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International networking on in vitro colon models simulating gut microbiota mediated interactions

Book of Conference Proceedings

1st INFOGUT Annual Meeting

26-28 May 2025, Dept. of Agricultural and Food Sciences, University of Bologna, Bologna, Italy.

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1st INFOGUT ANNUAL MEETING at a glance.

The first Annual Meeting of INFOGUT was held in Bologna at Dept. of Agricultural and Food Sciences, Alma Mater Studiorum – University of Bologna, Italy, from 26 to 28 of May 2025. The meeting showcased for the first time the results obtained so far after the first 8 months of the projects, working on *in vitro* models of the gut microbiota of humans and animals, healthy and diseased. The Meeting hosted more than a dozen of oral presentations and more than 20 poster presentations form experts and young researchers coming from all the countries of the consortium.

COST ACTION CA23110: INFOGUT

The Action will be managed by the Management Committee, supported by coordinators and WG leaders to address challenges and facilitate decision-making. Three WGs (WG 1 to 3) are dedicated to scientific innovations, one WG (4) to data management and AI applications, and one WG (5) deals with regulatory aspects, including IP and ethics, along with dissemination, and educational programs (i.e. towards ITCs and YRIs). The Action Management Committee will adopt the following policies to support the Action objectives: (1) fair regional contribution and involvement of members from ITCs at different levels of Action management, (2) balanced generational representation with an enrichment in YRIs at leading positions (3) gender and diversity equity, involvement of female members in decision-making bodies. The interrelations

between all WGs will promote information sharing during WG meetings and the annual Action conference. Overall, INFOGUT aims at advancing the state-of-the-art on *in vitro* and *in silico* gut models and generating guidelines to reproduce protocols in different laboratories.

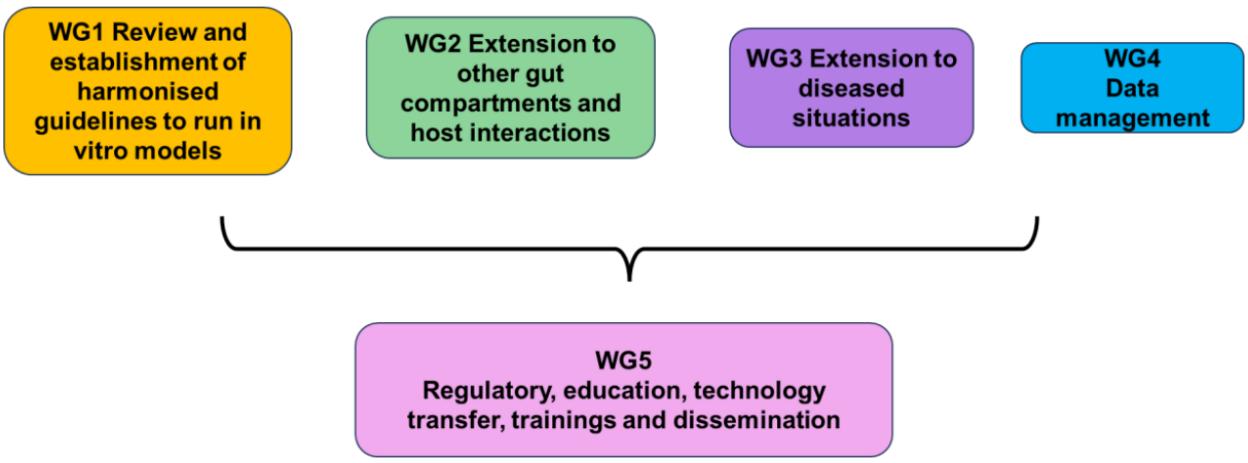

Figure 1. Organization of the project.

WG1: Review of existing *in vitro* human colon models and harmonized guidelines

WG1 Outline and introduction

WG 1 is committed to review and harmonize guidelines for using and analyzing *in vitro* gut models INFOGUT is expected to regulate and validate the many different protocols adopted by different research groups in the scientific community leading into harmonized guidelines, to substantiate the research activity, improve reproducibility, precision and accuracy of results, and reduce animal testing in food/feed science and technology. The guidelines are necessary to all comparative studies but also importantly to harmonize the protocols to run *in vitro* colonic fermentation (e.g. procedures for chemical preparation, media preparation, fecal collection and colonic model inoculation), and subsequent analytical protocols (e.g. microbiomics and metabolomics). This harmonization will ultimately give a precise, full, and robust description of colonic microbiota perturbations by any effectors.

WG1 Leaders and Team

WG1 is led by Lorenzo Nissen from University of Bologna (Italy) and Harsh Mathur from Teagasc Food Research Centre (Ireland). It is organized in 4 Tasks: Task 1.1. Harmonization of fermentation protocols (batch, continuous, multi-assays), that reviews and maps of the current *in vitro* model systems with the objective of identifying key parameters that require standardization across all models. Task 1.1 is led by Ieva Stundiene from Vilnius University (Lithuania), Edoardo Capuano from Wageningen University (The Netherlands) and Uri Lesmes from Technion Institute (Israel).

Task 1.2. Inoculum (faeces, mock communities), fermentation medium and control, that applies the standardized parameters across the range of *in vitro* colonic models available within the INFOGUT network to establish the consistency and repeatability of gut microbiota readouts under standardized conditions. Task 1.2 is leaded by Adamantini Kyriacou from Harokopio University (Greece) and Jekaterina Kazantseva from Microbiome Research at TFTAK (Estonia). Task 1.3. Non-human gut models, that reviews the available protocols and experimental conditions to propose standardized parameters for *in vitro* animal gut models. Task 1.3 is led by Jurgen Zentek from Freie Universitat Berlin (Germany) and Irene Chiesa from University of Pisa (Italy). The communication Team is leaded by Jaroslav Havlik from Czech University of Life Science (Czech Republic) and Aygun Israylova from Baku State University (Azerbaijan). Task 1.4. Guidelines and Consenus Statement, that prepares a peer review publication on the agreed standardized parameters and their application across the available *in vitro* colonic models, highlighting the key benefit of their application. The leaders of this task are still to be defined, while the other leaders are described in Figure 2.

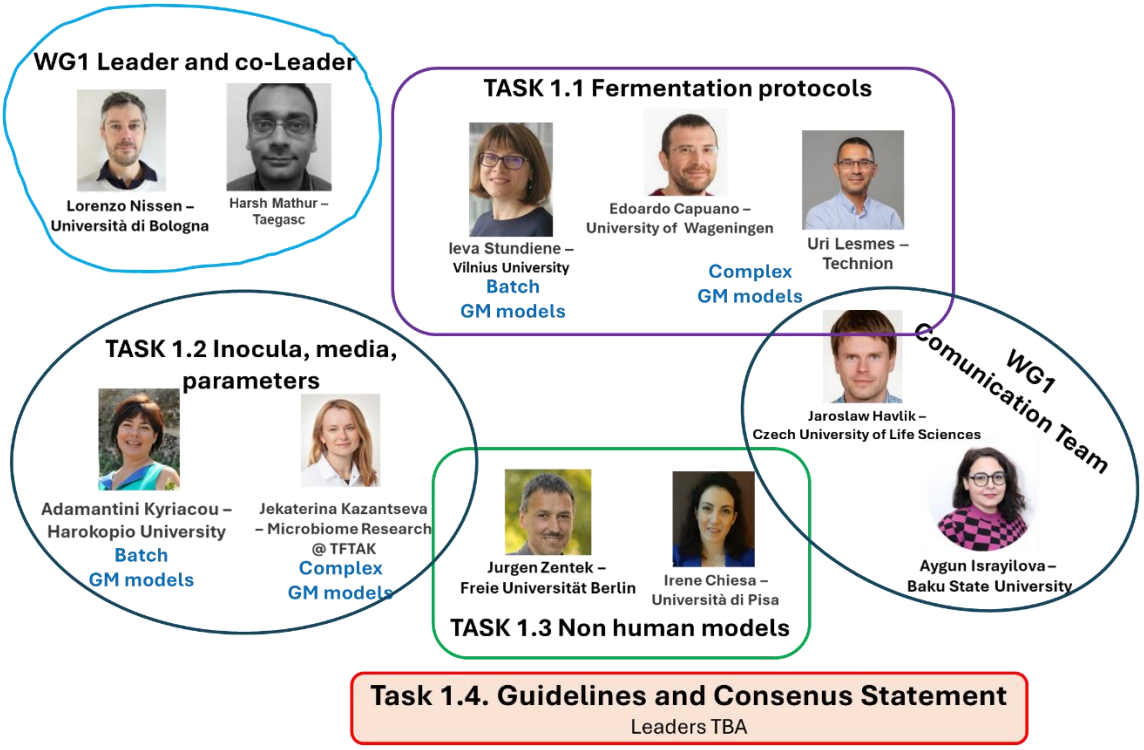

Figure 2. WG1. Organization and Leaders.

