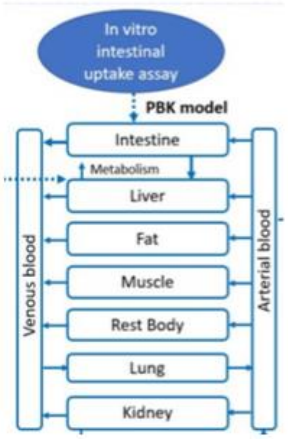
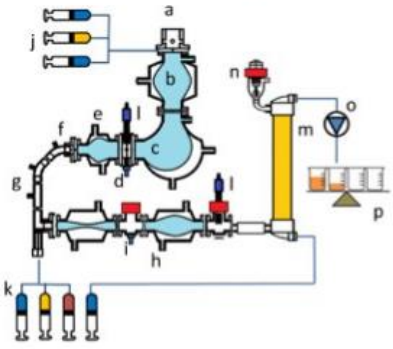
IN VITRO METHODS TO STUDY THE DIGESTION AND ABSORPTION OF SUBSTANCES IN THE GASTRO-INTESTINAL TRACT

Lisette Everaars





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Date:
6-2-2023 Leeuwarden

Abstract

Alternatives to animal testing are increasingly being used to assess the toxicological safety of substances and medicines, with a great deal of focus in recent years on alternatives for humans and small laboratory animals. Cattle, however, are also used for veterinary medicine research and education, but no complete alternative for testing with cattle is available so far. In analogue for rat and human, in vitro and in silico models could be developed for the cow as an alternative for animal testing. However, reliance on in vitro data entails a number of new challenges associated with translating the in vitro results to corresponding in vivo values. The RAAK-PRO project 'Generic bovine kinetic modelling platform, works on developing an in silico in vitro based model to predict the transfer of substances from feed to food (milk and meat from the cow) without testing in living cows. For this purpose, several in vitro assays for absorption are being developed. The aim of this study was to set-up a Caco-2 permeability assay to predict in vivo oral uptake values for the cow. Besides this, the application of an in vitro tiny-TIM digestion system for probiotic survival in the human GI-tract was studied, which can be used as an alternative for human studies.

During this study, Caco-2 cells have been cultured in a T25 flask with culture medium at 37°C with 5% CO_2 for five days, and so far, have grown sufficiently. Since the cell delivery took a long time, no further Caco-2 experiment could have been carried out, but these will be performed after this internship. A literature study towards the application of Caco-2 cells for transport studies and translating the in vitro to absorption into the cow has been included in this report and recommended to use for validation of the Caco-2 permeability assay. In addition to validate the reliability of apparent permeability data (P_{app}) results to translate in vitro data to in vivo data for the cow, it is recommended to compare expression of proteins responsible for integrity and for the transport of nutrients in the epithelial barrier.

To study the survival of a probiotic, digestion samples were taken from the intestinal compartment from the 6-hours long tiny-TIM digestive run system. One sample was taken before adding a high fat meal to the system, one sample every hour and one sample after the digestive run was completed (residue sample). After collecting the samples, they were diluted and anaerobically cultured for three days. After culturing, number of colonies were counted for every sample and the C.F.U. was calculated. The C.F.U. count of the samples taken from the digestive run without probiotic (blank run) had different results between the two runs regarding bacteria growth. Re-freezing of the blank TIM samples after the meal also has been frozen and thawed, may have had a high influence of the viability of the bacteria growth. Bacteria growth from samples of the digestion run with probiotic in a powder application, decreased during the time of the 6-hour during digestive run. The residue sample had a higher C.F.U. than the samples taken during the digestive run. The colonies of the blank run were larger and yellower then found colonies in the powder run. The colonies of the blank run did not seem present in the run with probiotic powder.

In conclusion, found colonies of the second blank run could be bacteria present in the meal itself and the probiotic bacteria could inhibit the growth of bacteria in the meal. To have more reliable results, it is recommended to perform the method of the second blank run again to gather more C.F.U. counts to get a reliable conclusion of the results of bacteria growth after a blank digestive run. However, a conclusion of the specific survival of the probiotic bacteria could not be made during this study. To determine the specific survival of the 4 different bacteria strains, present in the probiotic, it is recommended to perform a qPCR for specific DNA determination. The calibration curve of every different bacteria strains, all had a determination constant (R^2) value ≥0.95 which made the calibration curve reliable for further use in determining the DNA concentrations in the tiny-TIM samples in later studies.

Samenvatting

Alternatieven voor dierproeven worden steeds vaker gebruikt om de toxicologische veiligheid van stoffen en geneesmiddelen te beoordelen, waarbij de laatste jaren veel aandacht is besteed aan alternatieven voor mensen en kleine proefdieren. Ook koeien worden gebruikt voor onderzoek en onderwijs op het gebied van de diergeneeskunde, maar tot dusver is er geen volledig alternatief voor proeven met koeien beschikbaar. Naar analogie van rat en mens zouden voor de koe in vitro en in silico modellen kunnen worden ontwikkeld als alternatief voor dierproeven. De betrouwbaarheid van vitro data brengt echter een aantal nieuwe uitdagingen met zich mee in verband met de vertaling van de in vitro data naar overeenkomstige in vivo waarden. Het RAAK-PRO-project "Generic bovine kinetic modeling platform", werkt aan de ontwikkeling van een in-silico in vitro gebaseerd model, om de doorlaatbaarheid van stoffen van voer naar voedsel (melk en vlees van de koe) te voorspellen zonder tests bij levende koeien. Hiertoe worden verschillende in vitro assays voor absorptie ontwikkeld. Het doel van deze studie was het opzetten van een Caco-2 permeabiliteitstest om in vivo orale opnamewaarden voor de koe te voorspellen. Daarnaast werd in dit onderzoek de toepassing van een in vitro tiny-TIM verteringssysteem voor de overleving van probiotica in het menselijke GI-traject bestudeerd, dat als alternatief voor humane studies kan worden gebruikt.

Tijdens dit onderzoek zijn Caco-2 cellen gedurende vijf dagen gekweekt in een T25-fles met kweekmedium bij 37°C met 5% CO_2, en groeien tot nu toe sufficiënt. Aangezien de levering van de cellen lang duurde, konden geen verdere Caco-2 experimenten worden uitgevoerd, maar deze zullen na de stage worden uitgevoerd. Een literatuurstudie naar de toepassing van Caco-2 cellen voor transportstudies en het vertalen van de in vitro naar opname in de koe is in dit rapport opgenomen en wordt aanbevolen om te gebruiken voor validatie van de Caco-2 permeabiliteitstest. Naast het valideren van de betrouwbaarheid van de resultaten van de permeabiliteit (Papp) om in vitro gegevens te vertalen naar in vivo gegevens voor de koe, wordt aanbevolen de expressie te vergelijken van eiwitten die verantwoordelijk zijn voor de integriteit en voor het transport van voedingsstoffen in de epitheliale barrière. Om de overleving van een probiotica te bestuderen, werden verteringsmonsters genomen van het darmcompartiment van de 6 uur durende tiny-TIM verteringssysteem. Er werd één monster genomen voordat een vetrijke maaltijd aan het systeem werd toegevoegd, één monster elk uur en één monster na afloop van de digestie-run (residumonster). Na het verzamelen van de monsters werden ze verdund en voor drie dagen anaeroob gekweekt. Na de kweek werd voor elk monster het aantal kolonies geteld en de C.F.U. berekend. De C.F.U-telling van de monsters van de digestieve run zonder probiotica (blanco run) gaf verschillende resultaten tussen de twee runs wat betreft bacteriegroei. Het opnieuw invriezen van de blanco TIM-monsters nadat ook de maaltijd is ingevroren en ontdooid, kan een grote invloed hebben gehad op de levensvatbaarheid van de bacteriegroei. De bacteriegroei van de monsters van de digestie run met probiotica in poedervorm, nam af gedurende de tijd van de 6 uur durende digestie run. Het residumonster had een hogere C.F.U. dan dat van de monsters die genomen zijn tijdens de digestierun. De kolonies van de blanco run waren groter en geler dan de kolonies van de poeder run. De kolonies van de blanco run leken niet aanwezig in de run met probiotica poeder.

Conclusie: de kolonies in de blanco run kunnen bacteriën uit de maaltijd zelf zijn en kunnen de groei van bacteriën in de maaltijd beïnvloeden. Om meer betrouwbare resultaten te verkrijgen, wordt aanbevolen de methode van de tweede blanco runs opnieuw uit te voeren om meer C.F.U. data te verzamelen. Tijdens deze studie kon echter geen conclusie worden getrokken over de specifieke overleving van de probiotica bacteriën. Om de specifieke overleving van de 4 verschillende bacteriestammen aanwezig in de probiotica te bepalen, wordt aanbevolen een qPCR test uit te voeren.

Table of content

Abbreviations list

Abbreviation	Definition
ADME	Absorption, distribution, metabolism and excretion
BCRP	Breast cancer resistance protein
C.F.U.	Colony forming units
PAMPA	Parallel Artificial Membrane Permeability Assay
P_{app}	Permeability coefficient
$P_{app\ (AB)}$	Permeability coefficient apical to basal
PBPK	Physiologically based pharmacokinetic
PFZ	Peptone physiologic salt solution
P-gp	P-glycoprotein
QIVIVE	Quantitative in vitro to in vivo extrapolation
QSARs	Quantitative structure–activity relationships
SIA	Foundation Innovate Alliance (SIA)
TEER	Trans Endothelial electrical resistance

1.Introduction

Alternatives to animal testing for improving animal welfare, cost and decreasing worktime, are increasingly being used to assess the toxicological safety of substances and medicines. A great deal of focus has been seen in recent years on alternatives for humans and small laboratory animals (European Animal Research Association, 2022). The cow is one of the most common farm animals in the Netherlands. Cows are used for research into food production, animal welfare in livestock farming and in research into diseases that often occur in animals on livestock farms. Cattle are mainly used in veterinary drug research and education (Proefdiervrij, sd). So far, no complete alternative for testing with cattle is available. In analogue for rat and human, in vitro and in silico models could be developed for the cow as an alternative for animal testing. However, reliance on in vitro data entails a number of new challenges associated with translating the in vitro results to corresponding in vivo values. With the use of in vitro experiments such as cell culturing techniques, toxicity and chemical-specific parameters can be studied to predict in vivo absorption, distribution, metabolism and excretion (ADME) of medicines. Reliance on in vitro data entails a number of challenges associated with translating the in vitro results to corresponding in vivo exposures. This study wants to aid in the translation of in vitro data to in vivo situations to lower the use of cows in animal testing.

1.1 PBPK model

In vitro testing with cells can give information about the toxicity and efficacy of substances yet this does not say anything for the concentrations of when effects occur in the cow. Therefore, physiologically based pharmacokinetic (PBPK) modelling can be used to translate the in vitro data into relevant in vivo data. PBPK modelling provides an effective framework for conducting quantitative in vitro to in vivo extrapolation (QIVIVE). PBPK models are built based on the anatomical structure of a living system, with important organs or tissues listed as individual compartments interconnected through the mass transportation described by mathematical equations (Li M. Z., 2017).A PBPK model of a drug product can be used to quantitatively describe and predict drug concentration–time profiles and exposure in blood and individual organs, which are essential for efficacy/toxicity prediction and risk assessment. Their physiological structure facilitates the incorporation of in silico- and in vitro-derived chemical-specific parameters in order to predict in vivo absorption, distribution, metabolism and excretion (ADME) (Miyoung Yoon, 2012).

The foundation Innovate Alliance (SIA) project 'Generic kinetic bovine modelling platform, works on developing a model to predict the transfer of substances from feed to food (milk and meat from the cow) without testing in living cows. To study the absorption of substances for cows across the intestine no specific in vitro model has been described yet. In this project it will be studied whether relatively simple transport assays such as Caco-2, and Parallel Artificial Membrane Permeability Assay (PAMPA) are also applicable for the cow, or whether more advanced techniques for example cow intestinal organoids should be used. An accessible general assay to predict the absorption of substances is the use of Caco-2 cells, which is regarded as the gold standard for intestinal transport studies, and will be further investigated in this study (Keemink, 2018).

1.2 Parallel Artificial Membrane Permeability Assay (PAMPA)

The Parallel Artificial Membrane Permeability Assay (PAMPA) was first demonstrated by Kansy et al. (Kansy, Senner, & Gubermator, 1998) as a high-throughput screening tool to predict absorption of different compounds. Since then, several variants of PAMPA have been developed using different types of lipid barriers. The use of 96-well microtiter plates coupled with the rapid quantification by a spectrophotometric plate reader allows this system to screen a large array of compounds in a relatively short period of time (Li, Wainhaus, Uss, & Cheng, 2008). Compared to the Caco-2 permeability assay, PAMPA has advantages of cost and time effectiveness, a wider pH range and higher DMSO tolerance, and amenability to a high throughput. However, the limitation of PAMPA is that neither active nor efflux transporters are modelled by the artificial PAMPA membrane (Sun, 2017).

