Open source anaerobic and temperature-controlled in vitro model enabling real-time release studies with live bacteria

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Article history:
Received 17 August 2021
Received in revised form 24 January 2022
Accepted 2 February 2022

Keywords:
3D printing
Anaerobic
Probiotics
In vitro dissolution model
Oral drug delivery
Rapid prototyping

Abstract
In vitro release and dissolution models are widely used in the development phases of oral drug delivery systems to measure how an active pharmaceutical ingredient (API) is released from a dosage form. However, additional requirements for these models arise when evaluating probiotic dosage forms since they are often sensitive to temperature and oxygen levels. As a solution to this, we propose a custom-designed anaerobic in vitro release setup, made mainly by 3D printing and laser cutting, to function together with state-of-the-art pharmaceutical dissolution equipment – in this case, a microDISS Profiler™. The in vitro release model makes it possible to study the release rate of oxygen-sensitive probiotics in simulated intestinal conditions, while ensuring their survival due to the anaerobic conditions. This has not been possible so far since the available in vitro dissolution models have not been compatible with anaerobic conditions. With two different case studies, the developed model combined with a microDISS Profiler™ has proven capable of measuring the release of a probiotic and a small-molecule API from microdevices for oral drug delivery. Further, the model facilitated the survival of anaerobic bacteria present in the release medium.

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Specifications table

| Hardware name | Anaerobic and temperature-controlled in vitro release model |
|---------------|------------------------------------------------------------|
| Subject area  | • Medical (e.g. Pharmaceutical Science)                   |
|               | • Biological Sciences (e.g. Microbiology and Biochemistry)|
| Hardware type | • Biological sampling and characterization                  |
| Open Source License | CC BY SA 4.0                                      |
| Cost of Hardware | EUR 1319.11                                           |
| Source File Repository | https://doi.org/10.17632/9cyp4s22mc.2                     |

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https://doi.org/10.1016/j.ohx.2022.e00275
2468-0672/© 2022 The Author(s). Published by Elsevier Ltd.
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Hardware in context

In vitro release and dissolution models remain essential tools during the development phases of oral dosage forms [1]. One of the main challenges in this regard is to correlate the release and dissolution of the active pharmaceutical ingredient (API) in vitro, to the effect observed in vivo. Therefore, much effort has been devoted to improving the quality of the in vitro data by more accurate simulation of gastrointestinal (GI) parameters such as fluid composition and volume [2]. However, the increasing occurrence of more complex APIs, for example probiotics [live microorganisms that have a health benefit to the host when ingested in sufficient amounts [3]], leads to the realization that additional experimental parameters have to be included in the in vitro models. Additionally, probiotics are incorporated in increasingly complex drug delivery systems as an attempt to deliver live bacteria to different sites in the GI tract [4–6]. This increases the need for reliable in vitro methods to study the release of probiotics from such drug delivery systems and ensure that the probiotics stay alive during the in vitro study. The most common drug delivery systems for probiotics are the traditionally used capsules besides polymeric particles and microdevices [4,6,7].

Traditionally, a release test consists of two steps; the release test itself performed with a dissolution apparatus, and the subsequent analysis of the samples, typically performed with high performance liquid chromatography (HPLC) or UV spectrophotometry. In the latest dissolution equipment, these two steps have been merged into one. An example of one of the next generation dissolution testers is a microDISS Profiler™ (Pion, MA, USA). A microDISS Profiler™ is a fiber optic in situ UV monitoring system, specifically designed to monitor API concentration real-time in small volumes [8]. This release study approach can be used to follow the API release every 5 sec and it is far less time-consuming and labor-intensive than previous methods. However, it is difficult to evaluate the release of anaerobic probiotic strains from drug delivery systems in the currently available dissolution models. In order to do so, anaerobic conditions and more precise temperature control are required to ensure survival during release and accurate subsequent bacterial viability detection.

Some in vitro models have been developed to simulate the GI microbiota of humans and study probiotic strains in a suitable environment in vitro. The earliest example of such model is “The Simulated Human Intestinal Microbial Ecosystem” (SHIME) [9]. This concept has later been further developed to for example “The Smallest Intestine” by Cieplak et al. [10]. However, these models are not suitable for the evaluation of probiotic release from a drug delivery system since they focus on the survival of the bacteria without measuring the release rate.