WG1 Meeting Introduction and speakers

WG1 section was composed of three oral lectures and 11 poster presentations. The oral presentations were given by Fredrick J. Warren from Quadram Institute Bioscience Norwich (UK), by Mirjiana Rajic-Stojanovic from the University of Belgrade (Serbia) and by Irene Chiesa from the University of Pisa (Italy). The poster presentations were given by Davide Addazii from the University of Bologna (Italy), by Lorenzo Nissen from the University of Bologna (Italy), by Harsh Mathur from Teagasc Food Research Centre (Ireland), by Kamila Goderska from Poznań University of Life Sciences (Poland), by Fernando Camara-Martos from University of Cordoba (Spain), by Katerina Valentova from the Institute of Microbiology of the Czech Academy of Sciences (Czech Republic), by Claude Durif from Université Clermont Auvergne (France), by Ophélie Uriot from Université Clermont Auvergne–INRAE (France), by Charlotte Deschamps from Université Clermont Auvergne (France), by Akshay Bisht from Quadram Institute Bioscience Norwich (UK), by Ezgi Doğan Cömert from Hacettepe University in Ankara (Turkey), by Chandrama Roy Chowdhury from Czech University of Life Sciences in Prague (Czech Republic), by Senem Suna from the Bursa Uludag University (Turkey), by Inés Calvete-Torre from Instituto de Productos Lácteos de Asturias - CSIC (Spain) and by Joana Cavadas from Universidade Nova de Lisboa (Portugal).

WG1 ORAL PRESENTATIONS

Modelling the impact of diet on the human gut microbiota in health and disease

Frederick J. Warren

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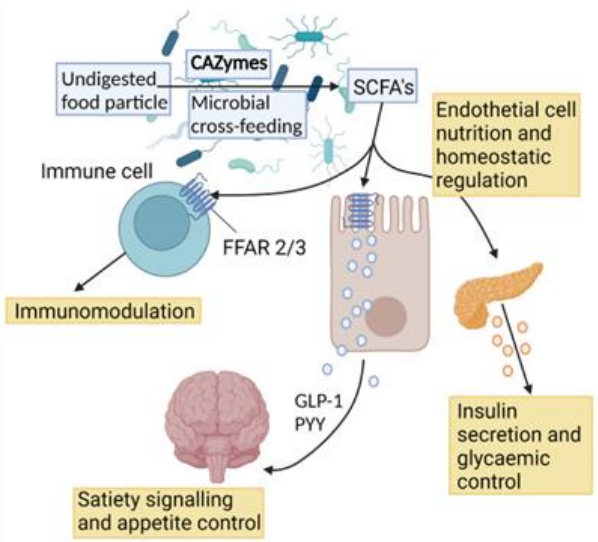

Research group presentation. The Warren Group at the Quadram Institute is a multi-disciplinary research team focused on understanding the impact of carbohydrates on the gut. The group's research spans a wide range of areas, from understanding how modifying carbohydrate quality in crop plants can impact digestion and the gut microbiome to exploring the impact of gelling carbohydrates in treatment of irritable bowel syndrome**.** Our main tools are *in vitro* gut models, combined with multi-omics analyses. We work closely with clinical partners to inform the design of our studies and obtain samples.

Introduction. The gut microbiome is a complex and diverse ecosystem distributed throughout the gastrointestinal tract. There are distinct microbial communities distributed within different regions of the GI tract, reflecting different ecological niches through gradients of pH, oxygen, mucous layer thickness and nutrient availability. The gut microbiome impacts the physiology of the host through a range of mechanisms. In this talk we focus on the role of SCFA's in triggering satiety hormone and serotonin release from enteroendocrine cells. We investigate the impact of dietary components on the gut microbiome and gut hormone signaling using a combination of the INFOGEST 2.0 protocol to mimic upper GI tract digestion and high-throughput 96-well plate-based model colon protocols combined with cell culture assays of

hormone release. The use of high-throughput methodologies allows screening of large numbers of stool donor samples from clinical studies to identify individualized microbiome responses.

Materials and Methods. Stool samples were collected from three different cohorts. Stool samples for studying the impact of inulin on gut hormone signaling were collected from the EoN clinical cohort at University Hospital Nottingham[1]. Stool samples from children with Severe Acute Malnutrition (SAM) were obtained through the MIMBLE trial[2]. Stool samples for studying the impact of high fiber wheat were obtained through healthy donors at Quadram Institute, Norwich, UK. The INFOGEST 2.0 procedure was followed for mimicking upper GI tract digestion[3]. Colonic conditions were simulated using a 96 deep-well plate format in an anaerobic cabinet held at 37 °C. Metabolomic profiles were measured using ^{1}H NMR, and microbiome composition was analyzed using shotgun metagenomic sequencing with the Illumina platform. GLP-1 and PYY releases were assayed by incubating the spent fermentation media with STC-1 cells. The released hormones were assayed by ELISA.

Results and Discussion. The first part of the talk focusses on the fermentation of inulin in clinical studies. *In vitro* fermentation results reveal clear differences between responder and non-responder groups identified in a clinical trial in their ability to degrade inulin. There are clear differences in fermentation pathways with responders producing more butyrate and non-responders producing more inulin. This correlates with microbiome composition and the abundance of key inulin degrading species. We demonstrate that the abundance of these species is related to habitual dietary FODMAP intake.[1] In the second part of the talk we demonstrate the use of gut models to study the microbiome of SAM patients. Inulin has been used unsuccessfully in clinical trials in SAM, but the reason for the failure is not known. In this study we demonstrated that following hospitalization, SAM patients receive large doses of antibiotics which reduce microbial diversity and render inulin unfermentable. Following 90 days of recovery, the microbiome has increased in diversity and inulin is now readily fermentable in a model system[4]. In the final part of the talk, we demonstrate the use of model colon systems to screen crop breeding programs for improved nutrition. Using a recombinant inbred population of wheat with elevated fiber, we demonstrate that increased fiber content in white flour is linked to increased beneficial microbial metabolites, primarily butyrate. Individualized responses are identified, and this is related to strain level differences in microbial community composition and the ability to degrade dietary fiber.

Conclusion. High throughput microbiome screening assays can be used in a wide range of clinical settings to explore microbiome impacts in a controlled setting. The findings of these studies can be used to inform the design of clinical trials, and to identify mechanistic links between microbiome composition and clinical outcomes.

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funded by the BBSRC Institute Strategic Programme Delivering Sustainable Wheat BB/X011003/1 and its constituent project BBS/E/F/000PR13650.

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2008.593150 (2024).

Exploring gut microbial dynamics in TIM-2: from fecal inocula to functional readouts

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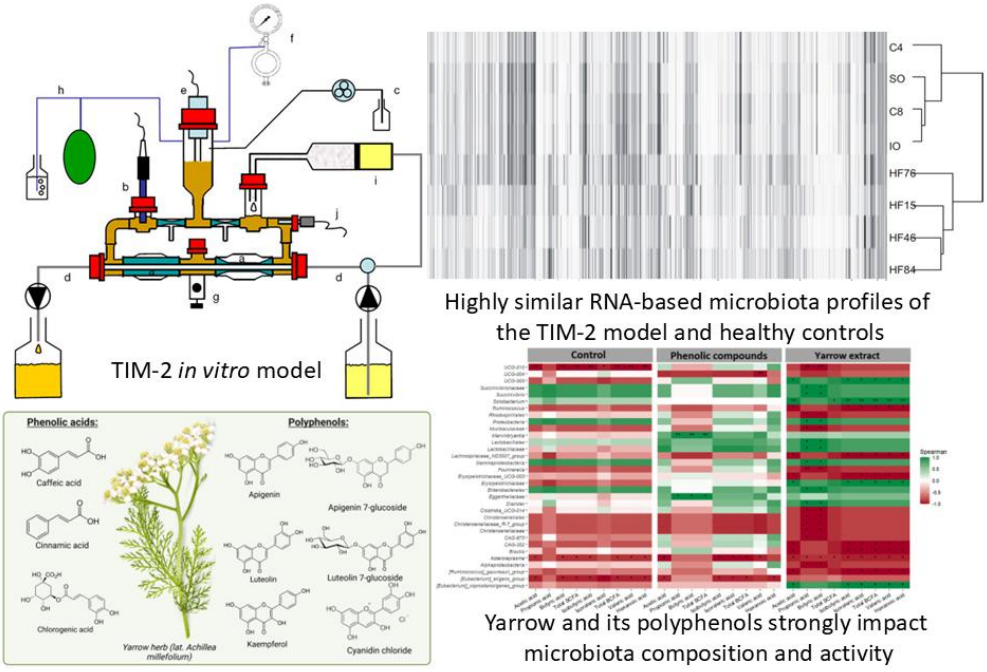

Research group presentation. The Department of Biochemical Engineering and Biotechnology at the Faculty of Technology and Metallurgy, University of Belgrade, focuses on applying enzymes and microorganisms to improve product quality and develop functional foods. Our research explores the effects of probiotics and prebiotics on gut, skin, and vaginal microbiota using *in vitro* systems and more complex models such as TIM-2 colon model (via collaboration). We analyze microbiota using molecular methods and bioinformatics tools, both *in vitro* and on clinical samples.

