase cell yield.
167 Subsequently, a starter culture was generated in liquid SA3X medium under anaerobic conditions
168 using the anaerobic isolator cabinet. The optical density (OD) of the cultures was then measured and
169 diluted in fresh SA3X medium to a starting OD of 0.05. The diluted cultures were incubated in
170 shaking Erlenmeyer flasks for 14 days to produce spores. In the production medium, spore yield
171 increases during the first week (from 0 CFU/mL to $>10^6$/mL); a 14-day time frame was selected to
172 ensure maximum reproducibility of spore yield. We found no significant difference in spore yield
173 between different culture volumes up to a liter-scale, demonstrating robustness of the method (data
174 not shown). After 14 days, cells were pelleted by centrifugation and washed 5 times with PBS/0.1%
175 Tween 80 to remove residual vegetative cell debris. All batches of the active substance yielded a
176 high spore count (**Figure 2**), meeting the acceptance criterion for spore count ($>1.6x10^6$ spores/mL).
177 Active substance was stored at -80°C.

178 The active substance was released for further processing after undergoing successful quality
179 control testing and passing all acceptance criteria as indicated in **Table 1**.

180 Active Product (AP)

181 The active product consists of a 500-µl suspension containing 10^4 or 10^7 CFU *C. difficile*
182 spores formulated in buffered 70/30% (v/v) glycerol and PBS encapsulated in liquid-filled direct-
183 release capsules for oral administration. The dosing of 10^4 and 10^7 CFU spores were selected based
184 on previously published experimental NTCD studies (19, 20).

185 To produce the active product, active substance was thawed and adjusted to the right
186 concentration in formulation buffer after which 500-µl volumes were dispensed in liquid-filled
187 direct-release Lonza Vcaps®. Capsules were stored at room temperature (15-25°C) and quarantined
188 until release.

189 The active product was checked for identity, potency, purity and safety, and released after
190 passing all acceptance criteria (**Table 1**).

191

192 Stability of Active Product

193 Stability testing was performed on the active substance and active product at multiple
194 timepoints according to a predefined stability plan (1, 3, 6, 12 and 24 months). Stability testing
195 consists of: 1) identity testing by *gluD* PCR and ribotyping PCR 2) potency testing by spore
196 enumeration (**Figure 3**) 3) purity testing by visual inspection of colonies on a TSS plate, colorimetric
197 measurement of protein and DNA content and aerobic plating and 4) capsule controls by visual
198 inspection of capsule integrity and measuring fill weight and volume (**Table 1**).

199 We observed stability, with limited or no decrease in plating efficiency over a period of 12
200 months so far for the NTCD active product, and up to 6 months for the TCD active product (**Figure 3**).
201 All other stability tests also met the predefined criteria (**Table 1**) at these timepoints. Stability of the
202 active substance aligns with the stability observed of the active product. The observed stability at
203 multiple timepoints across different production batches confirms robustness of stability across the
204 batches. The expiry date of the (N)TCD active product is adjusted to the stability data. Notably,
205 stability of the TCD product to date strongly resembles the NTCD product, suggesting a similar

206 stability profile. Based on these observations, we expect that the actual shelf life of the challenge
207 product extends well beyond the current stability data. Together this demonstrates that the active
208 product has a sufficiently long shelf life (stability) to enable its use in challenge studies following
209 production.

210 **In-process controls and quality controls**

211 The in-process controls (IPCs) and quality controls (QCs) performed to ensure identity, potency,
212 purity and safety during the production process of *C. difficile* challenge material are scheduled in
213 **Figure 1** and **Table 1**. A short description of the tests is given below.

214

215 *Identity*

216 *Species PCR.* Confirmation of the presence of cells/spores belonging to the species *C. difficile*
217 was obtained by performing a *gluD* PCR on isolated total DNA. The *gluD* PCR contains highly specific
218 primers which allow amplification of a 158-bp fragment of glutamate dehydrogenase gene (*gluD*
219 gene), after which the fragment can be detected using a specific *gluD* probe in a real time PCR assay
220 (12).