1.3 Caco-2 cells

Caco-2 cells are originated from a human colon carcinoma. These cells are widely used in intestinal absorption studies due to their exclusive ability to model human absorption characteristics and enable the validation of the application of Caco-2 cells in drug absorption studies (Bueno, 2015). Early studies revealed that differentiated Caco-2 cells expressed several morphological and functional properties characteristics similar to small bowel enterocytes (Lea, 2015). Importantly, Caco-2 cells are able to fully polarize into differentiated monolayers displaying brush border (microvilli) regions and tight junctions. The tight junctions and lipophilic makeup of the intestinal epithelium serve as a barrier to the absorption of drugs that are administered orally (Press & Di Grandi, 2008). Furthermore, Caco-2 cells express various phase 1 and phase 2 enzymes: CYP1A1 and CYP1B1, sulfotransferases 1A1 and 1A3 and transport proteins of the ATP-binding cassette family. For example: P-glycoprotein(p-gp), multidrug resistance-associated proteins, and breast cancer resistance protein (BCRP) (Meinl W, 2008). In an early study it was found that Caco-2 cells are able to glucuronidate various aglycones, including xenobiotics, steroid hormones, bile acids and drugs, which are substrates of different UGT isoforms (Abid A, 1995). Therefore, the Caco-2 cell line can be a useful intestinal model for drug glucuronidation and UGT regulation studies. However, levels of transporters and phase 1 metabolites such as cytochrome P450 (CYP) could be a difficulty to predict intestinal metabolism (Ohta, 2020).

1.4 Caco-2 Permeability assay

Since the permeability of humans takes place in the intestine, this could be a first step to respectively measure the absorption of substances in vitro. Applying the in vitro data combined with silico predictions, i.e., quantitative structure–activity relationships (QSARs) it could be able to develop a qualitative PBK model by predicting input values for kinetic parameters (Habka, RR Péry, Legallais, & Brochot, 2010). The permeability assay allows studying the major absorptive processes such as passive paracellular and transcellular diffusion and/or the study of active uptake and of efflux mechanisms (Alsenz & Haenel, 2003). In figure 1 a schematic explanation of this assay is shown, where medium is showed in pink, the cell monolayer in red, and the test compound in blue dots.

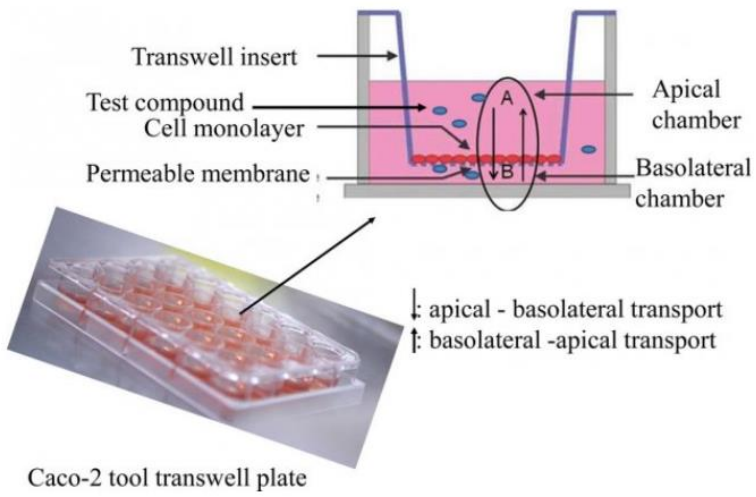

Figure 1: Schematic explanation of the overall technique of the permeability assay with an apical and basolateral chamber where a test compound is transported through a Caco-2 cell monolayer plated on a permeable membrane (Hylinkgroup.com, 2022).

1.5 Validation of a Caco-2 monolayer with the use of TEER

Trans Endothelial electrical resistance (TEER) is the measurement of electrical resistance across a cellular monolayer and is a very sensitive and reliable method to confirm the integrity and permeability of the monolayer (Srinivasan B, 2015). The classical setup for measurement of TEER (see figure 2), consists of a cellular monolayer cultured on a semipermeable filter insert which defines a partition for apical and basolateral compartments (Srinivasan B, 2015). The R_{TEER} in the figure is the total electrical resistance including the ohmic resistance of the cell layer. R_M is the cell culture medium, R_I is the semipermeable membrane inserts and R_{EMI} stands for electrode medium interface. (Srinivasan B, 2015).

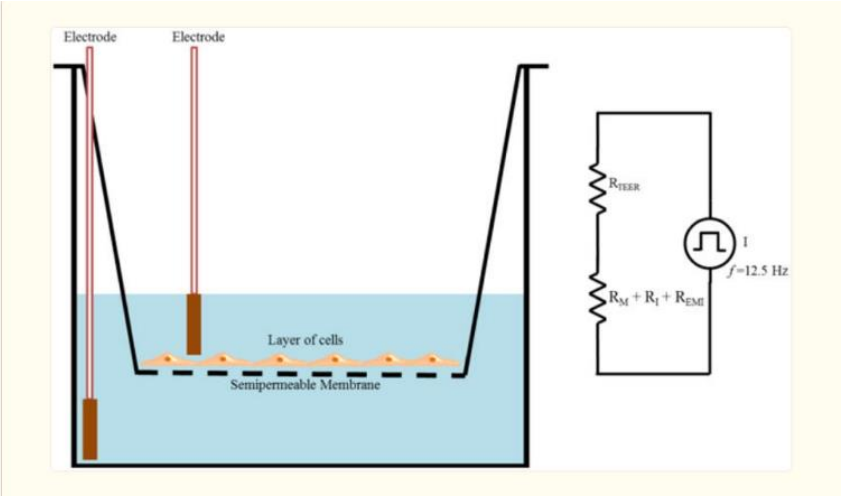

Figure 2: TEER measurement with chopstick electrodes. The total electrical resistance includes the ohmic resistance of the cell layer = R_{TEER}, the cell culture medium = R_M, the semipermeable membrane inserts= R_I and the electrode medium interface = R_{EMI}. (Srinivasan B, 2015).

1.5.1 Permeability coefficient (P_{app})

To measure the permeability rate of substances over the intestinal cells in vitro such as paracetamol, the Permeability coefficient (P_{app}) can be used. The apparent permeability coefficients (P_{app}) (cm/s) can be calculated using the following algorithm

$P_{app} = \frac{\frac{\Delta Q}{\Delta t}}{A * C0}$. Where P_{app} is the apparent permeability coefficient (cm/s), ΔQ/Δt (nmol/s) is the amount of the test compound transported to the receiver chamber in a certain time period(t), A is the Transwell membrane surface area (cm2) and C_0 is the initial concentration of the test compound in the donor compartment (μM) (Strikwold M, 2017).

1.5.2 Different mechanism for absorption

Most drugs are absorbed through the intestinal epithelium mainly by passive diffusion, which is driven by a concentration gradient. Lipophilic drugs mainly diffuse transcellularly, due to their high permeability across the plasma lipid membrane. Hydrophilic drugs with a low molecular weight might diffuse primarily via the paracellular route. In addition to passive diffusion, some drugs pass through the intestinal epithelium via active transporters. On the other hand, drug molecules, after entering the epithelial cells or reaching systemic circulation, can also be pumped back to the intestinal lumen by efflux transporters, such as P-gp (P-glycoprotein, MDR1) and BCRP (breast cancer resistance protein). See figure 3 for a simplified representation of the different mechanism involved in intestinal absorption of a drug molecule (Sun, 2017).

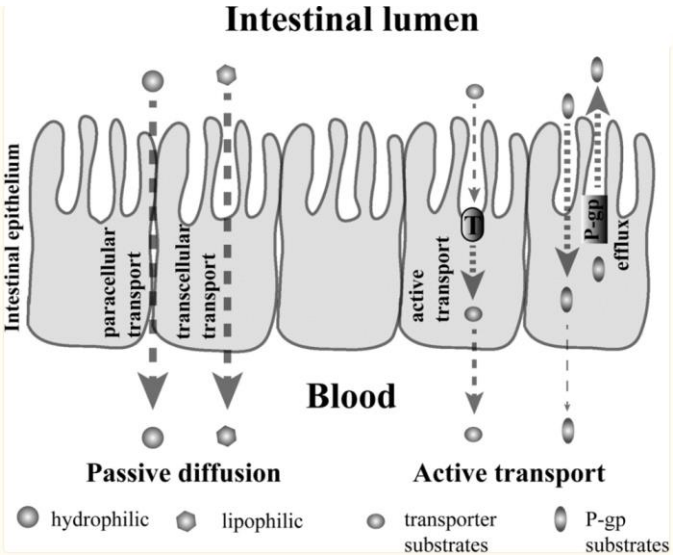

Figure 3: A simplified cartoon representation of different mechanisms involved in intestinal absorption of a drug molecule. After oral administration, drug uptake through the intestinal epithelium follows either passive diffusion or active transport. (Sun, 2017)

1.6 Tiny-TIM and Caco-2 permeability assay

A relatively new method to study the digestibility of substances before they are absorbed such as probiotics, is the use of tiny-TIM. This method models the digestive system of the human upper gastrointestinal tract. The system is computer controlled, with a two-compartmental in vitro system of the stomach and small intestine. It consists of a gastric compartment and one small intestinal compartment, which are connected by peristaltic valve pumps that successively open and close, allowing the chyme to transit over time through the tiny-TIM system (see figure 4) (TIM protocols, 2017). The system closely mimics the events in the lumen of stomach and small intestine in an accurately controlled way. The tiny-TIM can for example be used for dosage form testing on the release, bio-equivalence studies, drug–drug and drug–nutrient interactions and proof of concept studies. Samples are collected during the tiny-TIM experiments in time for analysis, giving data on the availability for absorption through the gut wall (bio-accessibility) of compounds during gastrointestinal digestion and transit. The accurate and reproducible simulation of the gastrointestinal conditions means that duplicate experiments are sufficient to reliably predict the in vivo situation. Since the Caco-2 permeability assay has already been used to predict the absorption of substances for humans, this assay could be a possibility to predict how intestinal cells react to substances such as probiotics that survives in the human intestine after oral intake.

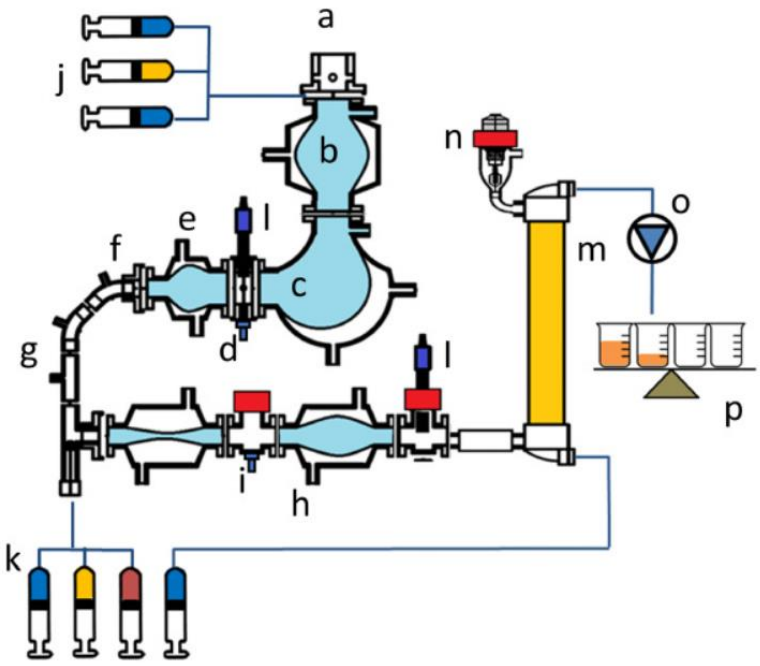

Figure 4: Schematic overview of the tiny-TIM model including an advanced gastric compartment (agc). a: meal input, b: corpus, c: proximal antrum, d: gastric port, e: distal antrum, f: pyloric valve, g: peristaltic valve, h: small intestinal compartment, i: small intestinal port, j: gastric secretions, k: intestinal secretions, l: pH electrodes, m: filtration system, n: level sensor, o: sample pump, p: sample bottles. The system consists of the advanced gastric compartment (agc) that is divided in corpus, proximal and distal antrum (b, c, e). The gastric and the intestinal compartments are each composed of a flexible silicon sleeve surrounded by tempered water within a glass jacket. The interplay established between the water inlet/outlet defines the contraction/distension pattern and therefore, the simulation of motility. The pyloric and peristaltic valves (f, g) mimic the function of the pylorus and successively open and close enabling the emptying of the gastric chyme into the small intestinal compartment. The single intestinal compartment (h) simulates the transit of the chyme through the upper small intestine. All fluids entering the small intestinal compartment are removed through a filtration system (m) and collected in sample bottles (p) at predefined time intervals (López Mármol, 2022).

1.7 Study goal, main research question and sub questions

The aim of this study was to set-up a Caco-2 permeability assay to predict in vivo oral uptake values for the cow. To succeed in this goal a research question has been formulated. The main research question is: How can the vivo oral uptake values of small chemicals be predicted for the cow, using the Caco-2 transporter assay? To answer this question different sub-questions were studied:

1)Which permeability assay(s) from literature are available and suitable to perform at the Applied Research Centre of VHL.

2) Which methods are necessary to evaluate the quality of the Caco-2 permeability assay.

3) How to translate data of the Caco-2 permeability assay to in vivo oral absorption data for a PBK model of the cow.