Introduction. The human gut microbiota plays a central role in maintaining host health and is increasingly recognized as a key etiological factor in the development or progression of a wide range of diseases that are rising in prevalence in modern societies. This growing awareness has fueled interest in identifying dietary components and bioactives capable of beneficially modulating the gut microbiota. Medicinal plants represent a particularly rich source of such bioactives, having been used for centuries in traditional medicine. However, despite their long-standing application, the interactions between medicinal plant compounds and the gut microbiota remain relatively underexplored, especially at the mechanistic level. Most active plant-derived compounds are characterized by poor bioavailability, meaning they pass largely unabsorbed through the upper gastrointestinal tract and reach the colon, where they become substrates for microbial metabolism. This colonic transformation is believed to play a central role in shaping the bioactivity of these compounds. Investigating these interactions between dietary components and microbiota in humans is challenging due to the enormous inter-individual variability in gut microbiota composition, which often obscures consistent patterns of response. *In vitro* models of the colon offer a valuable alternative by enabling controlled and reproducible experimental conditions, including standardized feeding profiles and donor-independent microbial stabilization. Additionally, daily dietary intake, which varies widely across individuals, can be precisely controlled in *in vitro* models, enhancing experimental reproducibility. These systems allow for detailed analysis of microbiota composition, function, and metabolic output in response to specific substrates or interventions. In our study, we assessed the stabilization of pooled microbiota from multiple healthy donors to create a representative inoculum that minimizes the influence of individual-specific microbiota differences. Furthermore, we outline key research insights derived from the use of TIM-2 in the study of complex microbial ecosystems, with a particular focus on microbial and mycobial responses to polyphenol-rich medicinal herb extracts, notably yarrow (*Achillea millefolium*).

Materials and Methods. The data presented originates from three separate studies investigating the gut microbiota using the validated TNO *in vitro* colon model (TIM-2), which simulates conditions in the proximal human colon. In each study, pooled fecal samples from 7 to 34 healthy adult donors were used to inoculate the TIM-2 model. Standardized fecal microbiota pools were prepared under anaerobic conditions and stored at −80 °C until use. For each experiment, the TIM-2 system was inoculated with 60 mL of fecal slurry and 90 mL of pre-reduced dialysate, followed by an 18-hour adaptation period using SIEM medium. Substrates were then administered for 72 hours at a constant flow rate of 3.3 mL/h. In the yarrow extract study, test conditions included daily supplementation with 330 mg of phenolic compounds,

delivered either as a yarrow extract or a defined phenolic acid mixture (apigenin, caffeic, and chlorogenic acids). Samples were collected from the lumen and dialysate compartments at baseline and every 24 hours (0, 24, 48, and 72 h). Short-chain fatty acids (SCFAs) were quantified by gas chromatography. Microbiota profiling was performed using both DNA- and RNA-based HITChip phylogenetic microarrays, enabling the assessment of total and metabolically active microbial communities. In the yarrow study, additional analysis was conducted using 16S rRNA and ITS-2 amplicon sequencing to evaluate bacterial and fungal shifts and taxonomic responses to treatment.

Results. The TIM-2 *in vitro* model effectively simulated colonic conditions and supported long-term cultivation of complex fecal microbiota derived from pooled samples of healthy adult donors. The system's semi-permeable dialysis membrane allowed continuous removal of fermentation products, mimicking physiological absorption in the proximal colon and preventing metabolite accumulation. Although a pronounced shift in microbial composition was observed post-inoculation, production of SCFAs was maintained, indicating preserved functional capacity of the microbiota. RNA-based HITChip profiling revealed that metabolically active microbial communities more closely resembled the original fecal microbiota than the total community inferred from DNA, underscoring the value of RNA-based approaches in functional microbiota studies. To investigate the effect of bioactive compounds, a 72-hour experiment was conducted with yarrow extract, a medicinal herb traditionally used for gastrointestinal complaints and recognized by the European Medical Agency. The effect of extract, rich in phenolic compounds such as caffeic acid, chlorogenic acid, and apigenin, was compared to a matched phenolic acid mixture. Analysis of 16S rRNA gene sequences from luminal samples showed that yarrow extract selectively stimulated butyrogenic bacterial taxa, including *Lactiplantibacillus, Eubacterium coprostanoligenes* group, and members of *Christensenellaceae, Eggerthellaceae,* and *Butyricicoccaceae*. This microbial shift was accompanied by a significant increase in SCFA levels, particularly propionate, indicating enhanced fermentative activity. While both treatments modulated microbial composition, the phenolic mixture had a more pronounced effect on *Eggerthellaceae* and *Collinsella*. Together, these findings suggest that yarrow extract exerts prebiotic-like effects by modulating both microbiota composition and its metabolic output. Beyond bacterial community dynamics, fungal populations were also examined using ITS-2 amplicon sequencing. Although *Candida* and *Saccharomyces* are among the most relevant members of the gut mycobiota, they were only sporadically detected, likely due to their low abundance in the initial inoculum. While our previous *in vitro* assays have shown that yarrow polyphenols may inhibit *Candida albicans* and promote the growth of *Saccharomyces boulardii*, these effects could not be confirmed within the complex microbial ecosystem of the TIM-2 model. These observations also underscore a potential limitation in studying mycobiota using *in vitro* systems: such models may unintentionally favor the growth of foodborne fungal spores, which are not typical colonizers of the healthy human gut. This highlights the need for improved fungal profiling techniques and careful consideration of donor background, inoculum preparation, and sample handling when investigating fungal dynamics *in vitro*.

Conclusions. The TIM-2 *in vitro* model proves to be a robust and reliable platform for investigating microbial responses to dietary bioactives under physiologically relevant colonic conditions. In this study, yarrow extract demonstrated distinct and beneficial modulatory effects on the gut microbiota, particularly by promoting SCFA–producing taxa and enhancing microbial metabolic activity. Importantly, RNA-based microbial profiling provided a more accurate reflection of the metabolically active microbiota compared to DNA-based assessments, offering deeper insight into functional shifts within the community. While the model effectively captures bacterial dynamics, the study also revealed limitations in assessing fungal populations, underscoring the need for methodological refinement and improved strategies for *in vitro* mycobiota analysis.

Acknowledgments. This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract No.451-03-68/2022-14/200135) and the Horizon Europe 2021–2027 research and innovation program, TwinPrebioEnz, grant agreement ID 101060130.