221 *Ribotyping.* To identify and confirm the PCR ribotype of the *C. difficile* challenge material
222 capillary ribotyping PCR was performed, in which the 16S-23S intergenic spacer regions on the *C.*
223 *difficile* chromosome are amplified by PCR and visualized on a capillary gel electrophoresis system
224 (14). The resulting banding pattern is matched to a library of reference patterns, using BioNumerics
225 7.6 software (bioMérieux, Marcy-l'Étoile, France).

226 *Complete genome sequencing.* A reference sequence for the challenge strains was generated
227 using long-read circular consensus sequencing (Pacific Bioscience Sequel II) and automated assembly
228 using established protocols (21, 22). Reference assembly of the 150-bp paired-end Illumina reads of
229 this sequence revealed no single nucleotide polymorphisms. Analysis of the complete genome
230 sequence allowed for assessment of genome characteristics such as the presence or absence of
231 specific toxin and antimicrobial resistance genes.

232 *Short read sequencing.* Short-read next generation sequencing (Illumina Miniseq, NextSeq
233 500 or Novaseq Xplus) was additionally performed on the working bank and active substance; the
234 results of a core genome analysis using Ridom SeqSphere+ (Ridom GmbH, Münster, Germany)

235 confirmed that the clade (clonal complex, CC) and complex type (CT) of the strain matches the
236 reference genome sequence.

237 *Potency*

238 *Total cell count.* To enumerate viable bacterial cells in the master and working cell bank,
239 serial dilutions were cultured on BHI (brain heart infusion) plates. The total cell count is based on the
240 geometric mean minus two times standard deviation of log transformed values obtained from the
241 colony forming unit (CFU) enumeration.

242 *Total spore count.* The quantity of viable spores was assessed by plating out serial dilutions
243 of the product onto BHI plates supplemented with 0.1% of the germinant sodium taurocholate
244 (Thermo Fisher Scientific, Lancashire, UK) and enumerating the CFUs after 48h. The total spore count
245 is based on the geometric mean minus two times standard deviation of log transformed values
246 obtained from the CFU enumeration. Production yield of active substance was targeted at > 1.6×10^6
247 spores/mL , but generally produced >10^7 spores/mL. In our studies, the active product was
248 formulated in capsules with a targeted dose of 10^4 or 10^7 spores.

249

250 *Purity*

251 *Visual appearance of colonies.* Routinely, *C. difficile* cells or spores were anaerobically
252 cultured on a TSS plate for 72h. Plates were visually inspected and all colonies on the plate should
253 macroscopically be morphologically consistent with *C. difficile*, which required confirmation by a
254 second person.

255 *Contamination with aerobic bacteria.* To confirm absence (<10 CFU/mL) of aerobic bacterial
256 contaminants, routine aerobic culture was performed on non-selective TSS agar plates.

257 *Measuring protein and DNA content.* To quantify remaining vegetative *C. difficile* cell remnants, e.g.
258 free protein or DNA, in the active substance and product, colorimetric measurements (Qubit dsDNA
259 HS Assay and Qubit Protein Assay, LifeTechnologies, Eugene, Oregon, US) were performed. The
260 limits set at ~ 10-fold the minimum value observed after repeated washing (please refer to **Table 1**).

261 *Safety*

262 *Presence of toxin gene.* A multiplex PCR for genes encoding toxin A (*tcdA*), toxin B (*tcdB*) and
263 binary toxin (*cdtA* and *cdtB*) genes was performed on total DNA extracted from the samples. As a
264 DNA isolation control, the 16S rRNA and *gluD* gene were also tested, which should show a positive
265 result.

266 *Antimicrobial susceptibility.* To ensure susceptibility to antibiotics which may be used for
267 rescue treatment (fidaxomicin, vancomycin and metronidazole), antimicrobial susceptibility tests are
268 performed. Susceptibility for fidaxomicin (Selleckchem, Köln, Germany) was tested using the agar
269 dilution method (23) whereas for vancomycin and metronidazole E-tests (bioMérieux, Marcy-l'Étoile,
270 France) were used. Breakpoints are defined based on EUCAST criteria (24).