In figure 4 the approach of how the goal of this study will be answered is presented. Besides this, the survival of probiotics in different application forms was studied in assignment of the company Winclove. The powder form was the application form studied in this study. To answer this study question, a high fat meal, prescribed from the TIM company was added to the tiny-TIM with or without (for the Blank) probiotics. To analyse the results, anaerobic culturing methods were used for counting the colony forming units (C.F.U.). Furthermore, qPCR was used for validating the cultured samples for the bacteria present in the probiotica. The overall methods of how these questions will be answered are described further on in the method chapter.

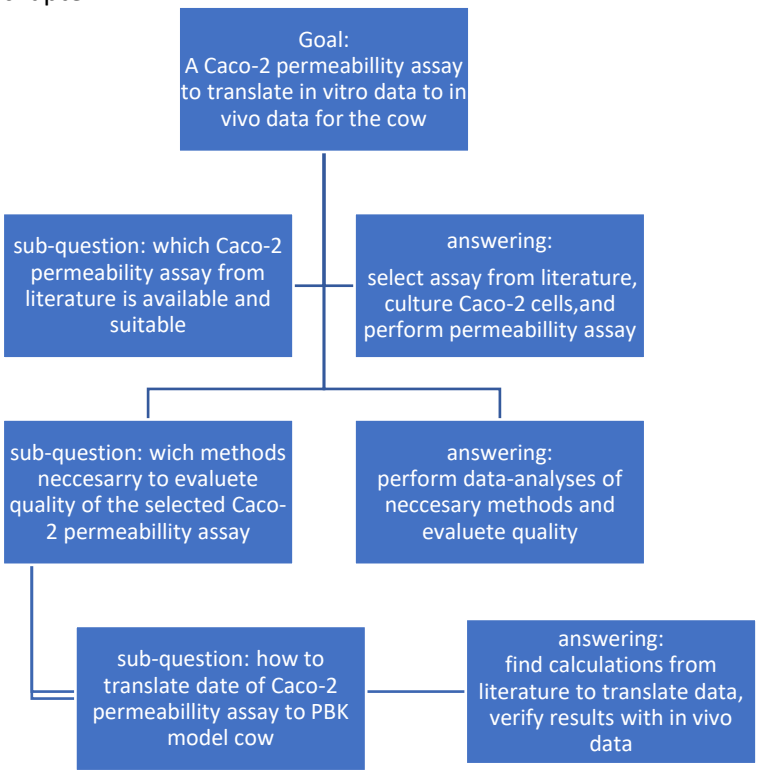

Figure 5: Shortly described Overview of Caco-2 permeability assay study goal, sub question and the overall method of answering these questions in this project.

2.Method

To set-up a Caco-2 permeability assay and to study the survival of probiotic bacteria, different methods were used such as cell culturing, anaerobic bacteria growth and qPCR. The exact methods and protocols that will be used in this project will be clear after the literature study.

2.1 Literature study toward Caco-2 permeability assay

The permeability assay of Caco-2 cells has three steps namely cell growth and feeding, transport study and subsequent sample analysis. In its traditional form, each of the three consecutive steps in the Caco-2 model is laborious, time consuming, expensive, and low throughput (Alsenz & Haenel, 2003).
A number of efforts have been undertaken by various authors and companies to simplify the model and/or increase its throughput. These approaches include reduction of the growth time of the cells, automation of the permeation study and reducing costs by either lowering the FBS concentration or by reducing the number of replicates run in the experiment.

To find out which permeability assay(s) from literature are available and suitable to perform at the Applied Research Centre of VHL, first a literature study has been carried-out by critically selecting articles based on the publication date and data of each assay using the databases of PubMed, Springer link, ScienceDirect and keywords such as: Caco-2, permeability assay, in vitro permeability assay, permeability drug testing, TEER and reviews Caco-2 permeability assays.

BCRP and P-gp were selected as criteria. First of all, BCRP is expressed on the apical membrane of small intestinal epithelial cells functions as an efflux pump with broad substrate recognition. Therefore, quantitative evaluation of the contribution of BCRP to the intestinal permeability of new chemical entities is very important in drug research and development. Secondly the efflux of Pgp decreases the bioavailability of a wide range of orally administered drugs. Drug permeability studies using the in vitro Caco-2 cell model commonly rely on small molecule modulators to estimate the contribution of Pgp to drug efflux.

2.2 Cultivation of a Caco-2 monolayer

The Caco-2 methods were based on literature studies. First a protocol was made to grow the received Caco- 2 cells to build up a stock number of cells. The culturing of cells took place in order to get enough cells with a good morphology to make a monolayer on a 24 Transwell plate for the transport experiment. A workflow for cultivation of a Caco-2 monolayer is described in appendix I.

2.2.1 Chemicals and biological materials

Dulbecco's medium w/ high glucose (DMEM, 1X) lot number: 241334, item number: 10938, storage temp: 2°C to 8°C, country of Origin: United Kingdom. Fetal Bovine Serum (FBS, f.c: 10% v/v)) lot number: 2485666RP, item number: A38402, storage temp: <= -10°C. Country of Origin: Mexico. MEM Non-Essential Amino Acids(NEAA) Solution((100X) (f.c 1%) lot number:2428393, Item number:11140, storage temp: 2°C to 8°C. Penicillin/Streptomycin stock solution: 10.000 U/mL / 10.000 µg/mL (f.c.1% = 100 U/mL penicillin and 100 (µg/mL streptomycin), lot number: 167967, item number: 15140, Storage Temp: -5°C to -20°C. L-glutamine Stock solution: 200 mM (f.c: 2mM(v/v)), lot number: 2444950, item number: 25030: , storage temp -5 to -20C. PBS(1x) lot number: 2458441, item number: 10010, storage temp: 15°C to 30°C. Company of these compounds: Thermofisher, Gibco™, United States. Dimethyl sulfoxide (DMSO, (ATCC 4-X), 0.25% Trypsin/0.53 mM EDTA in Hanks Balanced Salt Solution without calcium or magnesium, 1X (ATCC 30-2101) Company of these compounds: ATCC. Caco-2 cells (passage number 8) were kindly provided by RNO, Leiden, The Netherlands)

2.2.2 Start Caco-2 cell culture

Caco-2 cells were defrosted for approximately 2 minutes and centrifuged at 700*g* to get rid of the DMSO in the cell suspension. The cells were cultured in a T25 flask with culture medium containing high-glucose DMEM, supplemented with a 10%(v/v) heat inactivated fetal bovine serum with 1% L- glucose, 2 mM L-glutamine, 1% (v/v) minimal essential medium nonessential amino acids and 1% penicillin/streptomycin. Maintained in a 5% CO_2-humidified atmosphere at 37° for 24 hr. See appendix I for the detailed description of this procedure.

After 24 hr, 48 hr and 72hr the cells were examined to see if they were adhered and medium was changed after 72hr. The medium was refreshed by aspirating 80% of the medium and adding fresh culture medium. For passing the cells, when confluency was 80%, they were washed twice PBS and then trypsin was added to loosen the cells from the flask and incubated for approximately 5 minutes. To stop the activation of the trypsin, culture media was added and resuspended by gently pipetting the mixture up and down for 20-40 times (pipet set at 750 µl), or until the suspension was uniform. After centrifuging the cells for 5min at 700*g* the pellet was resuspend in culture medium. In three different T25 flasks, cell suspension was passaged in cell suspension to medium ratio 1:5, 1:6 and 1:8. In one T75 flask cells were passaged in cell suspension to medium ratio 1:5. Some cells will later on be frozen for further use.

For freezing the cells after different passages and having a confluency of 70-80%, cells will be washed twice with PBS, and to the flask trypsin would be added, to loosen the cells from the bottom of the flask. To the cell pellet freshly made freezing medium with a temperature of 4°C is added (DMEM medium +10% DMSO). In multiple vials, cell suspension is put in a 2mL cryovial and stored in a cryobox (mister Frosty) at -80 °C overnight. The morning after, the vials are stored in a box in -150°C for further use. In appendix I the detailed protocols for defrosting, passaging and freezing of the cells are described.

When the cell growth and morphology of the cells are good, the cells will be seeded on a polycarbonate 24 Transwell on an apical site and the basolateral chamber is filled with culture media.
The medium of both sites will be refreshed every two days for 21 days when cells are fully polarized (Hubatsch, Ragnarsson, & Artursson, 2007). The step-by step procedure for culturing the cells on the 24 Transwell is described in appendix I. After the polarization the integrity of the monolayer will be evaluated and the permeability of different compounds will be tested with the methods described further on.

2.3 Tiny-TIM

2.3.1 Tiny-TIM preparations

To determine the number of probiotic bacteria that survived digestion down to the small intestine, a high fat meal was prepared using the standard meal pre-made by the tiny-TIM company, as described in appendix III. The meal was stored at -20°C and thawed before adding it to the tiny-TIM system. Different reagents were prepared for the digestive run such as bile, electrolytes, bicarbonate, HCL and pancreatin. A cleaning solution was made for cleaning up the system after every run. After adding cleaning solution to the system and let it sit for 15 minutes, the system was washed with demi water and one time with 30% ethanol. The detailed description of every reagent needed for the digestive runs, and which one was premade or freshly made for each run can be seen in appendix IV.

The selected digestive protocol on the tiny-Tim used for each run was the General Fed HFM Dialyser (220421). ttim. in table 1 the setting for this protocol such as flow for each solution and runtime can be seen. A blank digestion run with only the high fat meal without addition of probiotic was performed in duplicate. A probiotic in powder application form was added to the high fat meal and then added to the digestion run with the tiny-TIM model. 1mL of sample from the intestine compartment was taken before adding the meal (with or without a probiotic application form) in the digestive run and one sample every hour during the 6-hour in vitro digestion run and one sample after. When taking the sample, they were stored on crushed ice until the entire run was over and then further used for CFU counting.

Table 1: Tiny-TIM protocol digestive run settings for the General Fed HFM Dialyser (220421). ttim used for each digestive run with or without addition of probiotica.

Experiment		**Flows**		**Sample scheme**		
Runtime(min)	360.00.	Acid	0.30	1-1min	6-150 min	11-300 min
HKW (min)	60	Acid+Water	0.5	2-30 min	7-180 min	12-330 min
pH Control	no	Enzymes	0.5	3-60min	8-210 min	13-360 min
		Bile	3	4-90min	9-240 min	
		Panc	3	5-120min	10-270 min	

2.3.2 Anaerobic culturing for counting colony forming units (CFU).

To determine the amount of survival of each probiotic application form, containing four different bacteria strains: Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis, Bifidobacterium animalis subsp. lactis and Lactococcus lactis.

The samples from the digestion run were diluted to have a number of colonies between 30-300 for CFU count. For the dilutions of each sample, they were diluted 1:10 in Peptone physiologic salt solution (PFZ). For the blank run without probiotic, dilution of 10^-1 and 10^-2 were used and cultured on MRS agar (Biokar), given by the Winclove company and incubated for 72h in anaerobic conditions.

For samples from a run where probiotic was added to the meal, dilutions of 10^-1 till 10^-6 were made. and 1:10 of the total volume was plated and cultured on MRS agar (Biokar) given by Winclove, for 72 hours. Only the dilutions of 10^-3-10^-6 were used for the CFU count. After counting the number of colonies on each plate For CFU calculation, or calculating CFU/mL, the following formula was used:

$$\frac{number\ of colonies}{0{,}1\ ml * dilution\ factor}.$$

For storage of the samples, a swipe of the culture of each plate was added to 1mL Peptone physiologic salt solution (PFZ) and stored at -20°C. The CFU determination was made according to a protocol described in Appendix IV of Makoto Yamagata, where the described medium has been replaced by the MRS agar (Biokar) used by Winclove. See appendix V for the protocol of this method.

2.3.3 QPCR

Besides the CFU method, cultured bacterial colonies on the plates were also identified by using a qPCR (stepOnePlus, Applied Biosystems) with specific forward, reverse primers and probes for the probiotic strains, to determine the ratio between the different strains in the samples with the use of calculating CT values. See table 2 for the primer sequences and probe sequences used for each bacteria strain.

Table 2: Overview of DNA sequences of each bacteria strain forward primer, reverse primer and probe.

Bacteria	Primer	Sequence
Bifidobacterium bifidum	Forward Reverse Probe	CAACGGCCGAGCGG GGAACGAACACGCGCA TGGGATTCCAGGCCAAACCCGTCC
Bifidobacterium animalis subsp. Lactis	Forward Reverse Probe	CTTCCCAGAAGGCCGGGT CGAGGCCACGGTGCTCATATAGA CGAAGATGATGTCGGAACACAAACACCCGG
Bifidobacterium animalis subsp. Lactis	Forward Reverse Probe	TTCAAGCCGACGTACTTGCT TGATTCGCATCATCGGTCCC TCGCCAATGCCGTCGACCAT
Lactococcus lactis	Forward Reverse Probe	TCGTCTGCCCAGATGTATTG GTCAAGTGTCGAGTGGCGTA CCATCTGCTATCCAGCCGATCGCCTCACAA

2.3.4 DNA ISOLATION

To determine the amount of each bacteria strain, DNA was isolated from the cultured samples and stored in -20°C, this was done by adding glass beads to each sample and letting them shake in the Bead Mill 4(fisherbrand), for 1 minute.