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MICRO-B: a gut microenvironment-mimicking bioreactor for the human gut microbiota *in vitro* culture

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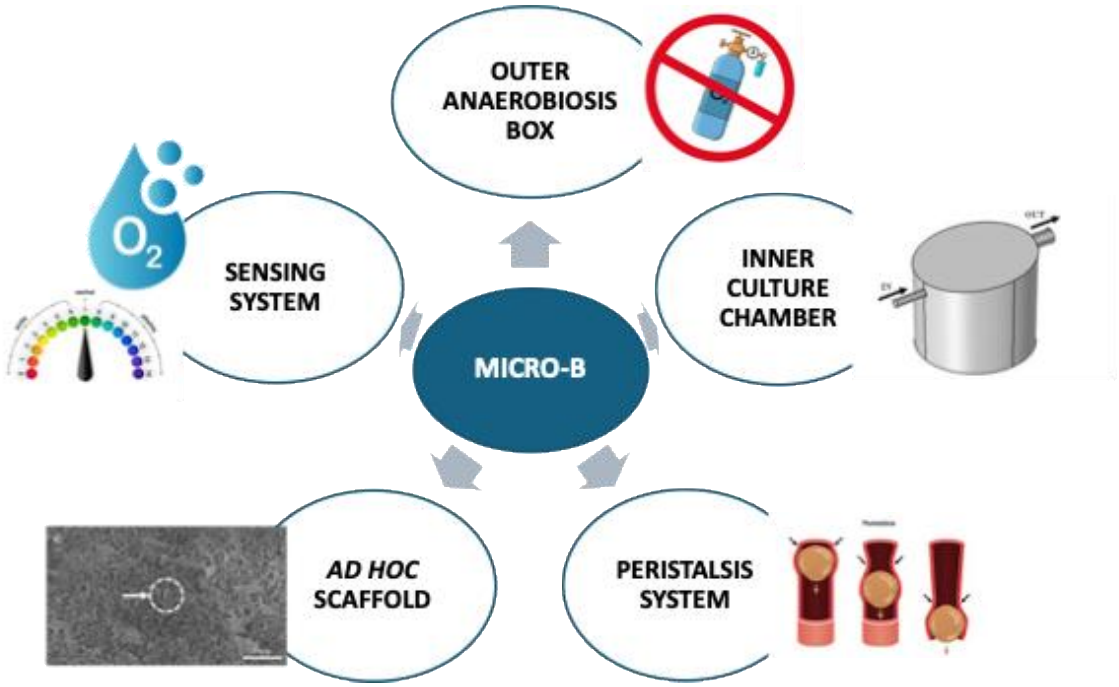


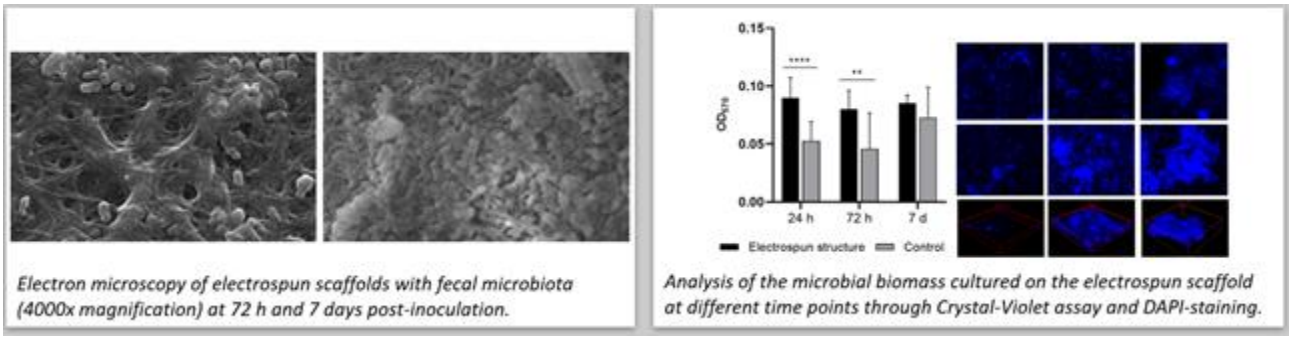
Research group presentation. MICRO-B rises from the interdisciplinary synergy between bioengineering and microbiology. The Biofabrication Group (Research Center "E. Piaggio", University of Pisa), led by Prof. Vozzi, combines advanced fabrication technologies, including 3D and 4D bioprinting, for tissue engineering, regenerative medicine and biotechnological purposes. Research activities involve the development of scaffolds, *in vitro* models, bioreactors, organs-on-a-chip, biosensors, and actuators using smart materials and biomaterials, as well as the fabrication of cutting-edge medical devices, robotic platforms for in situ bioprinting applications and AI enhanced *in silico* tools. The microbiology group of the University of Pisa, led by Prof. Ghelardi, brings its expertise on the gut microbiota, molecular microbiology, and intestinal health. The main research topics of Ghelardi's group include the human microbiota in health and disease, probiotics intended for human administration, and studying the physiology and virulence of Gram-positive bacteria.

Introduction. The human gut microbiota (HGM) is a heterogeneous microflora residing in the gastrointestinal tract. It establishes a symbiotic interaction with the host, and abnormal changes in its composition are associated with a plethora of disorders and diseases. Among the common HGM research approaches, *in vitro* models emerge as powerful tools for investigating the HGM's complex crosstalk with different anatomical regions, particularly focusing on the intimate contact between the HGM and the intestinal epithelium[1]. However, biomimicry is still an open challenge as existing analysis strategies, *in vivo* models and *in vitro* devices fail to comprehensively reproduce the complexity of the HGM itself, the gut microenvironment, and/or the kinematics of gut peristalsis. Therefore, bioengineering aims to develop novel and technologically advanced *in vitro* culture devices to overcome the bottleneck in this research area. Within this scenario, we present a scaffold specifically fabricated for the gut microbiota *in vitro* culture, designed to be host in MICRO-B, a dynamic

culture bioreactor that reproduces the gut (micro)environment and applies the physiological gut mechanical stimuli to the cultured biological sample.

Materials and Methods. MICRO-B is a patent-pending bioreactor (PCT deposit no.: PCT/IB2025/053466) for dynamic *in vitro* cultures composed of the ad hoc fabricated scaffold (see below) a deformable culture chamber, an external box generating and maintaining hypoxic environmental conditions, and a system that continuously applies a gut peristalsis-like stimulation to the sample. MICRO-B's components were developed by combining molding, subtractive and additive manufacturing techniques. The design of the components of the bioreactor was optimized through finite element simulations in Comsol Multiphysics and deformation tests. The bioreactor houses an electrospun gelatin scaffold specifically developed for the *in vitro* culture of the gut microbiota. The gelatin structures were fabricated as reported in our previous works[2,3]: 3.68% v/v GPTMS ((3-Glycidoxypropyl)-trimethoxysilane) and 10% gelatin (type A from porcine skin) were added to a 9:1 (v/v) glacial acetic and deionized water solution; which was then electrospun on a planar collector (35 KV voltage, 1ml/h feeding rate for 1.5 h and 10 cm distance between the spinneret and the collector). Finally, a fecal sample from a healthy donor was collected and prepared[2,3], and static *in vitro* cultures of the derived fecal microbiota on the gelatin scaffolds (either as is or mucin-coated) in an anaerobic atmosphere were performed up to 7 days. Biofilm biomass, viability and microbial biodiversity were evaluated.

Results and Discussion. The gelatin scaffold supported the longer period cultures (i.e., 7 days, Figure 1) of the fecal microbiota by promoting microbial adhesion and biofilm formations. Moreover, live/dead assay reported a higher number of living cells compared to the dead cells for each time point, except for day 7 (live/dead ratio slightly below 1). Interestingly, all microbial phyla composing the original fecal microbiota were preserved on the uncoated electrospun gelatine structures for up to 7 days, even the less abundant ones (*Verrucomicrobia*, *Nitrospirae*, and *Chlorobi)*. Moreover, the total bacterial load was maintained, and biodiversity was preserved.



Electron microscopy of electrospun scaffolds with fecal microbiota (4000x magnification) at 72 h and 7 days post-inoculation.

Analysis of the microbial biomass cultured on the electrospun scaffold at different time points through Crystal-Violet assay and DAPI-staining.

Figure 1. Representative results of the HGM *in vitro* culture on the electrospun gelatin scaffold.

Conclusion. Uncoated gelatin electrospun scaffolds were assessed as the best-performing support structures for the HGM *in vitro* culture since they showed better microbial adhesion, biofilm formation, microbial viability. Moreover, they supported the HGM *in vitro* culture for extended periods (up to 7 days) and preserved all the phyla present in human fecal samples, as well as the biodiversity of the microbial consortia. These scaffolds are currently used in HGM

in vitro culture experiments in dynamic conditions within the MICRO-B bioreactor. MICRO-B will enable the generation of more reliable HGM *in vitro* models that could be used to understand the HGM-host complex interplay and could boost the development of precision medicine.