271 *Microbial contaminants of the master cell bank.* To test for contaminants with
272 gastrointestinal pathogens, total DNA from the master cell bank was tested by qPCR for the
273 presence of gastrointestinal pathogens, i.e. the bacterial pathogens *Salmonella, Shigella,*
274 *Campylobacter, Yersinia*, *Aeromonas* and *Plesiomonas shigelloides spp*. and the viral pathogens
275 adenovirus, norovirus, rotavirus, sapovirus and astrovirus.

276 *Next-generation sequencing (NGS) for bacterial adventitious agents.* An additional
277 metagenomic analysis was performed to identify possible contaminants in a non-targeted manner.
278 Short-reads from the Illumina sequencing (see above) were analysed through the GenomeDetective
279 tool (EmWeb bv, Herent, Belgium). This analysis classifies reads to bacterial species and allows to
280 confirm both identity (assignment: *Clostridioides difficile*) and assess the level of selected bacterial
281 pathogens (25). As metagenomic analysis is prone to result in spurious identifications (for instance
282 because of DNA segments that are shared between bacterial species), acceptance criteria were
283 validated on the basis of analysis of simulated reads derived from reference genome sequence.

284

285 *Capsule controls*

286 *Capsule integrity.* Capsules from the beginning, middle and end of the manufacturing
287 process were inspected for integrity by visually inspecting the capsules: both capsule parts should be
288 aligned, show no deformations and no liquid should be leaking out when tilted.
289 *Fill weight and volume.* The fill weight of the active product capsules is measured on an
290 analytical balance; each capsule is filled with 500ul which should align with a weight of 700mg +/- 20
291 gram.

292 **Conclusion and Discussion**

293 To summarize, we demonstrate feasibility to produce *C. difficile* challenge material for use in CHIM
294 studies at small scale aligning to GMP principles including appropriate quality controls and release.
295 This application of GMP principles to an unconventional production process of a bacterial spore-
296 forming anaerobic challenge agent is a representation for the production of bacterial challenge
297 agents as described in the auxiliary medicinal product guidelines of EMA and FDA.

298 Challenge material is not classified as a medicinal product and specific international challenge
299 material production guidelines are lacking. The EMA identifies challenge agents as auxiliary
300 medicinal products (AxMPs) and states that when the AxMP is not authorized it shall be
301 manufactured according to GMP *or* at least an equivalent standard (26). In the United Kingdom, the
302 manufacturing of challenge agents is not regulated. In the US the challenge agent is considered a
303 biological product and is subject to regulation under federal law by the FDA. The challenge agent
304 must therefore 1) be manufactured under GMP conditions where possible, 2) satisfy the FDA
305 regulations of safety, purity and potency and 3) have detailed information on the provenance and
306 manufacture as part of the required Investigational New Drug Application (IND).

307 To ensure the quality attributes of identity and safety NGS was applied for full characterization
308 of the challenge strain. Additionally, applying NGS for the detection of adventitious agents enhances
309 the QC testing of challenge material facilitating the identification of potential other bacterial
310 pathogens in the active product beyond *Ph. Eur.* requirements. To safeguard the level of
311 contamination with adventitious agents, acceptance criteria must be predefined. This can be
312 challenging, as metagenomic analysis is prone to spurious identifications (e.g. due to DNA segments
313 shared between bacterial species). This is of particular concern for *C. difficile* spore challenge
314 material, as DNA isolation from spores is difficult and typically yields low-biomass DNA samples. We
315 established acceptance criteria for detecting adventitious agents through NGS by analysing
316 simulated reads generated from the reference genome using a well-characterized pipeline, resulting

317 in a cut-off of fewer than 0.3% of mapped reads aligning to pathogenic species. Such criteria may
318 require adaptation for other species. In the case of spurious NGS identifications, a quantitative 16S
319 micelle PCR (27) can provide additional value by enabling both the identification and quantification
320 of the bacterial composition.