2.3.5 Nanodrop

To calculate the amount of DNA in each sample the nanodrop was used. A blank measurement of DNA free water was used to confirm that the instrument is working well and that dried-down sample on the pedestals is not a concern. The samples were gathered from the -20 freezer, put on crushed ice and calculated by a ratio of absorbance at 260 nm and 230 nm by pipetting 1 µl from each sample on the Nanodrop both measurement pedestal. This is a secondary measure of nucleic acid purity. With the amount for pure DNA in a ratio of 1.8-2.2. When the ratio was appreciably lower, this could indicate the presence of co -purified contaminants. See appendix VI for the detailed protocol.

2.3.6 QPCR

Real-time quantitative PCR is the reliable detection and measurement of products generated during each cycle of the PCR process, which is directly proportional to the amount of template prior to the start of the PCR process. TaqMan reaction mix was prepared with a Tagma probe and forward and reverse primers of each bacteria strain. The Taqman reaction mix further consisted of DNase free water and a master mix with the concentrations of each compound given by the company Winclove. The Master mix contained Taq DNA Polymerase, dNTPs and $Mg\text{-}Cl^{2+}$. The qPCR reaction was as follows: 2 minutes enzyme activation at 50˚C initial denaturation at 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and specific primer annealing temperature for 1 minute at 60 °C. All qPCR reactions were run in duplicate or triplicate and a negative control was used for each run. For the step-by step procedure of this method see appendix VII and for the volumes used of each compound in the Taqman reaction mix. First a calibration was performed from isolated DNA given by the company Winclove. With concentrations between 50 ng/ µl and 1000 ng/ µl with the known concentration of isolated DNA of each bacteria strain given by Winclove (see table 3)

Table 3: Characteristics of the isolated DNA of the four bacteria strains where DNA was measured with a Nanodrop.

W-number	Isolated Strain	DNA conc (ng/µ)	260/280 ratio	260/230 ratio
W23B	Bifidobacterium bifidum	790.9	1.91	2.17
W51	Bifidobacterium animalis subsp. Lactis	533.7	1.89	2.12
W52	Bifidobacterium animalis subsp. Lactis	573.9	1.89	2.03
W58	Lactococcus lactis	815.9	1.87	2.34

3. Results

With the methods used to set up a Caco-2 permeability assay and to study the survival of probiotic bacteria, results were gathered from the literature study, the cell culturing the C.F.U. count of each tiny-TIM digestive run and from the DNA concentration calibration curves of each bacteria strain.

3.1 Literature study

By collecting and evaluating the information of the type of well plate, used filter, the presence of p-gp and BCRP and TEER values of the different assays, a selection has been made for an available and suitable permeability assay that can be applied at the Applied Research Centre of VHL for cellular transport mechanisms.

The results of this initial literature evaluation are as follows. The standard permeability assay selected from table 5 is the 24 wells with the use of DMEM medium instead of EMEM medium based on an earlier study where the two media has been compared. Here the dynamic change of TEER of the monolayer cultured in DMEM was more stable than that cultured in MEM, and AKPase activity of the former was stronger than that of the latter (Wu XW, 2013). The polycarbonate filter will be used to follow the differentiation process in the plates. This choice had been based on price comparison and because of the transparency of the filters. To study parameters like transcellular transport and permeability, 0.4 µm filters are recommended (Lea, 2015). The 24-well assay was selected because more experiments can be performed in one time compared to the 12 wells plate. Although the 96 wells plate has a high throughput, the speed that is required to perform the experiments could be a difficulty without experience of the procedure.

The selection of optimizations of Caco-2 permeability assay is presented in table 6. The article of the 5-day assay (Caldwell, 2014) does not have data about the morphology of the monolayer and is not suitable for compounds that are positive for P-gp. Therefore, this assay was not regarded appropriate. The 7 days assay (Li N. S., 2018) seems promising looking at the morphology and comparing it to the standard 21 proliferation assay. However, to accomplish these features the use of pig intestine was considered necessary. Because the main goal of this project is to decrease animal testing and the use of animal material, this assay has not been selected. The 7-days assay with a 96 wells plate (Alsenz & Haenel, 2003) had a difficulty by having to make a plate by itself and that it is not suitable for cellular transport mechanisms.

The Ready Caco-2 kit (Alsenz & Haenel, 2003) and the re-use of the monolayer (Pires CL, 2021) seemed to be the most promising assays suitable to perform at the Applied Research Centre of VHL. Since the Ready Caco-2 kit delivers a ready to use monolayer, this is less time consuming and requires less materials. However, making a stock of Caco-2 cells to make multiple monolayers, is more profitable for testing multiple drugs than having to use plate Ready Caco-2 kit of € 1.145.00 for maximal 24 compounds per plate. The Re-use of a monolayers gives the ability to test more compounds without having to remake a monolayer for each drug required for testing. This saves time and materials when testing multiple drugs in one proliferated Caco-2 monolayer.

Table 4: Overview of general Caco-2 permeability assays described in the literature with a proliferation time of 21 days

Cells passage	Wells plate	Filter	Read outs	P-gp/BCRP	Pros and cons	Reference
Caco-2 cell line (ATCC, cat. no. HTB-37)	12 Wells (BD Falcon or Millicell from Millipore)	Polyethylene terephthalate (PET) transparent membrane	TEER and lucifer yellow (global protocol without data)	-	Step by step explained, No data of minimal TEER value	(Angelis ID, 2011)
ATC (# HTB-37)	12 Wells	Millicell PET, 12 mm, pore size 0.4 µm	TEER above 600 Ω·cm2 Papp of Lucifer yellow CH flux less than 0.5 × 10−6 cm/s	P-gp+ BCRP	High TEER value	(Fang Y, 2017)
Passages 37–40	12 Wells	Transwell® inserts (0.4 µm pored polycarbonate membrane, 12 mm diameter	Between 500 and 1000 Ω cm2	-	High TEER value	(Strikwold M, 2017)
Iken BioResource Center (Ibaraki, Japan).	24 Wells	Polyethylene terephthalate (Transwell; 0.4 µm pore size, 0.3 cm^2 area	Relative TEER values	-	Higher output than 12 wells	(Shunji Imai, 2017)
	24 Wells 1.0-µM pore size, 0.33-cm2 area	HTS Multiwell™ insert system (Becton Dickinson Labware, Franklin Lakes, NJ) with PET	TEER 300 to 540 Ω·cm2.	P-gp+ BCRP	Higher output than 12 wells TEER value can reach higher than 500	(Li e. a., 2007)
	24 wells		TEER 24W > 1000 Ω·cm2		Ready kit, cells already proliferated high TEER value	(Readycell, 2019)
ATC (# HTB-37) passage number (31-43)	24 well	Polycarbonate membrane Transwell®	> 150 Ω·cm^2 before experiment <1% LY leakage	P-gp and BCRP	Higher output than 12 wells Low TEER requirement	(Fredlund L, 2017)
Not described	96 wells	Parallel artificial membrane	Teer>400Ω	P-gp No BCRP	Higher output Sample volumes enough to perform LC/MS a challenge for HPLC	(Anthony M. Marino, 2005)

Table 5: Selected information from article publications for alterations of the general Caco-2 permeability assays.

Assay	Cells	Proliferation time	Read outs/TEER	Cost	p-gp/BCRP	Pros and Cons	reference
5- Day cultured Caco-2 monolayers	HTB-37	5 Days	Lucifer yellow (Papp)< 0,1% TEER 300-500Ω	Lower proliferation time	Not for positive or borderline P-gp compounds no BCRP test.	Cons: different Papp values, no data on morphology/ metabolites Not for positive or borderline P-gp compounds	(Caldwell, 2014)
Fast screening model for drug permeability assessment based on native small intestinal extracellular matrix	Human colon adenocarcinoma cells Caco-2 (passage 45–55; Cat. No. HTB-37) were purchased from ATCC.	7 Days on SIS hydrogel	Cell viability TEER t 596 ± 48 Ω cm^2	SIS hydrogel, (pig intestine needed) but lower cost in incubation time	Gene expression measured for (PEPT1, OATP1A2, and P-gp) as well as (BCRP, MRP2)	Pros: unchanged metabolism activity of UGT, similar AP activity, linear correlation between the Papp value, fully confluent monolayer, cons: Different gene expression, medium replacing every other day, (SIS=animal product)	(Li N. S., 2018)
7-Day, 96-Well Caco-2 Permeability Assay	Caco-2 cells (passage 103–112	7 Days	Lucifer Yellow 0.5 (± 0.35) × 10−6 cm/ no TEER data	Semi-porous polycarbonate filter, 96 plate self-made	P-gp B CRP	Pros: 96 wells plate, high throughput cons: Self-made rectangular Teflon plate, not suitable for cellular transport mechanisms	(Alsenz & Haenel, 2003)

Ready- Caco-2 kit		15 Days (before ordering the kit)	TEER 24W > 1000 Ω·cm2 96W> 500 Ω·cm2 (Papp) 24W≤ 1 x 10-6 cm/s 96W≤ 1 x 10-6 cm/s Paracellular Flux 24W≤ 0.5% 96W≤ 0.7%	Not found	MDR1-Pgp and BCRP)	Pros: 24 wells or 96 wells plate, shorter proliferation time, ready to use Transwell inserts with semi-porous polycarbonate filter medium	(Ollé, 2022)
Re-Use of Caco-2 Monolayers	ECACC 09042001, Salisbury, UK) passages 95–105	21 Days (2 days for re-use	TEER >200Ω LY	Re-use is less time needed for more experiments	P-gp, no BCRP	Pros: Similar morphology and tight junctions' leakiness of the cell monolayers decreases from day 22 to 28	(Pires CL, 2021)

3.1.1 evaluation of the Caco-2 monolayer

To evaluate the Caco-2 monolayer and to test the permeability of different compounds, different methods will be used. The transepithelial/trans endothelial electrical resistance (TEER) rises as cells proliferate and reaches the highest value at high confluency, suggesting intactness of a cell layer. On the other side, a reduced TEER value is an indicator of a compromised barrier or loosening of the tight junctions. For this project an acceptable TEER value of the Caco-2 cell monolayers was chosen of >500 Ω.

Different reference compounds to test the different mechanism of absorption can be used to assess the suitability of the monolayer. Papp values for low and high permeability control compounds must meet their respective classification to be certified as "acceptable". Talinolol, a known P-gp substrate, could be used as a control compound with $P_{app\ (Apical\ to\ Basal)}$ =0.44 0.7*10^-6 cm/s. This compound should confirm that the cells are expressing functional efflux protein transport. Acetaminophen could be selected for control of passive transport (mainly paracellular but also transcellular) $P_{app\ (AB)}$ =30*10^-6 cm/s. Propranolol could be used for transcellular transport. $P_{app\ (AB)}$ = 17.110^-6 cm/s and Lucifer Yellow for paracellular transport.

To extrapolate the in vitro derived apparent permeability coefficients (P_{app}) from the Caco-2 transport experiments to in vivo oral absorption coefficients (k_a), relative P_{app} ratios will be calculated. Based on a known vivo oral absorption coefficients (ka)of acetaminophen in cattle's=0.472 ± 0.106. Acetaminophen could be a reference compound (RC) where the relative P_{app} ratio can be calculated with the following formula: ((P_{app} _acetaminophen / P_{app} new compound) * ka acetaminophen.

3.2 Caco-2 cell culture

The Caco-2 cells have been cultured in a T25 flask with culture media during this study for 5 days at 37°C with 5% CO_2. After thawing the cells, a number of cells were floating in the culture medium and were visual as small round dots. Pictures were taken with a magnification of 40X. The number of cells increased over the culture period of 96 hr and increased attachment to the bottom of the flask was visual. Pictures were taken after 24hr where confluency was 10%, after 48hr the cells had a confluency of 30%, after 72 hours the confluency was 50% and after 96 hours of culturing, the cells had a confluency of 80% and were passaged with different cell concentrations in culture media.

3.3 C.F.U.

To determine the colony forming units after every digestive run, 1 sample was taken of the meal before the digestive run, (with or without probiotic) every hour from the intestine compartment and a residue of the digestive run and directly put on ice. The formula used to determine the C.F.U.

$$\frac{number\ of colonies}{0{,}1\ mL * dilution\ factor)}$$

The results of the bacteria growth on the MRS ager plates after a digestion run with the tiny-TIM system without added probiotic, are shown in figure 6 and 7. The plated dilutions were 10^-1 and 10^-2. Sample H0 was taken before adding the meal to the tiny-TIM system and the R sample was taken of the residue left in the system after the run.

In the results of the first blank experiment (figure 6) there can be seen that there is a small difference between the dilutions and that bacteria growth was either not present or none of these plates had colonies between 30-300, which made C.F.U. count unable to perform. See figure 7 for the results of the second blank experiment. The C.F.U. calculated was 177.000 colonies/mL for the blank run 2H sample freshly plated (177/ 0,1 mL *10^2).

From a digestive run with a meal with a probiotic in a powder application form (figure 8), the plates with dilutions of 10^-3, had all colonies mainly higher than 300. Dilutions 10^-5 and 10^-6 had colonies lower than 30, so these dilutions were not used for C.F.U. count (table 6) The dilution of 10^-4 had the most plates between 30-300 colonies and were used for determining the C.F.U. See table 7 for these C.F.U. counts. Here it can be seen that the C.F.U. counts decrease in time, and that from the residue sample a higher C.F.U. count was found than from the samples during the digestive run.

