Chapter 25
One Compartment Fermentation Model

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Abstract In vitro colon model was first applied in an inter-laboratory dietary fibre (DF) fermentation study and adapted at VTT for whole foods and beverages, isolated dietary phenolic compounds and pharmaceuticals. The application of the models includes strict anaerobiosis, which ensures active anaerobic microbial community. Pooling of faecal samples from several donors ensures reproducibility between the experiments. The correlation of in vitro data with in vivo data is quantitatively challenging, but is qualitatively highly relevant. In this chapter we explain the applicability of the one compartment fermentation model, including the general protocol as well as the advantages and disadvantages of the system.

Keywords One compartment colon in vitro model • Human faecal microbiota • Anaerobic conditions • Microbial metabolites • Dietary fibre • SCFA

25.1 Description of the VTT One Compartment Fermentation Model

25.1.1 History of the Model

In 1995, VTT developed an enzymatic digestion model to simulate the digestion of DF in the upper intestine. The model was optimized for maximal starch digestion and to obtain non-digestible DF residue using physiological conditions (Aura et al. 1999). In the same year an inter-laboratory study of in vitro colon fermentation models was published by Barry et al. (1995). The physiological relevance of the model was ensured by a multidisciplinary approach involving specialists in nutrition and gastroenterology. The fermentation was expressed as the difference between the faecal control (inoculum, no substrates) and substrates under investigation (e.g. plant foods or other DF ingredients). Later the model was adapted for pure

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phenolic compounds (Aura 2005), fruit matrices and beverages (Bazzocco et al. 2008; Aura et al. 2013). The fermentation model from Barry et al. has been used in combination with the enzymatic digestion model from Aura et al. (1999) for cereal studies (Nordlund et al. 2012, 2013). Most recently the model has been coupled to a platform with bioinformatics tools to obtain non-targeted metabolite profiling of plant foods (Aura et al. 2013).

25.1.2 Special Features

Venema and van den Abbeele (2013) have pointed out recently a non-physiological slow rate of conversion in "static cultures". In reference to the unit operation the authors describe the "static" culture conditions (in contrast to the continuous "dynamic" culture conditions) (Venema and van den Abbeele 2013) often in context of one compartment cultures. When one compartment in vitro colon models are performed in well-buffered non-nutrient media with strictly anaerobic microbiota, accurately timed sampling and monitoring of pH changes, they appear to show also the distinctively higher rate of conversion. The time course studies under agitation bring out the dynamic processes, which occur from food components also in "batch" unit operations. Static, standing cultures without agitation cannot be used in conversions related to DF components or isolated phenolic compounds, because the suspension would sediment and enzyme–substrate interaction would be suppressed, especially in incubations with solid substrates such as DF components.

A distinctive characteristic of the VTT one compartment in vitro colon model is the use of a strong buffer with minerals as the main matrix of the medium (Barry et al. 1995) instead of nutritive medium with additional carbon sources (Hughes et al. 2008). The faecal material brings additional carbohydrates and a matrix to support the microbiota together with the buffer and mineral solution. It has been shown in previous studies (McBurney and Thompson 1989; Mortensen et al. 1991) that use of faeces from at least three donors secures the diversity of the microbiota and enables the reproducibility of the results. Therefore in the one compartment in vitro model applied at VTT a pooled human faecal suspension is prepared from samples from at least 3 but usually from 4 to 6 donors.

The faecal inoculum is dense (10–16.7 %, w/v), especially, when non-digestible carbohydrate fermentation is performed. The microbiota acts as a source of enzymes, which degrade and convert released components such as sugars to short-chain fatty acids (SCFA) or phenolic compounds to microbial phenolic acid or lactone metabolites.

One of the positive features of the VTT model is that the anaerobic conditions can be easily maintained as the system is closed and air-tight. This anaerobic environment is needed to avoid damaging of the strictly anaerobic microbiota during the preparation of the inoculum (homogenization, dilution and filtration) and its incubation with substrates. It also ensures that the microbial enzymes can perform degradation of DF constituents under the same conditions as in the human gastrointestinal tract.