3D printing and other rapid prototyping techniques (for example laser cutting) are becoming increasingly consumer-oriented, available, and affordable. These techniques introduce much diversity and flexibility as 3D printers can print with a large variety of polymers and laser-cutting allows for fast and precise cutting of materials such as sheets of acrylic or aluminum [11]. These easy-to-use rapid prototyping techniques have led to custom-build standard lab equipment as well as more advanced laboratory tools. 3D printing has for example been used to make a sample mixer [12], a micropipette [13] and a microsyringe autosampler [14]. 3D printing and laser cutting have also been used to fabricate in vitro models, for example a fully replicable retention model to study the intestinal adhesion of microdevices for oral drug delivery [15]. Additionally, 3D printing has been applied to develop a workstation for automated in vitro workflows [16]. Here, an impressive level of precision was achieved for pipetting and dilution series, which was confirmed by R² values close to 1 (0.9992). When applying 3D printing to produce laboratory equipment, it is important to be aware of the level of precision and accuracy required for the end product. 3D printers come with many different levels of resolution, which can be applied depending on the type of equipment to be fabricated.

In the present study, we develop and share designs for an in vitro model with an anaerobic and temperature-controlled environment to function together with state-of-the-art pharmaceutical dissolution equipment – in this case, a microDISS Profiler™ (Fig. 1). The custom-designed in vitro system makes it possible to study the release of probiotics from a dosage form in an environment simulating the conditions in the intestine (for example, 37 °C and anaerobic conditions). The release profile is of utmost importance for formulation development. It only becomes more relevant when working with different polymeric coatings to modify the release rate, for example for targeted delivery of probiotics. So far, this has not been possible with the available microbiological in vitro models since these models are not compatible with traditional dissolution equipment, such as a microDISS Profiler™. Here, we merge the requirements and needs from the fields of pharmacy and microbiology related to the evaluation of real-time release while ensuring the survival of probiotics in vitro.

Hardware description

The anaerobic and temperature-controlled in vitro release model consists of an environmental chamber with build-in magnetic stirrers (Fig. 2A) as well as two anaerobic jars with two sample chambers in each (allowing four replicates at a time) (Fig. 2B). This model can be combined with a microDISS Profiler™ to monitor API release in real-time.

To control the temperature, the environmental chamber is connected to a precision heat controller (Air-Thermal ATX, WPI, FL, USA) (Fig. 2A), with a temperature resolution of 0.1 °C. Heated air from a hose passes through an airflow regulator (small holes in a plate) and is thereby homogenously distributed around the two anaerobic jars (Fig. 2A). Cold air is drawn out of the chamber through another hose in the other side of the chamber. The thermometer from the precision heat controller is placed in front of the anaerobic jar where it feeds a temperature signal back to the heat controller for closed-loop control. An access door can be opened to load and unload the anaerobic jars (Fig. 2A).
Each of the two anaerobic jars contain two sample chambers (Fig. 2C). The UV-probes from a microDISS Profiler™ are fixed in the lid of the jars to facilitate measurement of API release inside the two sample chambers. Two small sampling holes in the lid of the jar are used for sampling at selected time points during the experiment. The anaerobic jar is designed with an empty space next to the two sample chambers, where an anaerobic sachet (Oxoid™ AnaeroGen™, Thermo Fisher Scientific, Waltham, MA, USA) is placed to establish the needed anaerobic conditions. To measure the API release from a drug delivery system, a sample is placed directly on a custom-designed stirrer at the bottom of each chamber. The stirrer is run by a stirrer driver under the jar body, which is rotated by a micro motor at a rate of 90 RPM (Fig. 2D). The stirrer driver runs the stirrer through magnetic force introduced by four magnetic balls in the stirrer driver and the stirrer (Fig. 2D).

In summary, the presented model setup provides:

- An anaerobic environment with stable temperature control over time.
- Four chambers for multiple API release experiments at the same time.
- Four miniaturized magnetic stirring mechanisms.
- Compatibility with state-of-the-art pharmaceutical dissolution model.