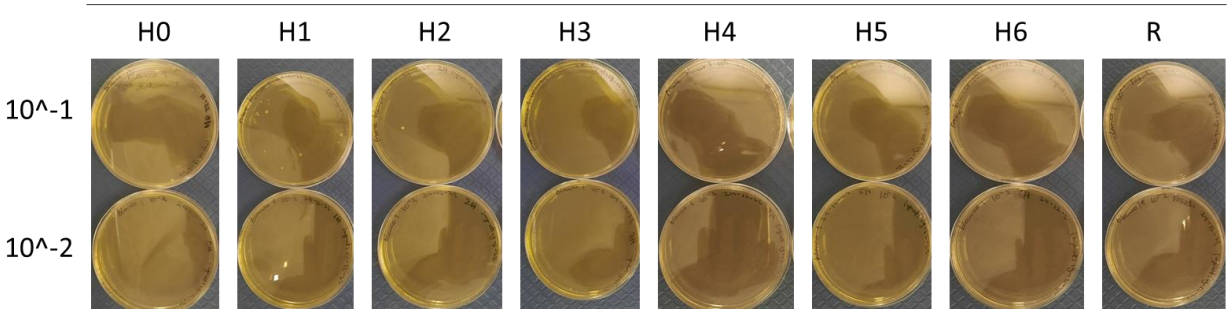

Figure 6: Results of a first blank digestive run with tiny-TIM, where 1mL sample was taken from a high fat meal before adding it to the tiny-TIM(H0) and every hour during the 6-hour run of the tiny-TIM digestion run(H1-H6). A sample of the residue of what was left in the compartments from the tiny-TIM sample was also taken(R) and frozen for approximately 3 weeks. After thawing the samples, each sample was diluted in 1:10 PFZ for dilution 10^-1 and for further dilution every dilution was added in 1:10 in new PFZ. 1:10 of each dilution were plated on MRS agar plates and incubated for 72H. Every plate had colony growth under the required amount for C.F.U. count. This figure shows a small difference between the dilutions and that bacteria growth was either not present or none of these plates had colonies between 30-300, which made C.F.U. count unable to perform.

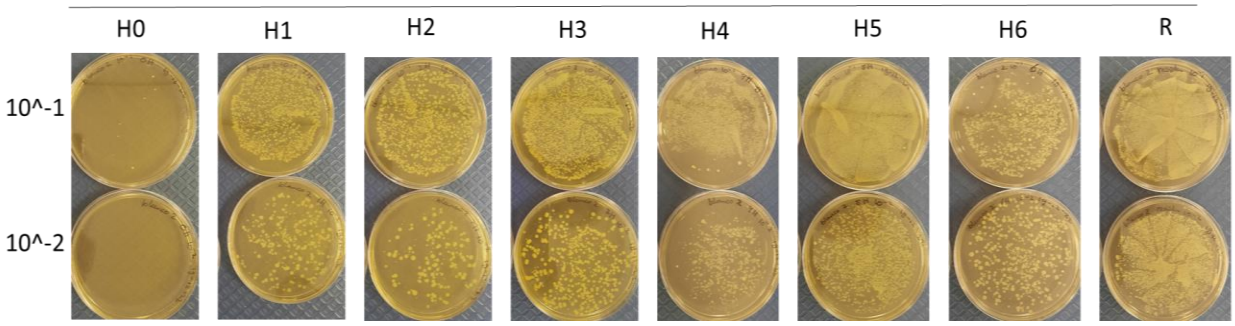

figure 7: Results of a second blank digestive run with tiny-TIM, where 1 mL sample was taken from a high fat meal before adding it to the tiny-TIM(H0) and every hour during the 6-hour run of the tiny-TIM digestion run(H1-H6). A sample of the residue of what was left in the compartments from the tiny-TIM sample was also taken(R). Each sample was diluted 1:10 in PFZ for dilution 10^-1 and for further dilution every dilution was added in 1:10 in new PFZ. Diluted sample were plated on MRS agar plates and incubated for 72H. The number of colonies increased rapidly after putting the meal (HO) to the digestive run, but it was not possible to identify the difference of number of colonies during time to identify. All of the plates with a dilution of 10^-1 had colonies over 300, and for dilution 10^-2 the sample taken after 2 hours of the digestive run(H2), was the only plate that had between the range of 20-300 colonies for C.F.U. count (which was 177). The C.F.U. calculated with this number of colonies, was 177.000 colonies/mL for the blank run 2H sample freshly plated (177/ 0,1 mL *10^-2).

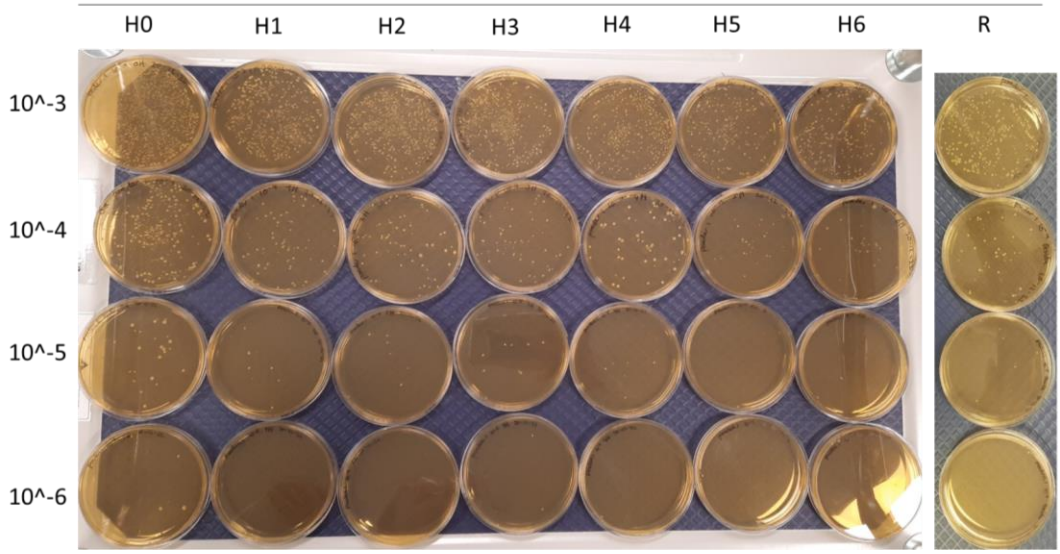

figure 8: Results of the first digestive run with probiotic powder in tiny-TIM. 1 mL sample was taken from a high fat meal with probiotic powder before adding it to the tiny-TIM(H0) and every hour during the 6-hour run of the tiny-TIM digestion run(H1-H6). A sample of the residue of what was left in the compartments from the tiny-TIM sample was also taken(R). Each sample was diluted 1:10 in PFZ for dilution 10^-1 and for further dilution 1:10 µl of every dilution was added in new PFZ. Diluted samples were plated on MRS agar plates and incubated for 72H. The plates with dilutions of 10^-3, had all colonies mainly higher than 300. Dilutions 10^-5 and 10^-6 had colonies lower than 30, so these dilutions were not used for C.F.U. count (table 7) The dilution of 10^-4 had the most plates between 30-300 colonies and was used for determining the C.F.U., see table 8 for these C.F.U. counts. Before the digestive run a small number of colonies had been formed (H0, 10^-4) The C.F.U. decreases in time, and that from the residue sample a higher C.F.U. was found than from the samples during the digestive run.

Table 6: Colonies counted from a second blank tiny-Tim digestive run. Samples were taken before putting a high fat meal in the digestive run, taken after every hour as well as when the digestive run had ended. The samples were diluted 1:10 in PFZ were dilutions 10^-3 till 10^-6 were plated on MRS agar plates anaerobically for 72 hours.

Hour (H) sample was taken	10^-3	10^-4	10^-5	10^-6
H0/start	>300	300	29	6
H1	>300	70	8	0
H2	>300	71	6	0
H3	>300	58	8	2
H4	>300	66	6	0
H5	256	26	0	0
H6	274	25	1	0
Residu	>300	45	5	0

Table 7: Calculated C.F.U./mL from samples with dilution 10^04 in PFZ taken before putting a high fat meal in the digestive run of a tiny-TIM system and taken after every hour during the digestive run as well as when the run was ended, diluted in PFZ plated on MRS agar plates anaerobically for 72 hours.

Hour(H) when sample was taken	C.F.U../mL
H0	30.000.000
H1	7.000.000
H2	7.100.000
H3	5.800.000
H4	6.600.000
H5	2.600.000
H6	2.500.000
Residu	4.500.000

3.4. QPCR

To determine whether the cultured colonies on the MRS agar plates from the samples during the digestive run are indeed the bacteria from the probiotic or different ones, a qPCR was used to identify and quantify the samples.

Therefore, first a calibration curve was performed from isolated DNA given by the company Winclove. Only the average CT values of DNA concentration between 50 ng/ µl and 400 ng/ µl were used for the calibration curve, because reliable qPCR measurement of DNA decreases with higher concentrations. Besides this, it was also expected that found DNA concentrations of the tiny-TIM samples would not reach these DNA values. All four calibration curves had a R2 value ≥0.95(figure 9) which made the found average CT value for each DNA concentration reliable for further use in determining the DNA concentrations in the tiny-TIM samples.

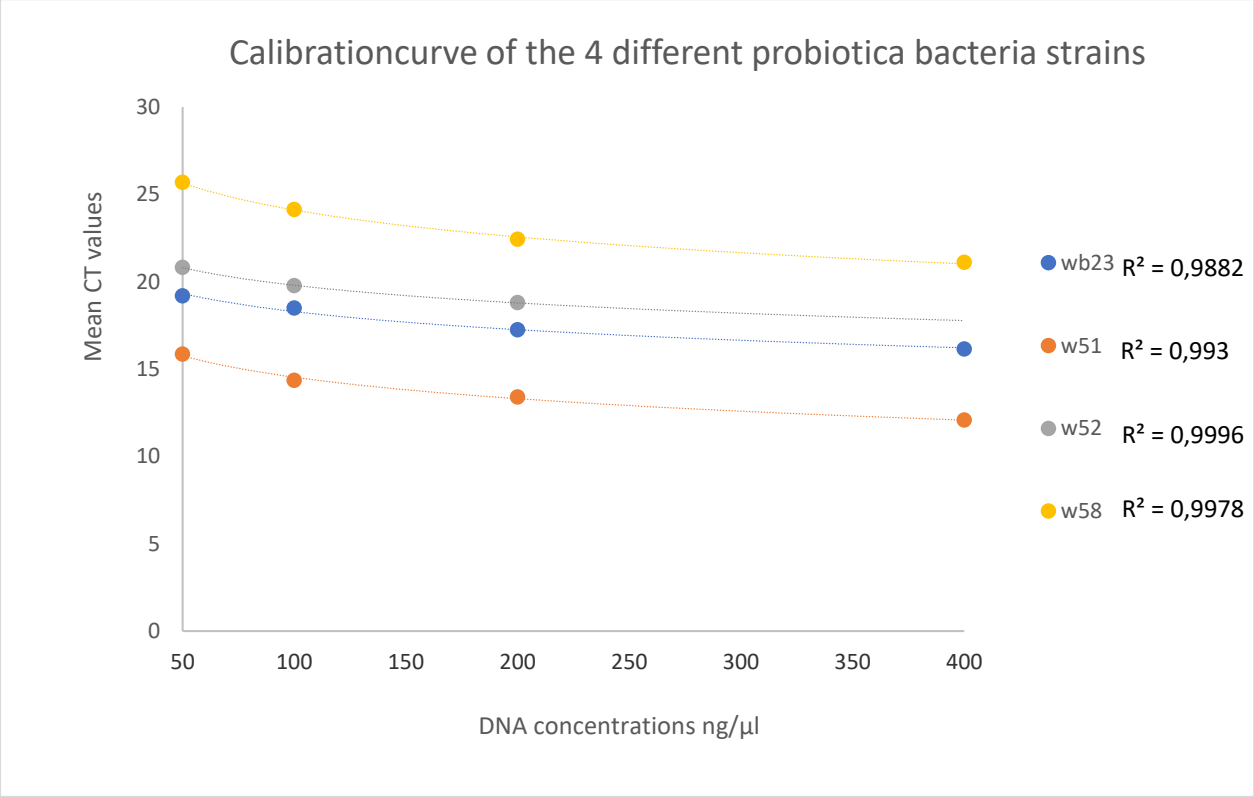

Figure 9: Calibration curve of the 4 different probiotic Bactra strains, with the Log R^2 to determine reliability of the results. In blue the average CT values of wb23 (Bifidobacterium bifidum) with R^2= 0,9882. In orange the average CT values of w51 (Bifidobacterium animalis subsp. lactis) R^2= 0,993. In grey the average CT values of w52(Bifidobacterium animalis subsp. Lactis) R^2= 0,9996 and in yellow the CT average values of w58(Lactococcus lacti) R^2=0,9978. All four calibration curves had a R2 value ≥0.95 so the found average CT value for each DNA concentration was reliable for further use in determining the DNA concentrations in the tiny-TIM samples.