321 Secondly, to ensure the quality attribute of safety, food-grade media and GMP-grade raw
322 materials– formulated to avoid the use of animal-derived products where possible, thereby
323 mitigating the risk of potentially important diseases such as transmissible spongiform
324 encephalopathies (TSE) - are required during challenge material production. However, the culture
325 media routinely used for *C. difficile* growth and sporulation contain animal-derived constituents, and
326 conventional spore purification techniques involve reagents that are not indicated for use in a spore
327 production process or for oral ingestion (e.g. non-ionic X-ray contrast agents). Therefore, during the
328 manufacturing process development, the normally used brain heart infusion (BHI) medium was
329 replaced by a soy peptone (SA3X) medium and for spore purification washing with PBS-0.1% Tween
330 was established. Moreover, the dedicated manufacturing area was adapted to include appropriate
331 control measures, equipment validation and a cleaning protocol to prevent cross-contamination.
332 Although GMP guidelines may stipulate production in a controlled environment (e.g. a clean room),
333 the inherent resistance and persistence of *C. difficile* spores present significant challenges for
334 decontaminating such facilities. Notably, the guidance document on the production of challenge
335 agents (9) states that a clean room is not mandatory, but the manufacturing area should be a part of
336 the contamination control strategy and the level of environmental control for particulate and
337 microbial contamination should be tailored to the manufacture of the challenge agent and
338 production step, considering the potential level of contamination of starting materials and the risks
339 for the final batch of the challenge agent. Since *C. difficile* requires anaerobic conditions during
340 production, an anaerobic isolator cabinet was needed as the manufacturing area, and to maintain a
341 robust and reproducible manufacturing process a qualified anaerobe cabinet (GMP grade A) was
342 selected. The isolator underwent full qualification (installation, operational and performance

343 qualification). Thereby this manufacturing area, provided not only the necessary environment for
344 spore production but also ensured environmental control, including regulation of particulate and
345 microbial contamination through controlled, HEPA-filtered, unidirectional airflow.

346 Thirdly, to ensure the quality attribute of potency, the route of administration, storage and
347 transport conditions of the challenge agent must be carefully considered. Oral capsules were chosen
348 as the route of administration to mimic the natural infection route, while simultaneously
349 maintaining control over the number of spores reaching the gastrointestinal tract. As *C. difficile*
350 spores need exposure to primary bile acids in the duodenum for germination (28, 29), liquid-filled
351 direct-release capsules (instead of enteric capsules) were chosen as they release the spores in the
352 stomach or shortly after. 70/30% (v/v) buffered glycerol/PBS was selected as the formulation buffer
353 for its optimal balance between feasibility to formulate the spore suspension and prevention of
354 downstream capsule leakage. The capsules were preserved at room temperature (15-25 °C) to avoid
355 structural changes in the content and integrity of the capsules which occur upon freezing. Extensive
356 testing of different capsule types demonstrated best performance of Lonza Vcaps® liquid-filled
357 direct-release capsules in terms of secure containment of liquids and semi-solids, stability and
358 preventing leakage at room temperature.

359 Applying the GMP principles to *C. difficile* spore challenge material for clinical use highlights the
360 need for collaboration and thorough risk analysis among various disciplines, including pharmacists,
361 microbiologists, clinicians and technicians. This collaboration has led to a highly controlled
362 production of *C. difficile* spore challenge material in an academic setting. Building on these efforts,
363 the *C. difficile* challenge material has been approved by the Medical Research Ethics Committee to
364 use for colonisation (with non-toxigenic *C. difficile* spores, ClinicalTrials.gov NCT05693077) and
365 infection (with toxigenic *C. difficile* spores, ClinicalTrials.gov NCT06702345) of healthy volunteers in a
366 proof-of-concept clinical trial to find a safe and infectious dose. Establishing a CHIM for *C. difficile*
367 will be a critical advancement in improving our understanding of *C. difficile* infections as well as