**Design files**

The 3D printed parts were designed with Solidworks 2014 (Dassault Systèmes SolidWorks Corporation, Waltham, MA, USA) computer aided design (CAD) software. All the design files are translated in IGES format and can be downloaded from the linked Mendeley data online.

**Design files summary**

See Table 1 for an overview of the Design Files.

**3D printing files**

All 3D printing files are provided in STereoLithography (STL) format and available on the Mendeley data online (Table 2). All parts were printed on a fused deposition 3D printer Prusa i3 MK2.5S (Prusa Research, Prague, Czech Republic) with a 0.4 mm nozzle, a layer height of 0.1 mm, and 30% infill. The 3D printed parts were fabricated from a biocompatible, transparent polylactic acid (PLA) filament (reprap.me, Hedehusene, Denmark).
Fig. 2. Overview of the anaerobic and temperature-controlled in vitro model for API release studies. A) A photo of the assembled in vitro model, including an environmental chamber connected to a precision heat controller and two anaerobic jars placed on stirrers inside the environmental chamber. B) CAD drawing of the environmental chamber with the two anaerobic jars inside. C) Two-chamber anaerobic jar design, with empty space for placement of an anaerobic sachet. D) Magnetic stirring mechanism at the bottom of each chamber in the anaerobic jars.

Table 1
Design file summary.

| Design file name          | File type | Open source license | Location of the file |
|--------------------------|-----------|---------------------|----------------------|
| jar_body. IGS            | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| jar_lid. IGS             | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| jar_base. IGS            | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| Stirrer_driver. IGS      | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| Stirrer. IGS             | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA01. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA02. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA03. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA04. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA05. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA06. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA07. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA08. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| PMMA09. IGS              | CAD       | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
| In Vitro Anaerobic ModelIGS | CAD | CC BY SA 4.0        | [Link](https://doi.org/10.17632/9cyp4s22mc.2) |
All the laser cutting files are in DXF format and available on the Mendeley data online (Table 3). All the parts were cut with 3 mm transparent poly (methyl methacrylate) (PMMA) plates (Goodfellow, Cambridge, England) by a tabletop laser cutter (Epilog Mini, Epilog Laser, Golden, CO, USA) with a speed, laser power and frequency setting of 7 %, 40 % and 5000 Hz, respectively.

Electronics

The stirring mechanism is supplied by four micro motors connected with a USB type A connector (Fig. 3). The micro motors have an operating voltage from DC 3 V to DC 9 V (48–140 RPM) and rotates ~ 90 RPM when supplied by DC 5 V. A single motor consumes 30 mA current while operating without an external load. Four micro motors need a current of ~ 120 mA provided by a standard USB power source.

Bill of materials

In addition to the 3D printed and laser-cut components, some items must be purchased from other sources. An overview of these components, the number required, as well as the total cost can be found in Table 4.

Build instructions

The assembly process requires an instant glue (Loctite super liquid 20 GR, Henkel, Düsseldorf, Germany) to ensure a firm bonding between the components. The diameter of screw holes in the 3D printed parts was designed for direct screw-in fix-

Table 2
3D printing files.

| Design file name | Number of required prints | Open source license | Location of the file |
|------------------|---------------------------|---------------------|----------------------|
| Jar_lid.STL      | 2                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| Jar_body.STL     | 2                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| Jar_base.STL     | 2                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| Stirrer.STL      | 4                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| Stirrer_driver.STL | 4                       | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |

Table 3
Laser cutting files.

| Design file name | Number of required prints | Open source license | Location of the file |
|------------------|---------------------------|---------------------|----------------------|
| PMMA01.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA02.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA03.DXF       | 2                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA04.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA05.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA06.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA07.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA08.DXF       | 1                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |
| PMMA09.DXF       | 2                         | CC BY SA 4.0        | https://doi.org/10.17632/9cyp4x22mc.2 |

Fig. 3. Illustration of the four micro motors and their electrical connection (USB type A connector). The USB cable has a color code of red, black, green and white which are + 5 V, ground, USB data + and USB data, respectively. Only the red and black wires are needed to power the motors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fabricate all the 3D printed and laser cut parts before initiating the assembly process. 