The quality of the anaerobic conditions in the VTT model was tested by cultivating samples from the fresh faecal suspension in aerobic and in anaerobic conditions and counting the microbial cells after the cultivation. The initial (0 h) anaerobic cell count (1.6×10^9 CFU/ml) exceeded that from the corresponding aerobic cell count (3.1×10^7 CFU/ml). After 4 h of anaerobic incubation at 37 °C, cell counts were changed to 3.2×10^9 CFU/ml and 2.3×10^7 CFU/ml, for the anaerobic and aerobic cell counts, respectively. The corresponding log 10-anaerobic-to-aerobic ratios were 1.2 and 1.3 for 0 h and 4 h, respectively, indicating that anaerobic conditions were not disturbed during the incubation. When frozen faeces were used as an inoculum in three experiments, the log 10-anaerobic-to-aerobic ratio was 1.5 ± 0.2, but cell counts were 10 % of the fresh inoculum. After 4 h in corresponding conditions both the cell counts showed fourfold increase, and the log 10-anaerobic-to-aerobic-ratio was still 1.3 ± 0.04. Based on these results it was concluded that fresh inoculum was the most vital for DF fermentations and that the inoculum preparation conditions need to be strict enough for maintaining the adequate difference between the anaerobic and aerobic microbiota (2–3 log difference). When the cultivated log 10-anaerobic-to-aerobic ratio is between 1.2 and 1.6, anaerobic microbiota dominates the population. If the ratio were 1, strictly anaerobic strains would be dead and only microaerophilic strains would stay alive. This would be non-physiological situation in terms of DF fermentations.

Faecal samples from each donor and the corresponding faecal suspension were obtained from the in vitro colon model and kept frozen at −70 °C before microbiological analysis. Partial 16S rRNA gene was amplified for the denaturing gradient gel electrophoresis (DGGE) analysis of predominant bacteria, *Eubacterium rectale–Blautia coccoides* group (Erec-group, *Lachnospiraceae*), *Clostridium leptum* group (Clept-group, *Ruminococcaceae*), *Bacteroides*, *Bifidobacterium* and *Lactobacillus* as previously described (Maukonen et al. 2006, 2012). Above mentioned bacterial profiles of individual donors were compared to the bacterial profiles of the corresponding faecal suspension. Even though the inter-individual similarity between the donors was low (Fig. 25.1), and partly different people were used as donors in different in vitro colon model experiments, the bacterial profiles of faecal suspensions were rather similar (Fig. 25.2). In predominant bacteria, Clept-group, lactobacilli and bifidobacterial profiles clear differences between faecal suspensions were observed. However, the observed differences corresponded to intra-individual temporal variation previously observed in healthy adults (Maukonen et al. 2012). Moreover, in the Erec-group (Fig. 25.2b) and in *Bacteroides* profiles there were no major differences between the different faecal suspensions. Erec-group bacterial populations have also previously been shown to be temporally rather stable (Maukonen et al. 2006).

In conclusion, the microbiological reproducibility of the in vitro model was good: the microbiological variation observed between the different in vitro colon model experiments was equivalent to intra-individual temporal variation. Since DNA-based methods were used, which target both living and dead bacteria, it cannot be reliably stated that the active microbiota has been the same in each experiment. The used DGGE methods target only the rDNA amplicons obtained from the population that exceeds 1 % of the species present in the analysed community

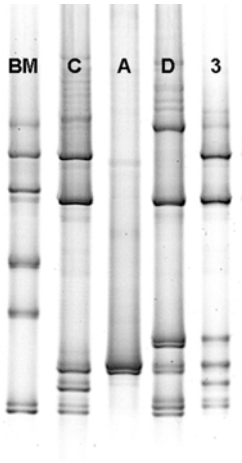

Fig. 25.1 Bifidobacterial profiles of samples used for in vitro fermentation (BM = bifidobacterial marker; A–C = donors; 3 = faecal slurry)