368 accelerating (novel) product development to battle the rising incidence and global burden of this
369 complex disease.
370

371 Contribution

372 *Writing- original draft* (lead): AH, *Writing original draft (supporting):* MR and WKS*, Writing-review*
373 *and editing*: EK, PS, PM, ES, IA, CH, CV, LP *Conceptualization:* WKS, MR, EK, ES, PM and AH,
374 *Investigation*: CH, CV, AG and ME, *Methodology*: ES, PM, MR, WKS, EK, CH, CV, AG and ME,
375 *Resources*: PS, PM, IA, ES, EK and WKS *Project administration*: LP, *Validation*: ES, PM, IA and PS,
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381

382 Declaration of competing interest

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386

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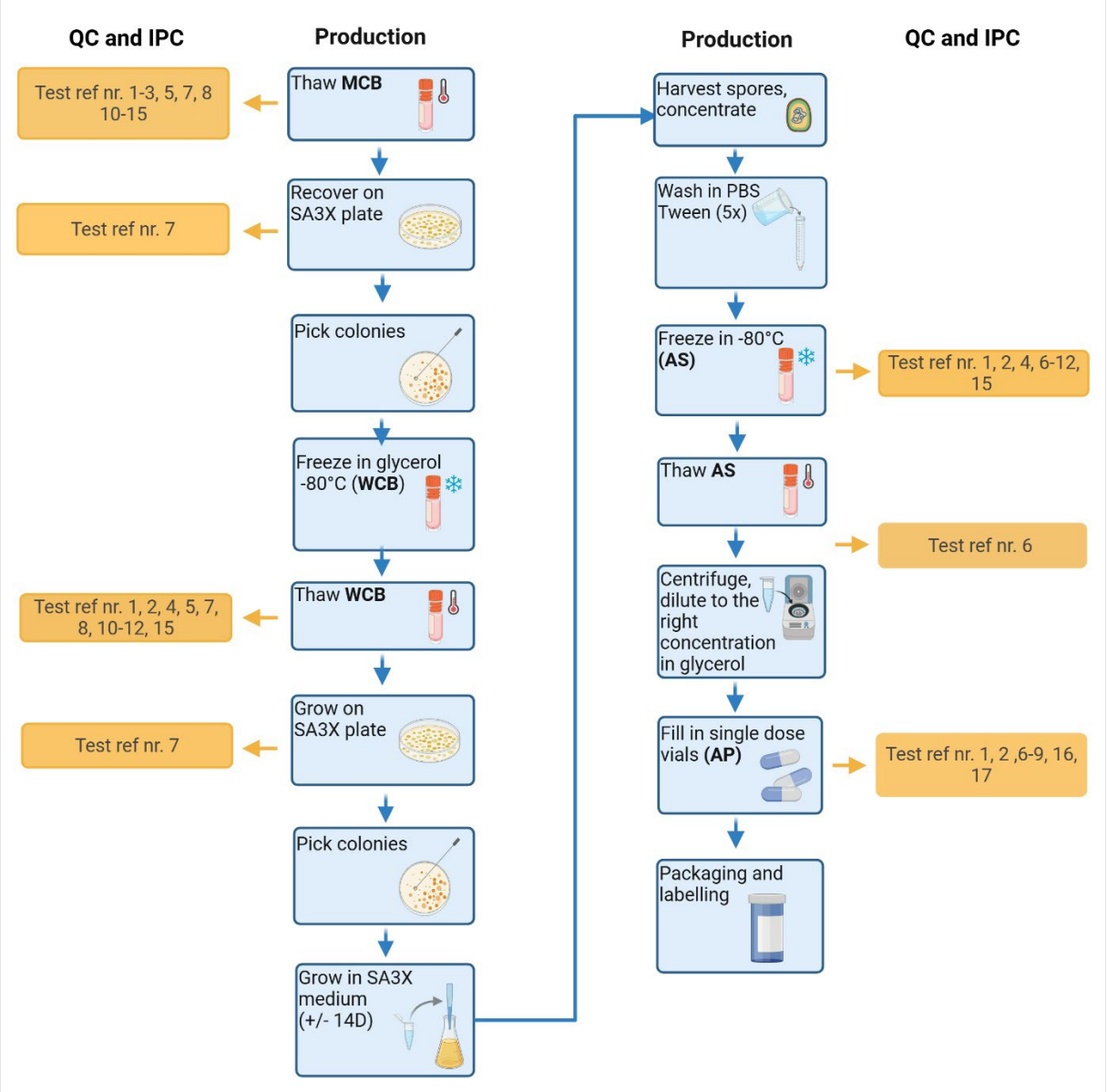
480 **Figure 1**