4. Discussion

In this study, the aim was to set-up a Caco-2 permeability assay to predict in vivo oral uptake values for the cow. Next to this research question the survival of probiotics in different application forms was studied in assignment of the company Winclove.

Caco-2 cells has been frequently studied for the use in absorption studies due to their exclusive ability to model human absorption characteristics and enable the validation of the application of Caco-2 cells in drug absorption studies (Bueno, 2015). A relatively new method to study the permeability of orally absorbed substances such as probiotics in humans is the use of a tiny-Tim digestive system. However, for the absorption of compounds in cows so far, no in vitro model is yet available. The methods used to study the Caco-2 experiments were a literature study and cell culturing. For the tiny-TIM probiotic study the methods that were used were anaerobic bacteria growth, C.F.U. count and qPCR. These methods are further described in the method chapter with their additional protocols described in the appendixes.

During this study, since the cell delivery took a long time, no further Caco-2 experiment could have been carried out because the culturing, proliferation and transport experiments of these cells take together longer than the internship. These methods will be performed after this internship. A start in culturing the cells have been made to create a stock number of cells available for cultivating the monolayers. Some protocols about the Caco-2 cells stated to refresh the medium every two days (Angelis ID, 2011) (Fong, Clifford W, 2021) (Keemink, 2018) (Caco-2 Permeability Assay, 2022). Since only around 20% of cells were adhesive in this time, and more cells were floating in the medium, it was decided to refresh the medium after three days. In other articles they described the time of medium change of three days and 80 % confluency after 4–5 days (Lea, 2015) (ATCC), which was comparative to our results of 80% confluency after five days of culturing. Furthermore, it is expected that some fully differentiated cells will be formed with microvilli after 12–14 days cultured on Transwells, while other areas contain less differentiated cells. According to an overall protocol, the Caco-2 monolayer will be homogenously differentiated on Transwells after 18–21 days (Lea, 2015). When monolayers have been cultured for 21 days, it is expected that physiological features such as microvilli, tight junctions (Press & Di Grandi, 2008) will be formed and that various phase 1 and phase 2 enzymes (Press & Di Grandi, 2008) will be expressed. Besides this, it was found that the TEER values of Caco-2 cells cultured on Transwells, increased with higher passages and that sucrase activity (measured in 21-day-old cultures) increased through about passage 36. TEER values are expected to decline around passage 60 (Briske-Anderson, 1997).

To extrapolate the in vitro derived apparent permeability coefficients (P_{app}) from the Caco-2 transport experiments to in vivo oral absorption coefficients (k_a), relative P_{app} ratios will be calculated. Acetaminophen could be a reference compound (RC) where the relative P_{app} ratio can be calculated with the following formula: ((P_{app} _acetaminophen / P_{app} new compound) * ka acetaminophen (Strikwold M, 2017). It is important that new compounds have the same transport mechanism as acetaminophen, where the exact transport mechanism is still a point of discussion. It is stated that acetaminophen is mainly transported by passive transport but the exact form of transport is still a point of discussion (Raffa, Pergolizzi, & al, 2014).

Proliferated Caco-2 cells also express P-glycoprotein (p-gp), multidrug resistance-associated proteins, and breast cancer resistance protein (BCRP) (Meinl W, 2008). An earlier study (Bach A, 2018) examined the gene expression of certain protein expressions in the colon of the cow. Where difference in protein expression between this data and the Caco-2 monolayers could be important of proteins responsible for participating in the maintenance of cell integrity such as HSPA1A and HSPB1, gene CLDN4 and OCLN are genes responsible for the integrity of the epithelium barrier and genes such as SGLT1 and MCT1 are involved in the transport of nutrients. The presence of these proteins could also be possible to be visualised with fluorescence protein markers (Lippincott-Schwartz, 2011).

It is widely recognized that correlations exist between in vitro drug dissolution and in vivo drug absorption, but there has been made limited progress towards the development of a comprehensive model capable of predicting in vivo drug absorption based on in vitro drug dissolution. In vitro-in vivo correlation (IVIVC) digital analysis could be a tool to predict in vivo drug absorption. This can be done by scaling in vitro passive and efflux permeabilities to in vivo intrinsic permeabilities, and by scaling these in vivo intrinsic permeabilities to whole organ permeabilities using a physiological value for the in vivo membrane surface area obtained from literature data (Lu, 2011) (Ball, 2013).

With respect to digestion, there has been a growing interest in the role of the human microbiome, specifically the gut microbiome, in the development of various health problems such as neurodegenerative diseases, cancer, cardiovascular diseases, inflammatory diseases and allergic reactions. (Bellali, Lagier, Raoult, & Bou Khalil, 2019). Probiotic could be a way to maintain a balanced intestinal flora. The World Health Organization (WHO) defines probiotics as live microorganisms that provide a health benefit to the host when present in sufficient numbers in the gut (Visciglia, 2022). In the food sector, a wide range of probiotic supplements is available in different application forms. Most supplements are offered in oral capsule or powder form. According to FAO guidelines, it is important that regardless of how the probiotic supplements are administered, they arrive alive in effective amounts in the large intestine (estito S, 2020)

Despite the fact that many types of probiotic supplements are already available, much is still unclear about the exact survival of probiotics in the intestines after ingestion and the effect of the application form on this. In this study the effect of one application forms of one probiotic was studied with the use of the tiny-TIM system (TIM protocols, 2017).

From the digestive run samples were taken from the intestine compartment of the system. One sample was taken before adding a high fat meal to the system, one sample every hour and one sample after the digestive run had been completed (residue sample). After culturing, number of colonies were counted for every sample and the C.F.U. had been calculated. The difference between the two blank runs was mainly that the samples after the first run were frozen for a long period and thawed prior to bacteria culturing. When researching the viability of bacteria in frozen high fat meal, the tiny-TIM company recommended the storage of the high fat meal at -20°C where expiration would occur after 6 months (TIM protocols, 2017). The colder the storage temperature, the higher viability of bacteria is maintained. Storage at −80 °C is significantly better than at −30 °C (Foschino, 1996). But even at −80 °C, significant viability losses can occur over a few months of storage (Foschino, 1996).The performance of the experiments was taken prior to the expiration date. However, less conclusive data are available on the thawing process. When analysing frozen foods, total plate survival will vary as a function of strain or medium.

Whatever the methodology used, re-freezing will almost certainly negatively affect viable counts (Champagne, 2011). Therefore, freezing of the samples after the digestive run and later thawing them for bacteria culturing, when the meal already has been frozen and thawed, may have had a high influence of the viability of bacteria.

The C.F.U. count of the digestive run with probiotic in a powder application decreased over time which was comparable to results of an earlier studies with other probiotics in a TIM system digestive run were samples were taken every 30 minutes during a 3 hours digestive run (Tompkins & Arcand, 2011) (Arroyo-López, 2014) (Cordonnier, 2015). The higher C.F.U. count of the residue sample could be explained because this sample was taken when the run was over and no dynamic movement of the meal, which could have influenced the concentration of bacteria in this sample.
The calibration curve of the 4 different bacteria strains Bifidobacterium bifidum, Bifidobacterium animalis subsp. Lactis, Bifidobacterium animalis subsp. Lactis and Lactococcus lactis. All had a R2 value ≥0.95 which made the found average CT value for each DNA concentration reliable for further use in determining the DNA concentrations in the tiny-TIM samples in later studies. Here only the average CT values of low DNA concentration were used for the calibration curve, because reliable qPCR measurement of DNA decreases with higher concentration (Forootan, 2017).

Besides this, it has also been expected that found DNA concentrations of the tiny-TIM samples will not reach DNA values higher than 400ng (Winclove, 2023). In this study, no qPCR data was gathered about the presence of the probiotic bacteria strains during the digestive run. However, comparing the colonies from the second blank run with the run of the probiotic powder, colonies of the blank run were larger and yellower than colonies found in the powder run. This could be caused by milk bacteria present in the high fat meal (Winclove, 2023).

The colonies of the blank run did not seem present in the run with probiotic powder. This could indicate that found colonies of the blank run are bacteria present in the meal itself and that the probiotic bacteria could inhibit the growth of bacteria in the meal. Earlier studies found that probiotic bacteria showed improved survival and efficacy when delivered through milk rather than in another medium (Lee, 2015). Given limited resources, the success of one microbe may mean that another dies off – there is only so much space and food available to feed the present bacteria. Survival of bacteria depends on the ability of bacteria to compete, which they do in several ways. Beneficial microbes like Lactobacillus acidophilus create short-chain fatty acids such as lactate that lower the pH of the gut space. This favours the growth of more lactobacillus while making it difficult for other, less helpful microbes to survive. Bacteria also can produce many different types of antimicrobials, such as hydrogen peroxide, antimicrobial peptides or bacteriocins that kill off competing strains. Besides this, the nutrient availability is limited in the gut, strains that grow and ferment available nutrients remove them from the environment, and inhibit the growth of other strains. However, the metabolites they produce, such as short-chain fatty acids or sugars liberated from other compounds can also drive the growth of other microbes. When one strain grows, it takes up physical space that will limit the growth of other strains. Certain bacterial species, such as Lactobacillus rhamnosus, can also create sugar ‘bubbles’ called biofilms that strengthen the physical defences of any complementary strains, improving nutrient availability and oxygen levels while keeping other strains out (Bäumler, 2016; Thursby, 2017) (Awad, 2014) (Stubbendieck, 2016) One or multiple of these factors could have been the reason that the bacteria visible on the plates of the second blank run were not visible on the plates with probiotic bacteria added to the high fat meal thru the tiny-TIM digestive run.

5. Conclusion

The aim of this study was to set-up a Caco-2 permeability assay to predict in vivo oral uptake values for the cow. Next to this research question the survival of probiotics in different application forms was studied in assignment of the company Winclove.

During this study, Caco-2 cells have been cultured for five days, and so far, have grown sufficiently. Since the cell delivery took a long time, no further Caco-2 experiment could have been carried, out but these will be performed after this internship. A literature study toward the application of Caco-2 cells for transport studies and translating the in vitro absorption data to in vivo absorption data for the cow has been included in this report and recommended to validate the Caco-2 permeability assay using the TEER to test the integrity of the monolayer and using several test compounds like Propranolol and Lucifer Yellow, to test transcellular and paracellular transport of the Caco-2 permeability assay. To translate the in vitro obtained P_{app} data from Caco-2 data to in vivo absorption data for cow, the reported first order in vivo acetaminophen oral absorption coefficients(ka) from the cow, can possibly be applied as a reference compound to translate in vitro obtained Papp values for other test compounds with transcellular transport.

In addition to validate the reliability of apparent permeability data (P_{app}) results to translate in vitro data to in vivo data for the cow, it is recommended to compare expression of proteins responsible integrity of the epithelium barrier of the colon. Furthermore, for validation of reliability of results of translating in vitro data to in vivo for the cow, it is recommended to compare expression of proteins responsible for participating in the maintenance of cell integrity, the integrity of the epithelium barrier and genes coding for proteins involved in the transport of nutrients. In addition to the expression of these proteins, the presence of these proteins could be visualised with fluorescence protein markers.

In this study, no qPCR data was gathered about the presence of the probiotic bacteria strains during the digestive run. Results indicated that found colonies of the blank run were bacteria present in the meal itself and that the probiotic bacteria could inhibit the growth of bacteria in the meal. It is recommended to gather qPCR data of the cultured colonies from the tiny-TIM samples. The calibration curve of every different bacteria strains, all had a determination constant (R^2) value ≥0.95 which made the found mean CT value for each DNA concentration reliable for further use in determining the DNA concentrations in the tiny-TIM samples in later studies. Re-freezing of the TIM samples after the meal also has been frozen and thawed, may have had a high influence of the viability of bacteria. To have more reliable results, it is recommended to perform the method of the second blank run again and possibly with higher dilutions to be able to gather more C.F.U. counts to get a reliable conclusion of the results of bacteria growth after a blank digestive run.

6. Sustainability

There are about 1.7 billion cattle on earth. They are not only a source of meat, milk and cheese, but also of methane gas, which is released during the digestion by ruminants. Partly because of this, cattle are single-handedly responsible for two-thirds of the climate impact of all livestock. Eating less or no meat, especially beef, is therefore said to be good for the climate (Cleene, 2019). However, as stated before cows are also used for different research purposes. The feed costs per cow are € 1,011.00 per annum, excluding other costs that do not formally fall under the livestock costs. These costs are related to the livestock, such as litter and rearing costs at third parties (Stevens, 2020). Large animal models for permeability investigation have been mostly overlooked, because of the prohibitive costs of maintaining these animals in a research environment. However, from a veterinary, public health and agricultural perspective, 3D epithelial cultures from livestock animals could prove to be highly beneficial, potentially providing insight into diseases and drug treatments (Derricott, 2017).