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WG1 POSTER PRESENTATIONS

Mutual interaction of silymarin flavonolignans with human gut microbiota

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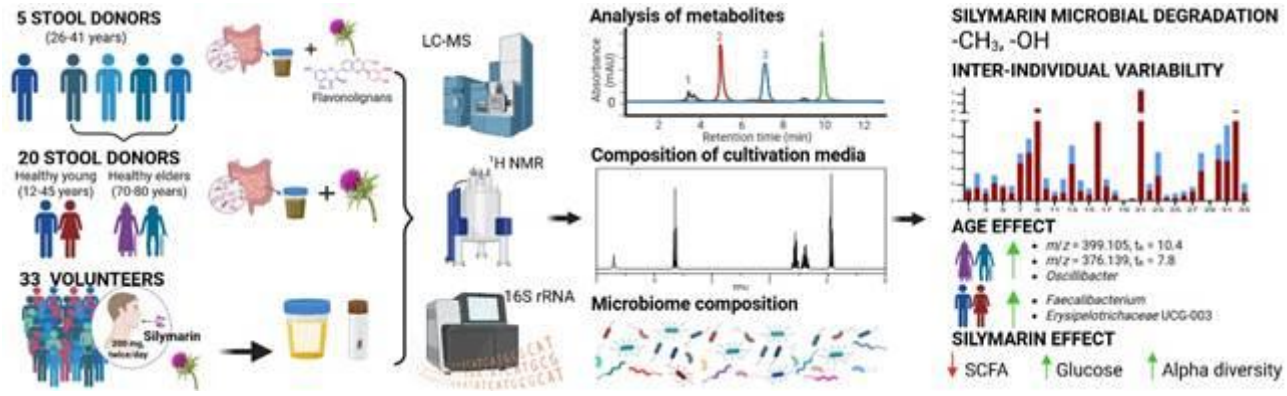

Research group presentation The Agromics Group, led by Dr. Jaroslav Havlík, specializes in metabolomics applied to food, health, and gut microbiota. They use *in vitro* models (batch and cellular), advanced analytical techniques (LC-MS, NMR), and omics approaches to study

digestion, polyphenols, and biomarkers. The group is involved in European projects on health and nutrition.

Introduction. Silymarin, an extract from fruits of the milk thistle *Silybum marianum* (L.) Gaertn., is made up of the flavonolignans silybin A and B, isosilybin A and B, silychristin A and B, silydianin and the flavanol taxifolin. Minor components include isosilychristin, 2,3-dehydrosilybin, 2,3-dehydrosilychristin and 2,3-dehydrosilydianin. The bioavailability of silymarin components is 20–50% due to low. The aim of the study was to evaluate the complex interactions of silymarin and its components with gut microbiota both *ex vivo* and *in vivo*.

Materials and Methods. Biotransformation of silymarin components was studied ex vivo, using batch incubations inoculated by fecal slurry and HPLC/MS. The interaction of the silymarin complex was investigated with a focus on aging populations. Using advanced techniques such as NGS, NMR and LC-MS, we analyzed the dual impact of the microbiome on silymarin metabolism and the effect of silymarin on the microbiome's structure and function. Finally, in 33 healthy adult male volunteers, who received 200 mg of silymarin orally twice daily for three months, formation of metabolites in urine and feces was evaluated by HPLC/MS and bacterial composition of feces was investigated by NGS.

Results and Discussion. At 200 mg/L the flavonolignans were resistant to the metabolic action of microbiota. At 10 mg/L, biotransformation of flavonolignans was much slower than that of taxifolin. Silybin, isosilybin, and 2,3-dehydrosilybin underwent mostly demethylation, silychristin was predominantly reduced. Silydianin, 2,3-dehydrosilychristin and 2,3-dehydrosilydianin were reduced and decarbonylation and cysteine conjugation proceeded. No low-molecular-weight phenolic metabolites were detected. Silymarin significantly altered the metabolism of the gut microbiota, decreasing short-chain fatty acid production and glucose utilization. Healthy elders (70–80 years) showed a significant increase in a specific catabolite associated with *Oscillibacter*. Conversely, healthy young donors (12–45 years) exhibited faster breakdown of silymarin components, particularly isosilybin B, which negatively correlated with higher abundance of *Faecalibacterium* and *Erysipelotrochaceae UCG-003*. In volunteers, a correlation was found between the number of metabolites and the composition of the intestinal microbiota.

Conclusion. Silymarin interacts with the microbiome, influences its composition and metabolic profile. The amount of metabolites in feces depend on the composition of the gut microbiota that is responsible for large inter-individual differences

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In vitro colonic fermentation, and the impact on the intestinal microbiota of allyl-and benzyl-isothiocyanate from mustards

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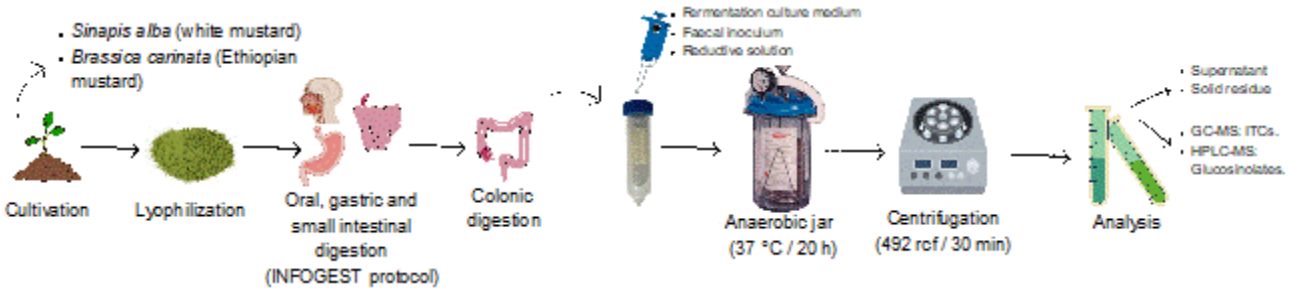

Research group presentation. BIOTRACE group (http://www.uco.es/biotrace/es/) has accumulated experience of more than 25 years dedicated to the study of the bioaccessibility of inorganic micronutrients and bioactive compounds. To this end, we have been working on the development and validation of *in vitro* gastrointestinal digestion models, the use of cell lines and colonic fermentation models with intestinal microbiota.

Introduction. Isothiocyanates (ITCs) are phytochemicals, naturally occurring in a wide variety of vegetables such as mustards. They are of great interest in human nutrition due to their antioxidant, anti-inflammatory, and anti-cancer properties. They are produced by hydrolysis of glucosinolates by the enzyme myrosinase present in the idioblasts of their vegetable cells. Nevertheless, ITCs can also be produced by the action of the intestinal microbiota colonizing the large intestine of humans. The metabolism of glucosinolates and ITCs production in the distal part of the digestive tract have been understudied.

Materials and Methods. Green parts (leaves) from two mustard varieties, white mustard (*Sinapis alba*) and Ethiopian mustard (*Brassica carinata*) were submitted to the assayed conditions of the INFOGEST digestion method following by an *in vitro* colonic fermentation. The effect of allyl- and benzyl-isothiocyanate (ITC), two bioactive compounds derived from mustard, was studied in large intestine. Genomic DNA was extracted from each sample after *in vitro* colonic fermentation. DNA sequencing was conducted.

Results and Discussion. ITC levels decreased in large intestine. Additionally, treatments with ITCs supplemented to vegetable matrices had a modulatory prebiotic effect on the intestinal microbiota, not only by altering the overall composition of the microbiota but also by promoting

beneficial bacteria (*Bifidobacterium*) and reducing potentially pathogenic genera (*Enterobacter, Klebsiella*). Results also suggest that ITC-rich environments can favor the increase of specific microbial populations with the ability to metabolize these molecules and other derivatives.

Conclusion. The simulation of the colonic fermentation reduced allyl- and benzyl- ITC levels from the non-bioaccessible fraction of *Brassica carinata* and *Sinapis alba*. The decrease of the ITCs concentration at the end of the colonic fermentation could indicate possible production and effect by intestinal microbiota. The shallow shotgun metagenomic analysis of the microbiota changes during the colonic fermentation simulation revealed that treatments with ITCs and cruciferous matrices had a significant impact on the modulation of intestinal microbiota. ITC-rich environments can favor the increase of specific microbial populations with the ability to metabolize these molecules and other derivatives. These findings are promising for the development of dietary or therapeutic strategies that use ITCs and specific foods to positively influence intestinal health.

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Acknowledgments/funding. This research was funded by the Project “Desarrollo y caracterización de alimentos funcionales obtenidos a partir de crucíferas cultivadas en condiciones ecológicas y nuevas tec-nologías de procesado (CRUCITECNO-ECOL). Ref: ProyExcel_00789 financed by Andalusian Government. Secretaría General de Universidades, Investigación Tecnología, Proyectos de Excelencia 2021.