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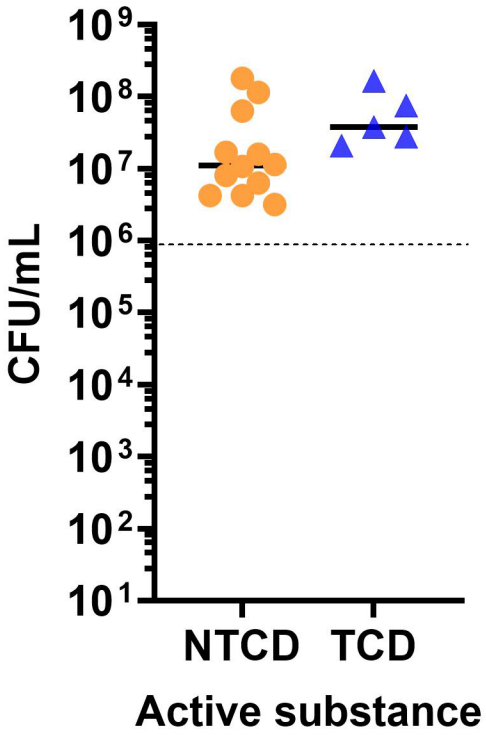
483 **Figure 2**



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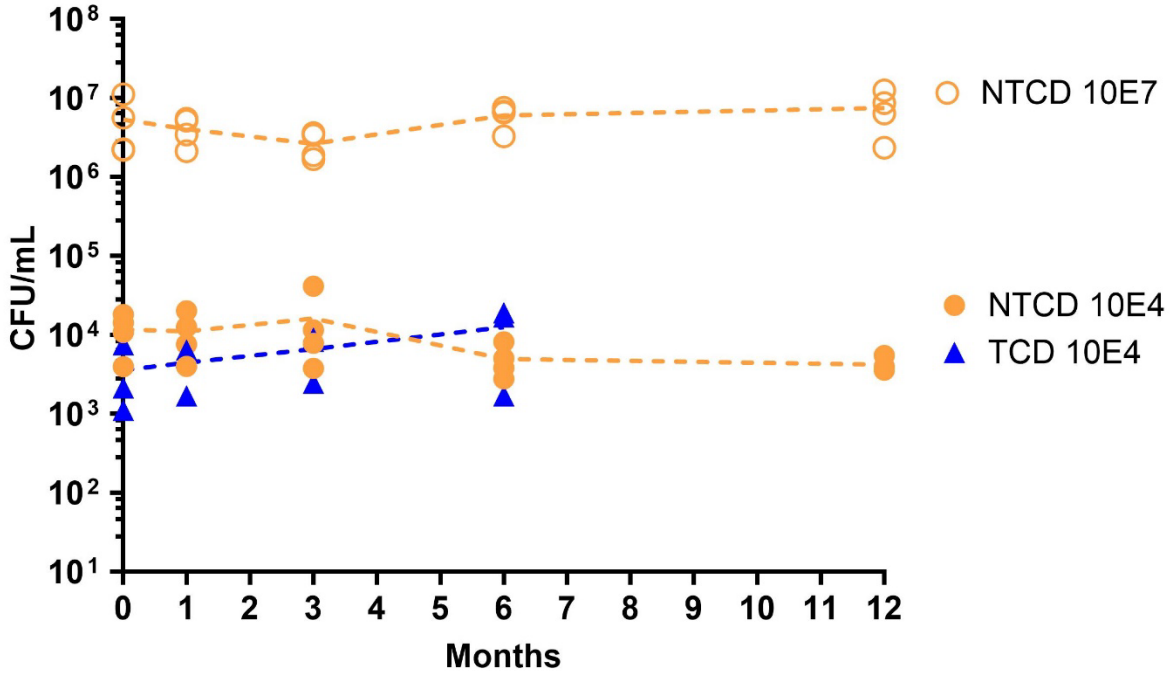
486 **Figure 3**