**Fig. 4.** Assembly instructions for **A)** magnetic stirring mechanism and **B)** anaerobic jars.

**Magnetic stirring mechanism assembly (Fig. 4A)**

1. Components needed: 8x M2 screws, 4x micro motors, 4x stirrers, 4x stirrer drivers, 32x magnetic balls and 2x jar bases.
2. Insert and glue four magnetic balls into four holes on the bottom side of the stirrers.
3. Insert and glue four magnetic balls into four holes on the stirrer driver.
4. To achieve the maximum stirring efficiency, it is essential to adjust the direction of the N and S poles on the magnetic balls before gluing.
5. Insert the micro motor shaft to a cross-shaped hole in the center of the stirrer driver. One small drop (~25 µl) of instant glue is needed inside the cross-shaped hole.
6. Fix two micro motors on the jar base with four M2 screws.
7. Repeat the assembly process to complete four stirrers, stirrer drivers, and two jar bases.
8. Connect four micro motors to one USB cable (Fig. 3).
**Anaerobic jar assembly (Fig. 4B)**

1. Components needed: 4x M3 screws, 1x thermometer (from the heat controller), 1x PMMA01, 2x assembled jar bases, 4x assembled stirrers, 2x jar bodies and 2x jar lids.
2. Mount the two assembled jar bases on PMMA01 with four M3 screws.
3. Insert the thermometer from the bottom of PMMA01 into a hole in front of the jar base and PMMA01. Leave the thermometer tip 20 mm outside of the hole. One small drop (~25 µl) of instant glue is needed to place the thermometer.
4. Place four stirrers in the bottom of the four sample chambers in the two jar bodies.
5. Close the top of the jar bodies with two jar lids.
6. Place the assembled jar bodies in the jar bases. **Fig. 5**

**Environmental chamber and temperature control assembly (Fig. 5)**

1. Components needed: 5x M3 screws, 4x M3 nuts, 4x rubber foots, 2x hinges kits, 2x jar assemblies, 1x knob, 1x heat controller (with warm and cold air hoses), 1x PMMA01, 1x PMMA02, 1x PMMA04-09 and 2x PMMA03.
2. Fix four rubber foots at the bottom side of PMMA01 with four M3 screws and M3 nuts.
3. Insert the bottom features of PMMA04 into the two slit holes in PMMA01.
4. Assemble the two PMMA03 to PMMA01 and insert the side features of PMMA04 into the slit holes in the two PMMA03 parts.
5. Assemble PMMA05 to PMMA01.
6. Insert the top features of PMMA04 into the two slit holes in PMMA02.
7. Fix PMMA01-05 with instant glue.
8. Assemble the hinge kits (each kit contains hinges, 4x M6 screws, 4x M6 nuts) to PMMA06 and PMMA07.
9. Assemble two PMMA09 parts by inserting their side features into the slit holes in PMMA07.
10. Insert the top features of PMMA09 into the slit holes in PMMA08.
11. Fix PMMA06-09 with instant glue.
12. Assemble the knob with a M3 screw.
13. Fix PMMA06 to PMMA01 and PMMA03 with instant glue.
14. Insert the warm air hose into a 65 mm diameter hole in PMMA05.
15. Insert the cold air hose into a 65 mm diameter hole in PMMA06. **Fig. 6**
Anaerobic sachet and microDISS Profiler™ UV-probes assembly (Fig. 6)

1. Components needed: 4x M4 set screws, 4x microDISS Profiler™ UV-probes (connected to a microDISS Profiler™ and a computer with AuPRO software) and 2x anaerobic sachets
2. Place the four UV-probes in the sample chambers in the anaerobic jar through the holes in jar lids.
3. Adjust the insertion depth of the UV-probes.
4. Fix the UV-probes with M4 set screws.
5. Insert the anaerobic sachets into the empty space inside the jars.
6. Seal the jar bodies with the jar lids.
7. Open the chamber door and insert the jars to the jar bases inside the chamber.