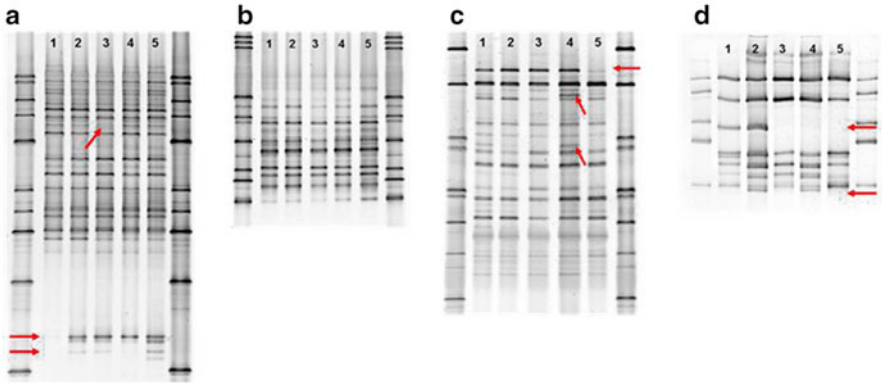

Fig. 25.2 Predominant bacterial (**a**), *Eubacterium rectale–Blautia coccoides* group (**b**), *Clostridium leptum* group (**c**) and *Bifidobacterium* (**d**) DGGE-profiles of faecal slurries used for five (1–5) different in vitro fermentations. *Red arrows* denote clear differences between samples

(Muyzer et al. 1993), therefore our results show that the most dominant bacteria within each bacterial group were similar. This study of microbiological reproducibility was a quality measure, which ensures that the metabolites are less dependent on the microbiota than from the substrates. Other microbial ecological studies are not recommended with the one compartment in vitro colon model, since the model is designed to study microbial metabolism of various substrates and for comparison of their metabolite dynamics.

Table 25.1 Variation of different microbial metabolites (mean ± standard deviation) measured from the faecal control between experiments (n: number of experiments) in the beginning (0 h) and in the end (24 h) of the incubation

Response	0 h	24 h	n	References
Total SCFA (mM)	11.7 ± 2.6	32.6 ± 5.7	5	1
Acetic acid (mM)	7.6 ± 1.5	20.5 ± 4.5	5	1
Propionic acid (mM)	1.7 ± 0.3	5.9 ± 1.1	5	1
Butyric acid (mM)	2.4 ± 0.9	6.2 ± 1.3	5	1
pH (initial 6.9) (range) pH (initial 5.5)	6.99–7.07 6.07 ± 0.05	6.67– 6.68 5.83 ± 0.12	2 3	1
3-Hydroxyphenylpropionic acid (μM)	7.7 ± 3.5	7.3 ± 2.4	3	2
3-Phenylpropionic acid (μM)	96 ± 15	235 ± 71	3	2
3-Hydroxyphenylacetic acid (μM)	5.4 ± 4.4	8.7 ± 1.7	3	2

1: Nordlund et al. (2012), Bazzocco et al. (2008), Aura et al. (2013) and Nordlund et al. (2013)
2: Aura et al. (2013) and Bazzocco et al. (2008)

25.2 Validation of the System

The validation of the model was performed by comparing the variability of metabolite concentrations in the faecal control (inoculum, no substrates) and in the substrate incubation in experiments performed on different days. The faecal control measurement provided information on the variation of the background fermentation of the remaining fermentable carbohydrates or phenolic compounds from the diet of the faecal donors. Table 25.1 shows comparison of 4 or 5 experiments in respect to major microbial metabolites and pH in the faecal control at 0-h- and 24-h-time points. The smaller the response, the higher is the variation of the response. The variation is smallest (14–25 %) for total SCFA, acetic acid, propionic acid and 3-phenylpropionic acid, whereas butyric acid varies 21–37 % between the experiments. pH shows only 1–2 % variation, for the initial pH 5.5 of the buffer; and 3-hydroxyphenylpropionic and -acetic acids show 65–114 % (0 h) and 23–27 % (24 h) variations. Furthermore, when the concentrations of the metabolites in the faecal control are compared between the experiments, the differences are dependent on the metabolite and its dynamics and particularly the diets of the donors of the faeces. The differences are balanced by pooling the inoculum from several donors. Therefore, the major phenolic metabolites in the in vitro model show similar variation between experiments (65–114 %) in phenolic microbial metabolites than corresponding urinary excretion profiles shown between different human individuals (70–113 %) (Vetrani et al. 2014).