Intestinal in vitro organoids, called enteroids, are 3D multicellular structures that resemble their in vivo counterparts more accurately than traditionally-used cell lines (Kasendra, 2018). In vitro models to predict the absorbance of compounds in the cow by the computer PBK model could be a good alternative for cow studies. In this study it was studied if Caco-2 cells could be an alternative for in vivo testing. Immortal cell lines such as Caco-2 cells are often used in research instead of primary cells. They offer several advantages, such as their cost effectiveness and user-friendliness. Furthermore, they provide an unlimited supply of material and bypass ethical concerns associated with the use of animal and human tissue. However, despite being a powerful tool, it could be a challenge to display and maintain functional features that are as reliable as primary cells. This may particularly be difficult to determine as often the functions of the primary cells are not entirely understood (Kaur, 2012). Therefore, it is important to study the accuracy of in vitro data for a reliable PBK model to replace the use of primary cells and in vivo experiments.

For probiotics, it is interesting to know that every adult carries 1.5 to 2 kilograms of bacteria. Most of these live in the intestines; they make up the so-called intestinal microbiota by the billions. These bacteria in our intestines are very important for our health. For example, they play a role in our metabolism and are important for maintaining our resistance. Through illness, stress or medication, for example, our intestinal microbiota can become disturbed, which can lead to complaints. Probiotics help restore the disturbance so that health issues will decrease (Winclove, 2023). To see which application form has a higher survival rate of the probiotic bacteria to restore the disbalance of the gut bacteria this study was performed.

The company Winclove which produces the probiotica used in this study, grants everyone in the world a good quality of life. The do this by developing indication-specific probiotics based on the power of diversity which is applicable to a broad target group. Furthermore, back in 2020, Winclove started a partnership with the non-profit organisation Justdiggit to offset its own carbon emissions by regreening the Tanzanian landscape. The focus is mainly on CO_2 reduction within the company itself. For example, the company has already switched to exclusively green electricity, packaging is being made more sustainable, staff are being encouraged to travel to work by public transport or on foot, and the company is investing in the most sustainable possible (new) premises with features such as solar panels (Winclove, 2022). So also, the promotion of probiotics has become part of their Sustainable Development Goals.

6. References

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7. Appendixes

I. Step by step protocol of cultivating Caco-2 cells (Li e. A., 2007) (ATCC) (Strikwold m, 2017

Amount compounds needed for 30mL culture meedium:

26,100 mL DMEM, (thermofisher,catalog number: 10938025)

3 mL FBS (ATCC,30-2020™) (f.c. 10% v/v FBS)

300 µl NEAA (Thermofisher, catalog number: 11140050, 100X) (f.c. 1% (v/v))

300 µl pen/strep (Thermofisher, catalog number: 15140163, 10.000 U/mL / 10.000 µg/mL) (f.c. 1% = 100 U/mL penicillin and 100 (µg/mL streptomycin))

300 µL-glutamine (Thermofisher, Catalog number: 25030081, 200 mM solution) (f.c. 2 mM (v/v))

Further needed for experiments:

3 mL trypsin (ATCC,30-2101™)

10 mL PBS (Thermofisher catalog number: 10010023)

500 µl DMSO (ATCC, 4-X™)

Defrosting and start culturing cells

1. To make the complete growth medium, add the following components to DMEM medium: 10% FBS 1% nonessential amino acids (NEAA), 2 mM L-glutamine, and 1% penicillin streptomycin. Place in a 37°C water bath for at least 15 min.
2. Prewarm centrifuge at 37°C
3. Put 8.5 mL culture medium at 37°C in a 10 mL tube and place in the water bath of 37°C.
4. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately < 2 minutes), until only a small amount of ice is present in the solution. Then thaw further by placing the vials in your hand/between fingers. Decontaminate the vial by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
5. Immediately after the cells are thawed, transfer the cell suspension (using a pipet boy with 5 mL stripette) to a 15-mL centrifuge tube containing 8.5 mL medium at a 45-degree angle, and mix the cell suspension gently by inverting the tube.
6. Centrifuge to pellet the cells for 5 minutes (speed = 700g).
7. Aspirate the supernatant (e.g., by 10 mL to a waste tube, by putting the tubes caps together so every last drop is gone)
8. In the same tube as the cell pellet, resuspend the cell pellet in 1 mL of media (use a 1000 µ pipet) by pipetting gently up and down (do this twice, so add another 1 mL and pipet gently up and down (use a clean 1000 µ pipet)). Do not let I foam!
9. Add 8 mL medium to the cell suspension. Gently invert the tube max 3 time to homogenise the cells.
10. Place the entire cell suspension into a T25 flask, make cross circulations on the table to homogenise the cell suspension in the flask) and label the flask with the cell line, passage number and date.
11. 24 h after seeding, check the cells under microscope for attachment, if attached to flask surface, slowly aspirate media to remove dead cells and add pre-warmed (37 °C) DMEM.

Passaging of cells.

1. Take a T25 flask out of the incubator and check for 80% cell confluency and add 3 mL Trypsin and incubate 5min in incubator (check if cells are detaching) Carefully loosen cells by tapping,
2. add 10mL culture media and resuspend well by pipetting over bottom of the flasks
3. Spin 5min at 190g, aspirate supernatant, loosen pellet and resuspend in 10mL culture media (1mL per T75 after splitting)
4. Add 11mL culture media to new T75 and add 1mL of cell suspension (note T-nr, Passage and date)
5. Incubate at 37°C at 5% CO_2

Freezing

1. Make sure the flasks are 80% full
2. Remove in 1 smooth movement in an empty 50mL tube.
3. Wash the cells with 10mL PBS 2X
4. Add 2mL Trypsine/EDTA to the T75 and incubate at 37oC max 5 min. Check under the microscope whether the cells are indeed detached from the bottom. The bottle can be tapped with the finger or tap the bottle gently on the table.

   6) Add 5mL of DMEM medium to the T75 bottle.

   7) Centrifuge the 50mL tube, 5 min at 1500 rpm at 4 °C degrees

   8) Remove supernatant and zip rats into the flow cabinet.

   9) Add at last moment to the pellet 5mL freezing medium (4°C DMEM medium (4,5mL) +10% DMSO(500ul)) (you can also combine 3 bottles and then add 15mL medium)

   10) Mix the medium and cells by pipetting up and down

   11) Add 1mL cells to each 2mL cryo tube

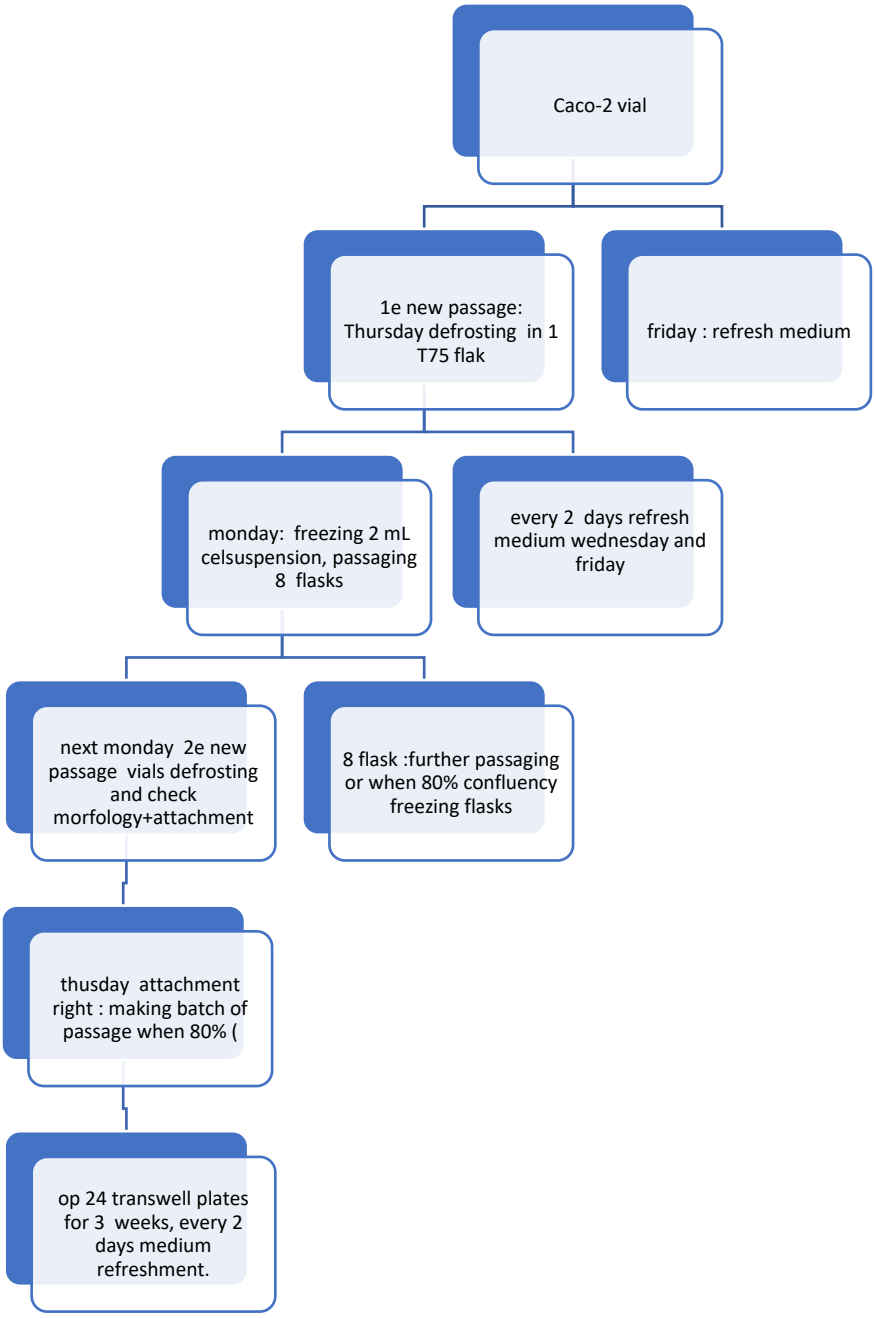
   12) Put the cells in the cryobox (mister Frosty) and put it in the -80 °C overnight

   13) Take out the cryo tubes and put them in a box in -150 °C and let them stand for at least 1 week before thawing.

Plating on 24 Transwell plate

1. Add **without touching the membrane** 250 µl PBS in the apical chamber and 750 µl in each well of a Polycarbonate Cell Culture Insert with 0.4micron pore size in 24-well plate with 0,47cm2 culture area in the basolateral chamber and put the plate in the incubator for 30 min.
2. Aspirate the PBS from the Transwells.
3. Make 2,256 *10^6 /mL cells cell suspension (0,47cm2 per well x10^5 cell per cm2) *24wells *2for 1 mL (Strikwold M, 2017) for one full 24 wells plate
4. Seed 0.5 mL of cell suspension to the apical chamber and add 1.5 mL media to the basolateral chamber.
5. Refresh the medium every 2 days subsequently. Media in both the apical and basolateral sides should be changed

Workflow


Caco-2 vial
1e new passage: Thursday defrosting in 1 T75 flak
friday : refresh medium
monday: freezing 2 mL celsuspension, passaging 8 flasks
every 2 days refresh medium wednesday and friday
next monday 2e new passage vials defrosting and check morfology+attachment
8 flask :further passaging or when 80% confluency freezing flasks
thusday attachment right : making batch of passage when 80% (
op 24 transwell plates for 3 weeks, every 2 days medium refreshment.

II. Description of prepared high-fat meal.

Materials needed are:

- Beakers/bowls that can hold > 2.2 L
- Knife
- Spatula
- Balance (+/- 0,1 g)
- Frying pan with Teflon coating
- Cooking apparatus
- Timer
- Kitchen Machine; SoloStar™II Saftpresse (specific setup pieces: 9 and
- Sample bottles for meal portions
- Labels
- Freezer (≤ -18°C)

Ingredients FDA recommended High Fat Meal (HFM)

Preferably ingredients should be obtained from Albert Heijn supermarket; AH own brand, unless specified otherwise.

Per batch the following ingredients and their amounts are required (Deviation: 1%)

1. 500 g chicken eggs size L (free-range eggs) measured without eggshells
2. 60 g breakfast bacon (Dutch: ontbijtspek)
3. 220 g slices of rusk (Dutch: beschuit) (brand: Bolletje)
4. 400 g potatoes (pre-cooked parts; freshly packed in plastic bag)
5. 80 g butter (Campina Botergoud or Landboter)
6. 1 Litre (1000 g) of full fat pasteurised milk (3.4% fat)
7. 100 g of margarine (70% fat of which ≥40% unsaturated fatty acids: Becel Gold).
8. Tap water (used after meal preparation prior to filling in portions)

Preparation one batch FDA recommended high fat meal (hfm)

- Weigh the eggs without egg shells
- Weigh the margarine in one portion of 40 g and one portion of 20 g

• (separated)

- Weigh the bacon, potatoes, milk and margarine separately
- Fry the potato slices with in 40 g margarine to golden brown on all sides
- Fry the eggs like 'scrambled eggs' in 20 g margarine
- Fry the bacon slowly in its own fat (empty frying pan) till golden brown
- Cut the baked bacon into small pieces of approximately 1x1 cm
- Divide and spread the butter on the rusk
- Cut/break the rusk in pieces of a size approximately 2x2 cm
- Mix the potato slices, the eggs, the bacon and the rusk in a big bowl with a

• spatula

- Add the fat from the bacon from the frying pan to the bowl
- Feed the mixture to the kitchen machine allow the processed mixture fall

• into a large bowl.