Operation instructions

1. Switch on the precision heat controller and set the target temperature to 37.0 °C.
2. Place the anaerobic jars on the stirrers inside the chamber, and add stirrers and sample of interest (can be mounted on the stirrers with double-sided adhesive tape as the microcontainer chips in the case studies) (Fig. 4B).
3. Add one anaerobic sachet together with an anaerobic indicator strip (Anaerotest™ Strips, MilliporeSigma, Burlington, MA, USA), to ensure an anaerobic atmosphere in the jar. Close the jar by applying the lid (Fig. 6).
4. Wait for 1–1.5 h for achieving anaerobic conditions and 37 °C inside the anaerobic jars (the temperature will be achieved shortly, but it requires up to 1–1.5 h to establish anaerobic conditions).
5. In the meantime, prepare a UV calibration curve on a microDISS Profiler™, as previously described in literature [17–19]. Briefly, measure the absorbance after adding various known amounts of a stock solution with the API of interest and build a linear calibration curve.
6. After preparing the calibration curve, mount the UV probes through the designated holes in the lid of the jar (Fig. 6).
7. Initiate the release study by adding 10 mL of release medium to each sample chamber (through the sampling holes in the jar lid) and press start in the microDISS™ software.
8. During the release study, liquid samples for bacterial survival detection can be withdrawn from the jar through the sampling holes in the lids by using a syringe (22G, 0.7 × 50 mm).
9. After use, the anaerobic chamber can be disinfected using ethanol and dried before re-use.

Fig. 6. Assembly instruction for anaerobic environment and microDISS Profiler™ UV-probes.
10. Bacterial safety: Non-pathogenic probiotics (as in the present case studies) should be discarded as biological waste, but do not require any other safety protocols. If pathogenic bacteria are introduced, they should be carefully handled and discarded according to their biosafety level, and other procedures for disinfection/sterilization might be required.

Validation and characterization

The functionality of the developed anaerobic and temperature-controlled in vitro release model was evaluated. We tested its performance regarding temperature control, anaerobic conditions and demonstrated the use of the hardware for two relevant case studies.

Stirring rate, temperature control and anaerobic conditions

To minimize the uncertainty between the chambers during release experiments, it is crucial to ensure that all four chambers have the same stirring conditions. The rotation rate of the four stirrer driver assemblies was characterized as shown in Fig. 7A. A standard USB 5 V powered all micro motors, which are rotating at a rate of 90 RPM. The difference between the rotation of the motors was 0.1 ± 0.07 RPM (mean ± SD, n = 6). Thus, the rotation rate in the developed model is comparable to the traditionally applied stirring rate of 100 RPM applied in a microDISS Profiler™ [20–22].

Reliable temperature control is critical when working with probiotics. Therefore, a precision heat controller was used to regulate the environmental chamber’s temperature. The controller has a variable airflow rate from 0.55 to 1.4 m³/min. The heat distribution inside the environmental chamber was analyzed by a FLIR One Pro infrared camera (Teledyne FLIR, Wilsonville, OR, USA) (Fig. 7B). From this analysis, it was visible that over-heating occurred before the airflow regulator, which regulated the air to ensure a homogeneous temperature distribution on the anaerobic jars. Furthermore, the infrared camera was used to detect the temperature inside the anaerobic jars through the holes for the UV-probes (Fig. 7C). The temperature inside the two anaerobic jars was monitored every 10 sec during the heating process. Based on the averaged temperature
curve, a temperature of 37 °C was reached inside the sample chambers after 10 min with a maximum airflow rate of 1.4 m³/min (Fig. 7D).

Additionally, the temperature variation was measured after 15 min, to ensure a stable temperature of approximately 37 °C (Fig. 8). Between the four chambers, the temperature varied less than 1 °C (between 37.0 and 37.7 °C).

The anaerobic conditions were evaluated using anaerobic indicator strips, which are developed to detect an anaerobic atmosphere. Before taking the equipment into use, it was ensured that anaerobic conditions could be obtained and last for the duration of the experiments (up to 150 min). Additionally, the anaerobic conditions were confirmed during each of the described case studies.