The true validation requires the same reference substrate for all experiments under investigation. Usually the space in the experiment is limited and the reference substrate is chosen according to the hypotheses of the specific projects. Rye bran and flaxseed meal were used in several experiments, the responses were measured

as SCFA production and were summarized in Aura (2005). The total SCFA formation at the end of the fermentation showed 10 % and 18 % variations for enzymatically pre-digested rye bran (83.3 ± 8.5 mM, n = 4) and pre-digested flaxseed meal (95.3 ± 17.5 mM, n = 3), respectively. Taking into account the different inoculum concentrations (10 % or 16.7 %) used in these studies, the variation was surprisingly low for SCFA production. In contrast, the 97 % variation of the enterolactone formation from predigested flaxseed meal (43 ± 42 nM, n = 3) was highly dependent on the inoculum concentration. This could be explained by the minor population of enterolactone-converting bacteria (Clavel et al. 2005) and therefore the extent of conversion was susceptible to the depletion by the low concentration of inoculum. Furthermore, the resilient structure of flaxseed meal and low nano-molar concentrations may have contributed to the high variation (Aura 2005).

25.3 Relevance to Human In Vivo Situation

The one compartment in vitro colon model measures only those conversions, which occur in anaerobic conditions by faecal microbiota. These reactions take place in caecum prior to absorption. The model does not take into account the membrane functions which occur in the colon epithelia, the intestinal epithelia and in the liver. The major metabolites from the DF intake are SCFA and phenolic microbial metabolites. The major SCFA formed in the colon is acetic acid, which does not have distinctive structural characteristics, which would be needed for follow-up of its route without a radiolabel. For example a correlation was sought for SCFA production between a fibre blend and its components in vivo and in vitro (Koecher et al. 2014). In vitro experiments showed SCFA formation and analysis of SCFA from faeces revealed an increase of SCFA content in faeces after consumption of the fibre blend versus fibre free diet, however, significant differences between the ingredients of the blend were not observed in vivo (Koecher et al. 2014). The authors speculated about the balance between production and absorption rates of microbial metabolites and concluded that the in vivo interventions and in vitro studies may not be directly correlated, but in vitro models are additionally informative (Koecher et al. 2014).

Phenolic compounds and their microbial metabolites may be better biomarkers for plant food and dietary fibre intake than SCFA. The metabolites in urine include also membrane-derived metabolites, such as glucuronidated and sulfated derivatives of phenolic compounds. In addition to this urine contains the microbial metabolites derived from the diet (Aura 2008). The closest and most non-invasive measure is the analysis of 24-h-urine, which describes the excretion of metabolites from the food and beverage intakes (Vetrani et al. 2014). The first challenge is that the urinary analysis describes the excretion of the metabolites from the whole diet from several precursors (Vetrani et al. 2014) which share the microbial metabolites (Aura 2008). If the diet is not controlled the background can be disturbed by the excretion of phenolic metabolites from the non-controlled components, and statistical differences are not obtained (Lappi et al. 2013).

Another issue is that a single component does not change the excreted metabolites in a significant way (Lappi et al. 2013), whereas a fully controlled diet high or low in polyphenols can show significant metabolite profiles typical for the polyphenols (Vetrani et al. 2014). Therefore, the metabolites cannot be connected to a single food or component, but refer to a whole diet. The third challenge is that the comparison of in vivo responses match qualitatively well with the microbial metabolites expected to be excreted from the food phenolic components (Aura 2008; Lappi et al. 2013; Vetrani et al. 2014). However, quantitative comparisons are problematic because of the high individual variation of microbial metabolites in vivo (Vetrani et al. 2014) and the diversity of hepatic metabolites, the quantification of which requires unavailable authentic standards for most of them for significant correlation.