- Clean the rotor of the kitchen machine and feed it to the bowl.
- Mix the meal again with a spatula
- Add the milk to the mixture
- Weigh the whole mixture and add tap water to bring the final weight up to

• 2180 g

- Mix the meal with the milk and the water with the spatula to a

• homogeneous mixture

- When mixing does not lead to a homogeneous mixture feed the mixture

• once again to the food processor

- Keep on mixing and divide the meal mixture in portions of 153 -155 g per

• sample bottle Label each sample bottle according:

-HFM

-Prepared: (preparation date, dd MMM yy)

-Expired: (+6 months after preparation dd MMM yy)

-Storage: ≤ -180 °C

III. Reagents prepared for tiny-TIM digestion runs.

All used chemicals are of an analytically pure quality. Special quality demands are mentioned specifically. Only distilled, demineralised or water of equal quality is used.

Name chemical	Brand
Acetic acid 96%	Merck (100062.1000) or comparable
Bile extract porcine	Sigma (B-8631-100G)
Calcium chloride-di-hydrate ($CaCl_2$•$2H_2O$)	Sigma (223506-500G) or comparable
Hydrochloric acid (HCl) 37%	Merck (1.00317.1000) or comparable
HydroxyPropylMethylCellulose (HPMC)	Sigma (H7509)
Potassium chloride (KCl)	Sigma (P3911-1KG) or comparable
RBS Phosphatfrei	Roth (art: 92432) or comparable
Sodium acetate-tri-hydrate	Sigma (S7670-1KG) or comparable
Sodium bicarbonate ($NaHCO_3$)	Merck (1.06329.0500) or comparable
Sodium chloride (NaCl)	Merck (1.06404.1000) or comparable
Sodium hydroxide (NaOH)	Merck (1.06498.1000 or comparable
Sodium citrate tribasic dihydrate ($C_6H_5Na_3O_7$•$2H_2O$)	Sigma (S4641-1KG) or comparable

Name enzyme	Brand
Lipase from *Rhinopus* Oryzae (lipase F-AP15)	Sigma (80612-25G)
Pancreatin	Sigma (P-1750) 4 xUSP
Pepsin	Sigma (P-7012) or comparable
Trypsin	Sigma (T-9201) or comparable
α-Amylase	Sigma (A-6380) or comparable

Preparation

All batch sizes (total amount) are standardized. If a larger or smaller batch size is required, the ratio of each individual ingredient to the total amount may not change.

Pre-made for runs: Gastric electrolyte concentrate (10 x concentrated GES)

Amount: 5000 mL (5320 g) relative density: 1.064

- Take: 4827 ± 10 g water
- Add: 400 ± 3 g sodium chloride
- 85 ± 1 g potassium chloride
- 8 ± 0.1 g calcium chloride di-hydrate
- Dissolve all salts by mixing.
- Store at room temperature.
- Expired after 3 months.

Pre-made for runs: HPMC 0.4% and bile 0.04% gastric solution

Amount: 2000 mL (~2000 g)

- Take: 2000 ± 10 g hot water (80 °).
- Add: 0.8 ± 0.01 g bile powder
- Stir for 2 min with a magnetic stirrer to dissolve the bile.
- Increase stirrer speed to until a vortex exists to the bottom.
- Add slowly: 8 ± 0.1 g HPMC in small portions in the vortex of the fluid.

Stir overnight at room temperature or until the solution is clear without lumps

- and air bubbles.
- Store refrigerated between 4 - 8°C.
- Expired after 1 month.

Pre-made for runs: Small intestine electrolyte concentrate (25 x concentrated SIES)

Amount: 10000 mL (10940 g) relative density: 1.094

- Take: 9077.5 ± 100 g water
- Add: 1750 ± 10 g sodium chloride
- 87.5 g ± 2 g potassium chloride
- 25 g ± 1g calcium chloride di-hydrate
- Dissolve all salts by mixing.
- Adjust the pH to 7.0 ± 0.5 with 1 mol/l sodium hydroxide.
- Store at room temperature.
  -Expired after 3 months.

Pre-made for runs: Sodium bicarbonate (1 mol/l)

Amount: 2000 mL (2108 g) relative density: 1.054 g/mL

- Take: 1940 ± 20 g water
- Add: 168 ± 2 g sodium bicarbonate
- Dissolve all salts by heating the solution to approximately 50°C.
- Allow the solution to cool down until room temperature.
- Store at room temperature.
  -Expired after 3 months.

Pre-made for runs: Hydrochloric acid (1 mol/l)

Amount: 1000 mL

★ Perform in a fume cupboard

- Take: 500 ± 50 mL water (in a 1000 mL cylinder)
- Add: 85 ± 2 mL 37% hydrochloric acid (in a 100 mL cylinder)
- Fill up to: 1000 mL with water
- Transfer into a 1 L bottle and mix.
- Store at room temperature.
  -Expired after 3 months.

Sodium hydroxide (1 mol/l)

Amount: 250 mL (260 g)

- Take: 10 ± 0.1 g sodium hydroxide (in a 250 mL volumetric flask)
- Add: 200 mL water (approx.)
- Cool down the solution to room temperature.
- Fill up to: 250 mL with water
- Store at room temperature.
  -Expired after 3 months.

Pre-made for runs: Sodium citrate buffer (pH 7.0; 0.1 mol/l)

Amount: 1000 mL

- Take: 29.4 ± 0.5 g tri-sodium citrate-dihydrate (in 1000 mL volumetric flask)
- Add: 900 mL water (approx.)
- Homogenize
- Adjust pH to pH 7.0 (± 0.2) with 1 mol/l hydrochloric acid or with 1 mol/l
- sodium hydroxide.
- Fill up to: 1000 mL with water
- Store at room temperature.
  -Expired after 3 months.

Pre-made for runs: Sodium acetate buffer (pH=5.0; 1 mol/l)

★ Perform in a fume cupboard

- Amount: 1000 mL
- Take: 87 ± 1 g sodium acetate-tri-hydrate (in 1000 mL volumetric flask)
- Add: 900 mL water (approx.)
- Dissolve the sodium acetate-tri-hydrate.
- Add: 22 ± 0.5 g acetic acid
- Homogenize
- Adjust pH to pH = 5.0 (± 0.1) with 1mol/l sodium hydroxide or 1 mol/l
- hydrochloric acid.
- Fill up to: 1000 mL with water
- Store at room temperature.
  -Expired after 3 months.

Pre-made for runs: Cleaning solution (0.1 M sodium hydroxide in 2 % RBS)

Amount: ~10000 mL

- Take: 40 ± 2 g sodium hydroxide
- Add: 9800 ± 100 g water
- Dissolve the sodium hydroxide.
- Add: 200 ± 10 g RBS Phosphate
- Homogenize
- Store at room temperature.
  - Expired after 3 months.

Freshly made: Bile solutions with bile powder (only for TIM-1 water experiments) Fed state (4% w/v)

Amount: 250 mL (252 g)

- Take: 242 ± 5 g water
- Stir with a magnetic stirrer at room temperature.
- Add: 10 ± 0.1 g bile powder (in small portions until all is added and the
- solution is clear).
  -Use on the day of preparation.

Pre-made: Pancreatic solution

Amount: 250 mL (255 g)

- Take: 237 ± 3 g water
- Add: 17.5 ± 0.2 g pancreatic powder
- Mix during 10 min on a magnetic stirrer.
- Centrifuge the solution during 20 min at 12.500 G at 4°C.
- Use the supernatant for the experiment on the day of preparation.
- Store between 0-5 °C.

Pre-made: Trypsin solution (2 mg/cup)

Amount: 100 cups

- Take: 200 ± 5 mg trypsin (in volumetric flask of 100 mL)
- Fill up to: 100 mL with un-concentrated SIES
- Distribute the solution in portions of 1 mL in Eppendorf cups using a pipette.
- Store below -18°C.
  - Expired after 1 year, or shorter depending on the expiry date of the trypsin batch.

The enzymes lipase and pepsin will be subdivided into portions to size for

standard Tiny-TIM experiments according the following equation:

equation 1

$$Size\_subportion\,(mg/cup) = \frac{Amount\_units\_necessary\,(units/cup)}{Activity\,(units/mg)}$$

pre-made: Gastric lipase, fed state and fasted state (6000 units/cup for 300 mL)

- Take: 6000 units lipase per cup (according equation
- When the analysed value is not available, use the lowest value of activity that is given
- on the label of the package)
- Store refrigerated between 4 - 8°C.
  -Expired after the expiry date of the used lipase batch.

*Pre-made: Pepsin, f*ed and fasted state (1440000 units/cup for 300 mL)

- Take: 1440000 units pepsin per cup (according equation 1). When the
- analysed value is not available, use the lowest value of activity that
- is given on the label of the package)
- Store below -18°C.
  -Expired after the expiry date of the used pepsin batch.

Pre-made: Salivary Amylase fed state (14000 units/cup for 300 mL)

- 14000 units amylase per cup (according equation 1.) When the analysed value is not available, use the lowest value of activity that is given on the label of the package)

★ For meal:

In Eppendorf cup

Take: 1800 units amylase per cup (according equation 1. When the

analysed value is not available, use the lowest value of activity that

is given on the label of the package)

 Store below -18°C.

Expired after the expiry date of the used amylase batch.

IV. Protocol for anaerobic culturing for counting colony forming units (C.F.U.)

1. After running the tiny-Tim and collecting the samples, make dilutions of 10^-1-10^-7 in 1mL physiological salt solution
2. Spread 0,1 mL of dilution 10^-1-10^3 for blank CFU count onto nerobic MRS agar (Biokar)

3. Spread 0,1 mL of dilution 10^05 until 10^-7 for the different probiotic application forms onto anaerobic MRS agar (Biokar)
4. Incubate the plates at 37 °C for 48 hours in an anaerobic jar
5. Count the plates with 30-300 colonies
6. take the amount you plated (0.1 mL) and multiply by the dilution factor (0.01)
7. Divide the counted colonies from the dilution by the result from Step 6 to have the CFU.

V. Protocol for Nanodrop

Start computer and login with your own account. Double click on the desktop NanoDrop™ 2000 software icon and select the application of interest select Nucleic Acid application and chose DNA If the wavelength verification window appears, Ok. Add 1ul of DNAse free water and ensure the arm is down and click op

OK. wipe the upper and lower pedestals using a dry laboratory wipe and the instrument. Add again 1ul of DNAse free water and press blank. Now you are ready to measure your sample DNA amount
Note: The arm must be down for all measurements.

1. Enter a Sample ID in the appropriate field, load the first sample as described for the blank above and click.
2. After the measurement: - Simply wipe the upper and lower pedestals using a dry laboratory wipe and the instrument is ready to measure the next sample.
3. Raise the sampling arm and pipette 1µl of the sample onto the lower measurement pedestal.
4. Lower the sampling arm and initiate a spectral measurement using the software on the PC. The sample column is automatically drawn between the upper and lower pedestals and the measurement is made.
5. When the measurement is complete, raise the sampling arm and wipe the sample from both the upper and lower pedestals using a dry, lint-free laboratory wipe.

VI. Protocol qPCR

1. Put samples on ice, to thaw samples slowly.
2. Isolate DNA by putting a glass beat to each sample and let shake for 1 minute in the bead beater 4.
3. Count number of DNA with the use of a Nanodrop (see appendix III)
4. Make a DNA concentration of 100 ng/µl. Add a volume of DNA free water until total volume is 5µl.
5. For each qPCR you will have a positive control for each tested bacteria (4 in total) and a negative control for each of the primer sets of the 4 bacteria tested without DNA.
6. Make the reaction mix showed in table 4 each well has 9,6 µl of reaction mix. Put each compound on ice, except for DNA free water.
7. Add 2 µL DNA dilution (or DNA free H2O for negative control) in each well.
8. Seal the plate with clear adhesive film and make sure that the edges are sealed properly.
9. Place the plate in a CVP-2 centrifuge Vortex (mix) for 30 seconds at 1000 RPM, then centrifuge (spin) for 30 seconds at 15000 RPM.
10. Tap the plate lightly to mix the master mix with the added DNA, and control if all sample is placed in the bottom of each well.
11. Load experiment, on the and select the appropriate template. Check that the file name and data destination are correct. At data destination, the Cloud and Network Drive must be selected.
12. Click on Method, check that the temperatures are correct and the volume Click on Plate, check that the plate layout is correct for the samples and the targets.
13. Click Start run. You will get a pop-up asking if you have changed the plate, if you have done this click on Start run again. The qPCR reaction will now run.
14. Once the qPCR reaction is complete, insert the USB stick into the device and then click Transfer file. Select USB and click Transfer. The file is now saved on the USB stick. This is used as a backup. Click on Done, and switch off the device using the button on the back.

Tabel 8: pipetting schedule of compounds needed for making the Taqman reaction mix

Taqman reaction mix	Each sample in µl
Mastermix	5.0
Forward primer	0.4
Reverse primer	0.4
Taqman Probe	0.25
DNAse free H2O	2.55
Total	9.6

The qPCR run: 2 minutes activation at 50˚C
10 minutes denaturation at 95˚C
40 cyclus of [15 seconds denaturation at 95˚C and 1 minute primer annealing at 60˚C]