To confirm that the developed anaerobic jars could be applied to work with oxygen-sensitive bacteria, the survival of *Bifidobacterium thermophilum* (*B. thermophilum*) (DSM 20209, DSMZ, Germany) was evaluated and compared to the survival in a traditional anaerobic jar (2.5 L, Thermo Scientific™ Oxoid™ Anaerojar™, Thermo Fisher Scientific, Waltham, MA, USA). *B. thermophilum* is known as an oxygen-sensitive bacteria (sensitive to anything higher than 10% oxygen), and it is mostly classified as anaerobic [23]. To study the survival, samples were taken 30, 60 and 90 min after initiating the study. A dilution series was plated on bifidus selective medium (BSM) agar (Sigma-Aldrich, St. Louis, MO, USA) and the survival was determined by counting the colony forming units (CFU) (Fig. 9). From these results, it could be observed that *B. thermophilum* survived in both the 3D printed anaerobic jar and in the traditional anaerobic jar. Thereby, we could confirm that the developed anaerobic *in vitro* model can achieve sufficient anaerobic conditions to work with oxygen-sensitive bacteria as *B. thermophilum*. Although the setup provided acceptable anaerobic conditions for the present purpose, the duration of the anaerobic conditions could potentially be improved by different sealing techniques (e.g. paint, sealant or multiple shells). As an alternative to anaerobic sachets, anaerobic conditions could be achieved by replacing the oxygen inside the anaerobic jars with nitrogen. This method has previously been applied by Cieplak *et al.* [10] to study the survival of probiotic strains *in vitro*. However, anaerobic sachets provide a larger degree of flexibility since no permanent installations are required in the lab.

![Fig. 8. Temperature inside the four sample chambers 15 min after the heater was turned on. The temperature was measured to be between 37.0 and 37.7 °C.](image)

![Fig. 9. The survival of *B. thermophilum* after 30, 60 and 90 min in the 3D printed anaerobic sample jars (red) and in a traditional anaerobic jar (orange). Mean ± SD, n = 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
Case study 1: Release of small-molecule APIs in the presence of simulated microbiota

In case study 1, we demonstrate how to use the developed in vitro model to assess the release of a small-molecule model API, mesalazine (also known as 5-aminosalicylic acid (5-ASA)) (Sigma-Aldrich, St. Louis, MO, USA), from microscale drug delivery systems, namely microcontainers. The microcontainers were fabricated on silicon chips, loaded with 5-ASA powder, and coated with an enteric lid, namely Eudragit® L100 (Evonik Industries, Essen, Germany) (Fig. 10). Such polymer is commonly applied to protect APIs from the harsh gastric environment (enzymes and low pH). The fabrication of microcontainers and techniques for loading and coating, are well described in literature [17,20,24].

To simulate the presence of bacteria in the small intestine, the release of 5-ASA was studied in the presence of an anaerobic bacteria *B. thermophilum*. The possibility to measure the release of small-molecule APIs while studying the survival of bacteria in the same medium can also be an advantage in relation to co-delivery of antibiotics and probiotics.

For the release study, individual calibration curves were prepared for each sample chamber as previously described for the traditional use of a microDISS Profiler® [21]. After preparation of the calibration curves, the microcontainers were placed on the stirrers in the sample chambers, and the chambers were left for acclimatization to establish anaerobic conditions and reach 37 °C (1–1.5 h). Following acclimatization, the experiment was initiated by adding 11 mL of phosphate buffered saline (PBS) containing *B. thermophilum* to the four sample chambers before starting the UV measurements in the AuPRO software. The release of 5-ASA was measured in real-time for 150 min (Fig. 11A), and samples were taken at 30, 90 and 150 min to study the survival of *B. thermophilum* (Fig. 11B). The release data showed that a 100 % 5-ASA release was observed after approximately 120 min, and that the data could be collected with minimal standard deviations. The CFU counts confirmed that the oxygen-sensitive bacteria *B. thermophilum* survived in the anaerobic jars after 30, 90 and 150 min.

Case study 2: Release of probiotics

As a different case study, we demonstrate how the developed anaerobic and temperature-controlled in vitro release model can be used to measure the release of probiotics from microdevices. The microcontainers were loaded with a spray-dried powder of *Lactobacillus rhamnosus* GG (LGG) [6], and the microcontainers were coated with Eudragit® L100 as described in the first case study. In contrast to 5-ASA that could be measured directly, the release of LGG was determined indirectly by measuring the UV absorbance of the skim milk powder in the spray-dried product, as previously shown by Kamguyan et al. [6].