In vitro gastrointestinal model to study stability of encapsulated and non-encapsulated *Latobacillus sp.* and *Lactococcus*

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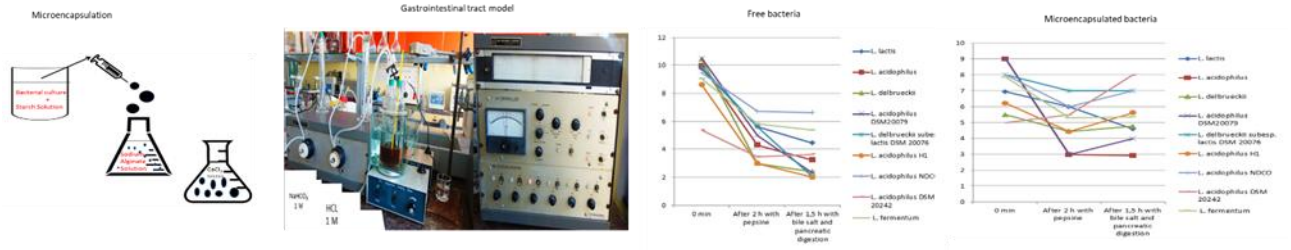

Research group presentation. The Group of Fermentation and Biosynthesis (Faculty of Food Science and Nutrition, Poznań University of Life Sciences, Poznań, Poland) aims to study the interactions between food, probiotics and digestive microbiota of humans with their environment. For 20 years, it has developed a well-known expertise in *in vitro* gastrointestinal tract simulation in human, with an *in vitro* model of the oral, gastric, small and large intestinal compartments.

Introduction. Probiotics are defined as living microorganisms that, when administered in adequate amounts, can improve microbial balances in the intestine and exert positive health effects on the host. Beneficial effect on the prevention of intestinal infections, cardiovascular disease, cancer and anti-allergic effects. Because of these beneficial health effects, these microorganisms are increasingly incorporated into dairy products and used for various therapeutic purposes. Probiotics could be bacteria, moulds, and yeasts, but most of them are bacteria; among them lactic acid bacteria, typically Gram-positive cocci and rods *Lactobacillus* and *Bifidobacteria,* specific components of the intestinal microflora. Probiotics get their beneficial effects when viable cells of probiotics are in the human intestine. In this study, a gastrointestinal model was used to represent the pH variation and gastric enzymes that participated in the digestion process. Eight *Lactobacillus* strains and one *Lactococcus* were studied; these strains were encapsulated using alginate sodium.

Materials and Methods. Probiotic organisms were microencapsulated following the method of extrusion using alginate compound. Microencapsulated and free probiotic bacteria were inoculated into 200 ml of MRS. Stimulated gastric juice and bile and pancreatic salt were used to test the tolerance of free *Lactobacillus* and *Lactococcus* species and microencapsulated bacteria to acid pH and enzymes. For the enumeration of microencapsulated probiotic organism, the bacteria were released from capsules after total digestion using sodium citrate solution. Samples were taken to count the number of live bacteria (CFU/g) using the plate count method on MRS plates incubated at 37° for 48 h. Bacterial cell viability was determined on MRS agar duplicate plates at different dilutions.

Results and Discussion. The results indicated that microencapsulated probiotic bacteria improved the viability of probiotic bacteria compared with free cells at 2 h of exposure to acidic conditions and also at 2 h of exposure to bile and pancreatic salt. Initially, free cells had an average count of 9.23 log CFU/ml of viable probiotic bacteria, but after 2 h of exposure to acid and pepsin, the average viability of cells was reduced to 4.73 log CFU/ml, a reduction rate of more than 48% for viable bacteria. The average percentage of viable cell reduction in

microcapsules was 20.64 % after 2 h with acid and pepsin and 26.27 % of reduction after bile salt.

Conclusion. In our study, the drop in quantity of encapsulated bacteria was lower in comparison with the drop in quantity of free bacteria. All free probiotic bacteria tested showed a loss in viability when exposed to gastric conditions. This feature is strongly strain-dependent. At least 73% of microencapsulated cells are able to survive passage through the stomach and the duodenum. And in the case of free cells, it is around 40% of free cells which could reach the intestine. Acid and pepsin solution was slightly more lethal to free and microencapsulated bacteria than bile solution. The next step of our research will be testing probiotic viability in the colon gut model.

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OPTIMATRIX v2.0: Optimised protocol to mitigate microbial blooms in the micro-Matrix bioreactor platform used as an *ex vivo* human distal colon model

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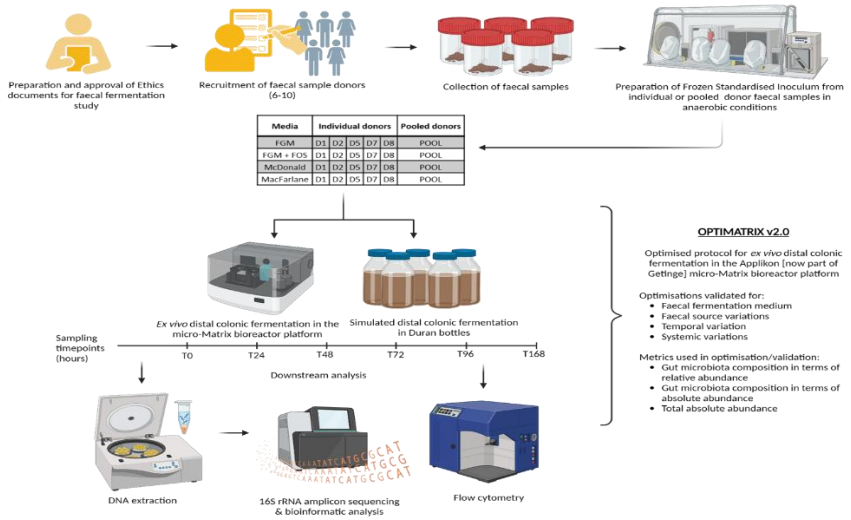

Research Group presentation. The Mathur lab in Teagasc Food Research Centre primarily works on plant-based as well as dairy fermented foods, and their impact on the gut microbiota.

Introduction. We previously reported optimisation of the methodology to mitigate *Escherichia coli* blooms and associated loss of microbial diversity when using the micro-Matrix bioreactor platform as an *ex vivo* model of the human distal colon. Here, we provide further critical

insights that we have gained in this regard through follow-up experiments. We tested four separate fecal fermentation media compositions for the purposes of such *ex vivo* distal colon model experiments and found that the media composition described by MacFarlane et al. is the most suitable for mitigating such microbial blooms, and concurrently, maintaining microbial diversity. We also tested if pooled or individual donor fecal samples were more suitable and found that pooled samples performed better in terms of maintaining gut microbiota diversity in such batch culture model experiments using the micro-Matrix system. Finally, we determined that prolonged experiments, i.e. for durations of up to 96 h, may be warranted with a view to affording particularly fastidious gut microbes an opportunity to grow and compete with their less fastidious counterparts. Essentially, we provide critical insights into: i) Optimal fecal fermentation media to minimise blooms and preserve diversity in ex vivo colon model experiments; ii) Optimal fecal inoculum source and duration of experiments.

Materials and Methods. The effects of different fecal fermentation medium, sampling time, systemic effects and fecal donor sources on the gut microbial community were determined using relative abundance metrics derived from 16S rRNA-based amplicon sequencing. Microbial compositions at the genus level for the gut microbial community in the fecal fermentation medium was determined for T0 (start of experiment), T24 (24hrs into the experiment), T48 (48hrs into the experiment), T72 (72hrs into the experiment), T96 (96hrs into the experiment), T168 (end point of the experiment). This was conducted for a pooled fecal slurry from 8 donors, alongside individual donor fecal samples from 5 donors. The following fecal fermentation media were tested: FGM, Fooks & Gibson media [1, 2]; FGMFOS, Fooks & Gibson media supplemented with 2.5% w/v FOS; MCF, Modified media derived from MacFarlane et al. [3]; and MCD, Modified media derived from McDonald et al. [4]. A flow cytometric approach was also undertaken to quantify the total abundance in fecal fermentation samples from the micro-Matrix bioreactor platform employed as an *ex vivo* distal colon model. This data was merged with relative abundance data from 16S rRNA-based amplicon sequencing to obtain absolute abundances for key gut microbial genera of interest.