25.4 Quality in Relation to Other Models with the Same Applicability

When microbial metabolism of rutin (quercetin-rhamnoglucoside) and chlorogenic acid was studied in a one compartment fermentation model using four individual donors (Rechner et al. 2004), the patterns of the metabolite formations varied according to the subject. The formation of 3,4-dihydroxyphenylacetic or -propionic acid from rutin and chlorogenic acid, respectively, showed maxima at 8–10 h using 10 % (w/v) suspension (Rechner et al. 2004). In contrast, using the diluted suspension (5 %, w/v) pooled and homogenized under strict anaerobic conditions, the corresponding value was shown in 2 h and complete dehydroxylation was observed in 8 h (Aura et al. 2002). The inoculum in VTT one compartment system has to be diluted 1 % in order to observe the deglycosylation of quercetin derivatives or anthocyanins in different experiments (Aura 2005). It is likely that strict application of anaerobic conditions enables faster conversions, when vulnerable anaerobic microbial suspension is used as a source of activity.

When in vitro colon models using monogastric pig and human faeces as inocula were compared, human inoculum showed more efficient fermentation for several sources of DF, whereas pig inoculum was more efficient in cellulose digestion. The pig inoculum produced less SCFA and more gas compared with the human inoculum. These differences were attributed to an adaptation of human microbiota to a more diverse diet compared to the pig feed and an adaptation of pig microbiota to a cellulose-rich diet (Jonathan et al. 2012).

25.5 General Protocol

The latest description of the model is from Nordlund et al. (2012). In vitro colon model for measurement of SCFA and phenolic acids was started by weighing 100 mg per 10 ml of incubation suspension (dry w/v) of plant foods or their

fractions to the bottles, and hydrating with 20 % of the volume of medium 1 day before inoculation. Human faeces were collected from at least 3 (usually from 4–6) healthy volunteers, who had not received antibiotics for at least 6 months and had given a written consent. Freshly passed faeces were immediately taken in an anaerobic chamber or closed in a container with an oxygen consuming pillow (Anaerocult Mini; Merck, Darmstadt, Germany) and a strip testing the anaerobiosis (Anaerotest; Merck, Darmstadt, Germany). Faecal suspension was prepared under strictly anaerobic conditions. Equal amounts of faecal samples were pooled and diluted to a 12.5 % (w/v) or 20.8 % (w/v) suspension, depending on the application and 80 % of the volume of the incubation suspension was dosed to the fermentation bottles to obtain a 10 % or 16.7 % (w/v) final faecal concentrations as described previously (Aura 2005). Lower concentrations can be used. For example 5 % suspension is suitable for isolated components, because the metabolite responses from faecal control are high in more dense inoculum and the substrate concentration should be below the saturation point of the substance to keep the substrate in the solution and not to suppress the activity of the microbiota.

The fermentation experiments were performed in triplicate and a time course of 0, 2, 4, 6, 8 and 24 h is followed using the same inoculum for all the substrates. Incubation is performed at 37 °C in tightly closed bottles and in magnetic stirring (250 rpm). Faecal background is incubated without addition of the supplements (Aura 2005). Headspace is sampled for the measurement of gas pressure (Nordlund et al. 2012) and the liquid space is sampled for SCFA and phenolic acid metabolite analyses (Nordlund et al. 2012; Aura et al. 2013).

25.6 Controls: Positive and Negative

Monitoring of metabolite background in the faecal control without substrate is crucial. In addition to this it is recommendable to use inactive microbiota as a negative control (Aura 2005). Relevant reference compounds/ingredients should be used. For instance when fermentation rate is the focus of the study, reference substrates such a "rapid" or "slow" standards can be used to make comparisons between different experiments. The controls should also be performed at least in three replicates for adequate statistical evaluation.

25.7 Read Out of the System

The read out of the in vitro colon model is caused by the interaction between microbial enzymes and the precursors and expressed as time course of metabolite formation. The precursors can be carbohydrates and the products are then SCFA or gas formation (Nordlund et al. 2012). Other precursors can be flavonoids, proanthocyanidins or plant-derived phenolic acids (ferulic acid, chlorogenic acid), which lead to the formation of benzoic acid derivatives or of hydroxylated phenylpropionic, -acetic

or -valeric acids (Aura 2008; Aura et al. 2013; Nordlund et al. 2012). Plant lignans are the precursors for enterodiol and enterolactone (the enterolignans) (Heinonen et al. 2001) and isoflavonoids for equol or O-desmethylangolensin production (Heinonen et al. 2004; Possemiers et al. 2007). Furthermore ellagitannin conversion by intestinal microbiota results in formation of urolithins (Cerda et al. 2004).