The release of LGG was studied in two steps; first in simulated gastric fluid (FaSSGF pH 1.6, Biorelevant®, London, UK) and afterwards in simulated intestinal fluid (FaSSIF pH 6.5, Biorelevant®, London, UK). By performing the release experiment in two steps, it was possible to study the protective properties of the applied Eudragit coating and confirm if a release occurred after reaching intestinal pH values. The release of LGG in FaSSGF was measured for 30 min, where after the medium was changed to FaSSIF (Fig. 12A). During the release study, samples (0, 30, 60 and 90 min) were taken from the four anaerobic chambers to determine the survival of the released LGG by plating on De Man, Rogosa and Sharpe (MRS) agar (SSI Diagnostica, Hillerød, Denmark). The release data confirmed that the protective coating worked as expected, by allowing only a minor release of the probiotic powder (approximately 10 %) during the first 30 min in the gastric step (Fig. 12A). The small release was confirmed by the CFU counts, where no bacteria could be found in the samples from 0 and 30 min (Fig. 12B). When changing the medium to FaSSIF after 30 min, the release rate increased and a complete release of LGG powder was observed after approximately 90 min (Fig. 12A). From the samples for CFU counts, LGG could be detected in the release medium after 60 and 90 min (Fig. 12B), which correlates with the release measured by the UV probes. The observed viability,
corresponding to \( \sim 2 \times 10^7 \) CFU/g powder, is in accordance with the survival of LGG previously observed after release from microcontainers sealed with the same coating technique [6]. For the sample taken at 90 min, a large standard deviation could be observed compared to the other time points. However, from the individual measurements, it was apparent that the large standard deviation was due to one of the triplicates, whereas the other two samples are in line with the CFU/g powder detected after 60 min (Fig. 12B).

In summary, the demonstration of the *in vitro* model shows:

- Achievement of anaerobic conditions and stable control of temperature over time.
- The model was successfully combined with a state-of-the-art pharmaceutical dissolution model (microDISS™) in order to
  - study the survival and release of probiotics from microscale drug delivery systems.
  - study the release in the presence of an intestinal bacteria.

Conclusively, the developed model provides a unique tool for representative viability assessments of anaerobic bacteria while monitoring the release in real-time. This has not been possible before, since the focus of microbiological *in vitro* models has been mainly the study of probiotic viability in physiologically relevant systems [9,10,25], but have never been combined with equipment for real-time monitoring of drug release, such as a microDISS Profiler™. Additionally, the developed anaerobic *in vitro* model provides a unique opportunity to study the release of more sensitive next-generation probiotics, of which many are extremely sensitive to factors such as oxygen [26,27]. Furthermore, the possibility to study the release of small-molecule drugs and probiotics while studying the viability of bacteria can be beneficial in relation to *in vitro* evaluation of co-delivery devices loaded with both antibiotics and probiotics.
Potential modifications

Potential modifications of the model could include additional alignment features on the anaerobic jar parts, which can eliminate angular adjustments during the experiments (Fig. 13). This is important in order to achieve the optimal conditions for stirring and release rate measurements. Additionally, a more physiologically relevant release medium could improve the model. This could be achieved by addition of a larger selection of bacteria to create a consortium, or by application of different biorelevant media. This would make it possible to study the in vitro release in the presence of a relevant gut microbiota. Finally, the high precision heater could be replaced with an open source low-cost temperature-control, for example OpenTCC [28], as a low-cost alternative.

Since we did not have any motor stuck issues during our experiments, there was no current protection for the motor power connection. For safety reasons, we suggest users add one inline type fuse holder (01550104ZXU from Digikey) with a 500 mA fuse (BK/AGA-1/2 from Digikey) (Fig. 14).

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.

Acknowledgements

This study was supported by the Novo Nordisk Foundation (NNF17OC0026910) as a part of the project Microstructures, Microbiota and Oral Delivery (MIMIO), the BioInnovation Institute Foundation (Grant No. NNF20SA0063552), as well as the Danish National Research Foundation (DNRF122) and the Villum Foundation (Grant No. 9301) as a part of the Center for Intelligent Drug Delivery and Sensing Using Microcontainers and Nanomechanics (IDUN). The funding sources had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the report or in the decision to submit the article for publication. The authors thank Tine Rask Licht and Katja Ann Kristensen from The National Food Institute, Technical University of Denmark for indispensable theoretical and practical support with the microbiological aspects of the work.
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