Results and Discussion. The main outcomes of the study indicated that modified MCF and MCD caused far fewer microbial blooms than FGM and FGMFOS when used in colon model experiments using the micro-Matrix bioreactor platform. In addition, it was shown that pooled fecal samples performed better than individual donor samples in terms of being less susceptible to microbial blooms. It was also shown that regulated environmental parameters of pH was also likely to be an important contributory factor on microbial blooms as such blooms were less common in micro-Matrix experiments when compared to corresponding experiments conducted in Duran bottles without pH control.

Conclusion. 1) We recommend users to use the modified MCF medium for mitigating any problems encountered with microbial blooms and loss of microbial diversity. 2) We additionally recommend researchers continue using pooled FSI instead of individual donor fecal samples; 3) We further recommend that users conduct micro-Matrix experiments for slightly prolonged durations (at least 72 hours and up to 120 hours when possible) instead of 24 hours; 4) Limitations: other media formulations; more replicates; interventions

Acknowledgments/funding. AM and HM are researchers in Teagasc Food Research Centre. AM was supported by the Marie Skłodowska-Curie Career-FIT PLUS Fellowship (MF20210247); this Fellowship has received funding from the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie Grant Agreement (as per Article 29.4 of the grant agreement). This research was funded by grant number MF20210247, Science Foundation Ireland (SFI) under [grant 1424 number SFI/12/RC/2273_P2] and by Enterprise

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Effect of feed ingredients on microbiota composition and skatole production in a new *in vitro* model of the adult pig colon

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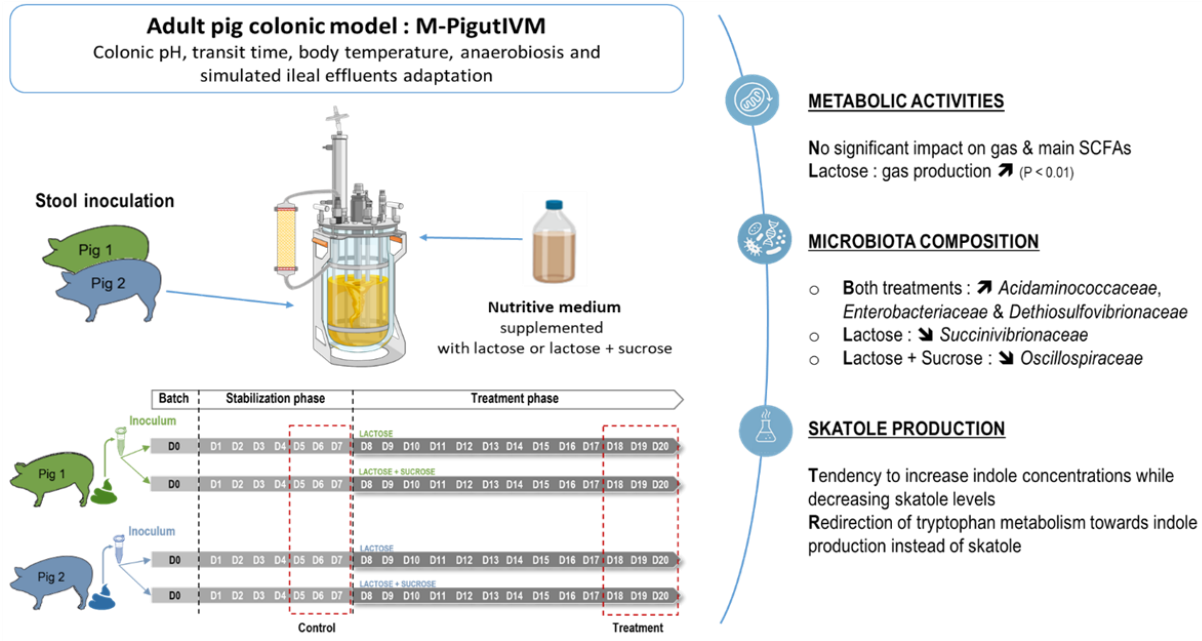

Research group presentation. The MEDiS Research Unit's (Clermont Auvergne University-INRAe, Clermont-Ferrand, France) aims to study the interactions between food and digestive microbiota of animals and humans with their environment. For over 30 years now, MEDiS has developed a well-known expertise in *in vitro* gut simulation in both human and monogastric

animals, with an original platform gathering complex and dynamic *in vitro* models of the oral, gastric, small and large intestinal compartments.

Introduction. Skatole (3-methyl-indole), a compound associated to boar taint and unpleasant odours in pork meat, is metabolised from tryptophan by the pig intestinal microbiota. This study aimed to evaluate *in vitro* the impact of feed ingredients (lactose or lactose + sucrose) on the pig microbiota composition and metabolic activities and their ability to reduce skatole, using a dynamic model mimicking the healthy adult porcine colon environment (MPigut-IVM).

Materials and Methods. Based on *vivo* data, the MPigut-IVM was set-up to reproduce the main physicochemical (pH, transit time, anaerobiosis), nutritional (ileal effluents composition, bile salts) and microbial (lumen and mucus-associated microbiota) parameters of the adult pig's large intestine. Two bioreactors were inoculated with a fecal sample from a healthy male pig (n=2) and supplemented with lactose or a lactose/sucrose mixture (0.4 g per day) during 13 days, after an 8-day stabilization period. Skatole production was followed by liquid chromatography. Gut microbiota composition and its main metabolic activities were monitored by 16S Metabarcoding/qPCR and short chain fatty acid (SCFA) plus gas measurements, respectively.

Results and Discussion. Sugars had no significant impact on SCFA concentrations and ratios, but lactose alone increased gas production ($P < 0.01$). Both sugars, and particularly lactose + sucrose, tended to reduce skatole concentrations while increasing indole levels ($P > 0.05$). This was associated with a slight reduction of the abundance of the skatole-producing *Olsenella scatoligenes* ($P > 0.05$). Both feed ingredients induced a decrease in bacterial α-diversity ($P < 0.05$).

Conclusion. Feed ingredients appeared to reduce skatole production by redirecting tryptophan metabolism toward indole production, without major changes in microbial activities. *In vitro* dynamic models, such as MPigut-IVM, represent a powerful platform for microbiome studies in the adult pig colon environment. Further developments should consider coupling the *in vitro* model with intestinal epithelial or immune cells to integrate host interactions.

Acknowledgments/funding. This research was funded by MIXSCIENCE company. We acknowledge MEDiS technical team for great assistance and Mr RONGIER's farm for stool collection.

Effect of a more realistic fermentation medium on microbiota composition and metabolic activities in two well-established *in vitro* human colon models

Ophélie Uriot[1], Galal Ali Esmail[2], Sylvain Denis[1], Walid Mottawea[2,3], Salma Sultan[2], Emmanuel N. Njoku[2], Mariem Chiba[2], Susan Tosh[2], Riadh Hammami[2,4], Stéphanie Blanquet-Diot[1]

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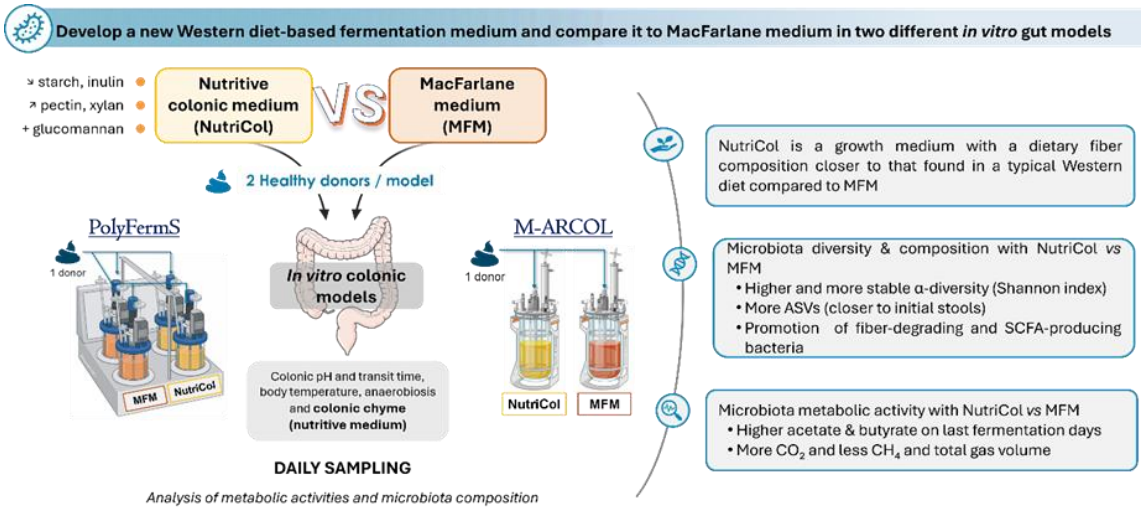

Research group presentation. The MEDIS unit (Clermont Auvergne University-INRAe, France) and the DIGESTiv platform specialize in gut microbiota research and have developed advanced *in vitro* models simulating the human and animal gastrointestinal tract (from oral to colon models). The Hammami Lab (University of Ottawa, Canada) and the N*u*Gut Research platform focus on diet–microbiota–host interactions, developing targeted microbiome modulation strategies (pro-, pre-, postbiotics, and bioactive compounds). Their research combines *in vitro* models, multi-omics, and bioinformatics to explore gut-brain and gut-immune axes in health and disease prevention.