The responses should be compared always within the experiment, using the adequate number of intermediary time points, at least in triplicates and in respect to the inoculum, the responses of which is dependent on the diet of the donor. Moreover, the quantitative results should not be extrapolated between experiments and the responses should be related to the clearly indicated faecal control read out within the experiment. Thus the accepted microbial metabolite can only be one which shows at least two- to fivefold higher responses than the background metabolites from the faecal control. Therefore the results show specific metabolite profiles from each substrate. These semi-quantitative results can be obtained from non-targeted metabolomics platform for identification of new metabolites. Quantitative measures are achieved using the targeted approach, which limits the profiling to those metabolites, which are available as authentic standards. A unit to be measured should be on molar basis, because structural transformations affect the molar masses and responses based on weight are not comparable.

The one compartment colon model can be applied to comparison of fermentation rates of different sources of DF or ingredients having different characteristics (Kaur et al. 2011; Koecher et al. 2014), whereas changes in microbial population requires a semi-continuous unit operation (Hughes et al. 2008).

25.8 Summary of Advantages, Disadvantages and Limitations of the System and Contingency Plan

Table 25.2 summarizes the advantages, disadvantages and limitations of the one compartment in vitro colon model and is divided into characteristics of the operation of the model (unit operation, microbiota, anaerobiosis, pH monitoring and control, timing and sampling, stirring) and into those of the outcome (data analysis, comparison of substrates, prediction of human metabolism, and absorption). It is important to judge the limitations in context of the hypotheses that are studied in the one compartment model.

In conclusion, the one compartment fermentation model experiments perhaps do not mimic human pH changes or include absorption as the computer-controlled continuous models do, but the one compartment incubations are very suitable when the perspective is in the food matrix or its chemistry. In vitro colon models do not have membrane functions required for full mimicking of the xenobiotic metabolism. However, in vitro one compartment digestion models explain the effects of food matrix on release of components or bioconversion of food components to their microbial metabolites and they can elucidate factors affecting these processing and predict in vivo bioavailability. This field of food biochemistry is essential to elucidate phenomena, by which food chemistry turns into nutrition.

Table 25.2 Advantages, disadvantages and limitations of the one compartment in vitro colon model

Characteristics	Advantages	Disadvantages/limitations
Unit operation: batch	Metabolic studies: targeted and non-targeted measures with the same background	Microbial ecological studies are not applicable
Microbiota	Anaerobic human microbiota can stay alive: authentic activities for DF fermentation	
Anaerobic environment	Easy to operate and maintain	Requires accuracy at all stages of the process
pH monitoring/control	Monitoring gives the effect of food on pH of the incubation	Inconvenient to regulate manually since the use of strong buffer is inadequate. Low pH can suppress conversions. Labour intensive
Timing/sampling	Accurate in respect to replicates and samples in comparison	Requires resources
Stirring	Stirring enhances enzyme–substrate interactions	
Data analysis	Replicates with the same control enable comparison with substrates Non-targeted metabolomics coupled with bioinformatics and compound libraries makes the statistics and data filtration easy	Semi-quantitative Identification of new components is labour intensive
Comparison of substrates	Time course studies reveal sensitively initial rates of fermentation and dynamic metabolite profiles within one experiment	Not applicable for comparison between experiments without reference substrates Requires significant structural/chemical differences between substrates Extent of fermentation is a robust measure
Prediction for human metabolism	Predicts qualitatively bioavailable metabolites with distinctive structural changes from dietary precursors Time course studies reveal dynamic processes and intermediary products: role of microbiota in the nutritional metabolomics	Quantitative prediction is difficult, because membrane-bound converting enzymes are missing and individual variation is high in vivo. Product inhibition can occur at later time points
Absorption		Missing

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