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489 **Table 1**

Test ref. nr	IPC/ QC	Description	Method	Acceptance criteria	Applied to			
			Identity		MCB	WCB	AS	AP
1	QC	Species PCR*	*gluD* PCR	Positive	Y	Y	Y	Y
2	QC	Ribotyping*	Capillary ribotyping	RT identical to the GLP clinical isolate	Y	Y	Y	Y
3	QC	Sequencing	NGS (complete genome/ long read)	Species: *Clostridioides difficile* ST: identical to the GLP clinical isolate	Y	N	N	N
4	QC	Sequencing	NGS (short read/Illumina)	Species: *Clostridioides difficile* ST: identical to the GLP clinical isolate	N	Y	Y	N
			Potency		MCB	WCB	AS	AP
5	QC	Cell count	CFU count	>100 CFU/ml	Y	Y	N	N
6	QC	Spore count*	Spore enumeration	S: > 1.6 x 10^6 spores/mL P: targeted dose 10^4 (range 10^3-10^5) P targeted dose 10^7 (range 10^6-10^8)	N	N	Y	Y
			Purity		MCB	WCB	AS	AP
7	IPC	Visual appearance of colonies*	Visual inspection of colonies on TSS plate	Visual appearance consistent with *C. difficile* species	Y	Y	Y	Y
8	QC	Contamination with aerobic bacteria*	Aerobic culturing on a TSS plate	No growth of aerobic bacteria (<10 CFU/mL)	Y[1]	Y	Y	Y
9	QC	Measure protein and DNA content*	Colorimetric assay	Protein: < 10 ug/uL dsDNA: < 10 ng/uL	N	N	Y	Y
			Safety		MCB	WCB	AS	AP
10[2]	QC	Presence of toxin	Multiplex qPCR for toxin A, B and binary toxin	*cdtA, cdtB:* NTCD and TCD neg *tcdA, tcdB:* NTCD neg, TCD pos gluD, 16S rRNA: NTCD and TCD pos	Y	Y	Y	N
11	QC	Antimicrobial susceptibility testing (MZ en VA)	E-test	MZ: <0.5 mg/L VA: <0.5mg/L	Y	Y	Y	N
12	QC	Antimicrobial susceptibility testing (FDX)	Agar dilution	<0.5 mg/L	Y	Y	Y	N
13	QC	Microbial contaminants	Presence of pathogenic bacterial contaminants	PCR negative for *Salmonella. Shigella, Campylobacter, Yersinia, Aeromonas* and *Plesiomonas shigelloides spp.*	Y	N	N	N
14	QC	Microbial contaminants	Presence of pathogenic viral contaminants	PCR negative for norovirus, astrovirus, sapovirus, adenovirus and rotavirus	Y	N	N	N
15	QC	NGS for adventitious agents	NGS for DNA	Positive assignment as *Clostridioides difficile*, no discovery with >0.3% of mapped reads to pathogenic species	Y	Y	Y	N
			Capsule controls		MCB	WCB	AS	AP
16	QC	Capsule integrity*	Visual inspection	Capsules are properly locked Capsules are whole and not dented	N	N	N	Y
17	QC	Fill weight and volume*	Weight	Volume: 500 uL Fill weight: 0.70 gram (+/-0.02 gram)	N	N	N	Y

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