Introduction. *In vitro* systems simulating the human colon are essential for studying gut microbes, yet the fermentation medium often fails to replicate the true colonic environment. This study aims to develop a new fermentation medium, called Nutritive Colonic (NutriCol) medium, to better reflect the colonic environment based on typical Western diets.

Materials and Methods. NutriCol was compared to the widely used MacFarlane medium (MFM) in two *in vitro* colon models: PolyFermS (proximal colon), and M-ARCOL (average colon, lumen and mucosa). NutriCol contains more pectin and xylan and less starch and inulin than MFM. Both models were inoculated with healthy donor stool and fed MFM or NutriCol to assess microbiota structure, diversity, and metabolites.

Results and Discussion. NutriCol significantly increased microbial α-diversity in PolyFermS (Shannon's, $p \leq 0.01$), with no significant change in M-ARCOL. PERMANOVA revealed clear differences between microbiomes grown with NutriCol and MFM, with higher ASV counts under NutriCol. SCFA production was significantly influenced by NutriCol, increasing acetate and butyrate levels in the M-ARCOL, with a similar non-significant trend in the PolyFermS. This increase was linked to the enrichment of SCFA-producing bacteria like *Butyricicoccus* and

Lachnospiraceae. Additionally, NutriCol was associated with lower intestinal gas levels than MFM in the M-ARCOL.

Conclusion. NutriCol better reflects Western dietary fiber intake, supported greater gut microbiota diversity and SCFA production Though model- and donor-dependent, NutriCol offers an improved tool for simulating human colonic conditions, enhancing microbiome research and understanding.

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Acknowledgments/funding. We thank Dr. Ayman Elsayed for his technical support. This study was supported by a Strategic Research Grant from the University of Ottawa.

CANIM-ARCOL a new *in vitro* model of the dog large intestine capturing size-related effects

Charlotte Deschamps[1], *Sylvain Denis*[1], *Delphine Humbert*[2], *Sandrine Chalanco*[1], *Emmanuelle Apper*[3], *Stéphanie Blanquet-Diot*[1*]

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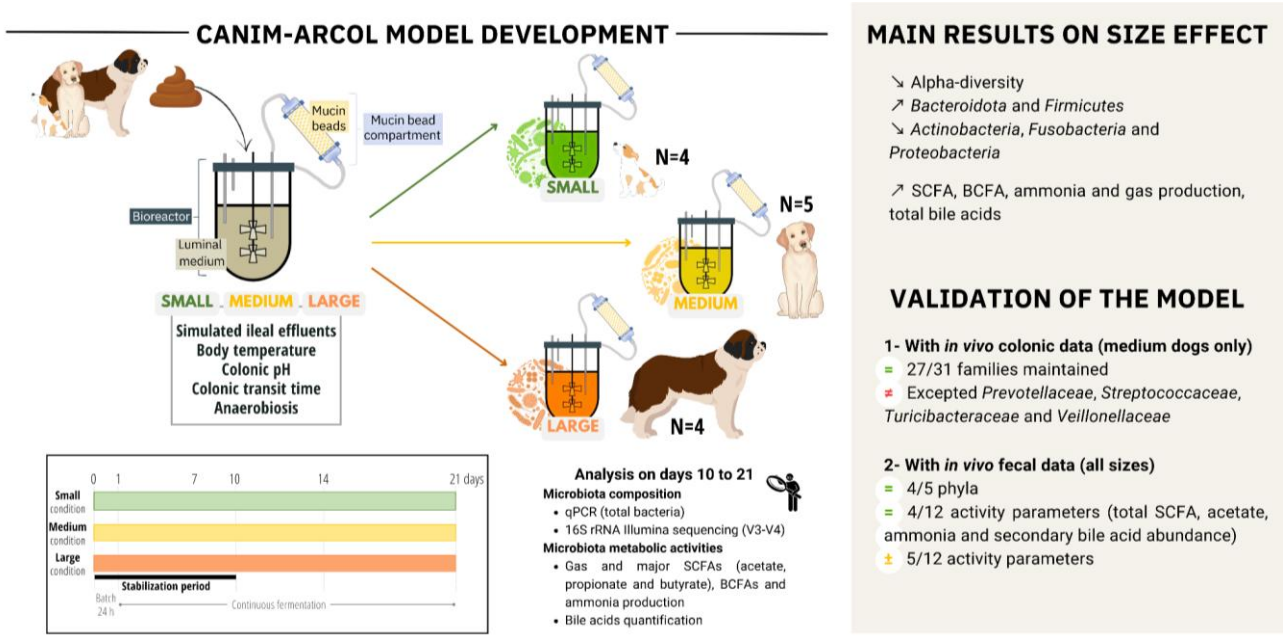

Research group presentation. UMR 454 MEDIS UCA-INRAE (Clermont-Ferrand, France) aims to study the role of the intestinal microbiota in human and animal health. The lab has extensive expertise in *in vitro* digestion and fermentation, with a dedicated platform including static batch models and dynamic systems reproducing the stomach and small intestine (TIM-1 and ESIN v2), the ileum (M-ARILE), the large intestine (M-ARCOL), or the entire gastrointestinal tract (M-SHIME), that can be combined with epithelial cell culture (Caco-2 TC7, HT-29 MTX). These models have been optimized for over 25 years and adapted to both human and animal (pig and dog) situations, to various age groups (infants, adults and elderly), physiological states, food matrices (from water to complete meals), and pathologies (such as obesity and IBS).

Introduction. Body weight is an important determinant of variations in canine digestive physiology, mainly related to the large intestine. *In vitro* gut models are increasingly used as an alternative to animal experiments for technical, cost, societal and regulatory reasons.

However, up to now, none of the available canine *in vitro* gut models has been adapted to reproduce size-related digestive parameters.

Materials and Methods. A new *in vitro* model of the canine large intestine was developed, the Canine Mucosal Artificial Colon (CANIM-ARCOL), simulating the main physicochemical (pH, transit time, anaerobiosis), nutritional (ileal effluent composition, bile acids profiles) and microbial (lumen and mucus-associated microbiota) parameters of this ecosystem. The model was adapted to three dog sizes, i.e. small under 10 kg, medium between 10 and 30 kg and large over 30 kg. To validate this new model regarding microbiota composition and metabolic activities, *in vitro* fermentations were performed during 20 days in bioreactors inoculated with stools from 13 dogs (4 small, 5 medium and 4 large), and results were compared to canine *in vivo* data from the literature.

Results and Discussion. After a 10 days of stabilization period, microbiota profiles clearly clustered depending on dog size. Especially, *Bacteroidota* and *Firmicutes a*bundances were positively correlated with body weight both *in vitro* and *in vivo*, while opposite trends were observed for *Actinobacteria* and *Proteobacteria*. As observed *in vivo*, microbial activities as followed through gas, short-chain fatty acids and ammonia concentration measurement, but also bile acid dihydroxylation, increased with dog size *in vitro*. The new model also provided useful data on mucus-associated microbiome, poorly described up to now in dogs.

Conclusion. In line with the 3R regulation, the CANIM-ARCOL represents a powerful platform to study the fate of food and veterinary products in the canine digestive environment, help to elucidate their mechanisms of action in relation with colonic microbiota and promote innovation in these fields. In a next future, this model will also help to move toward personalized nutrition or medication, by capturing interindividual or breed variabilities in gut microbiome and considering dog body weight.

Acknowledgments/funding. This research was co-funded by Lallemand SAS and Dômes Pharma companies.

Effect of antibiotic residues on the gut microbiota of piglets: insights from an *in vitro* colon model

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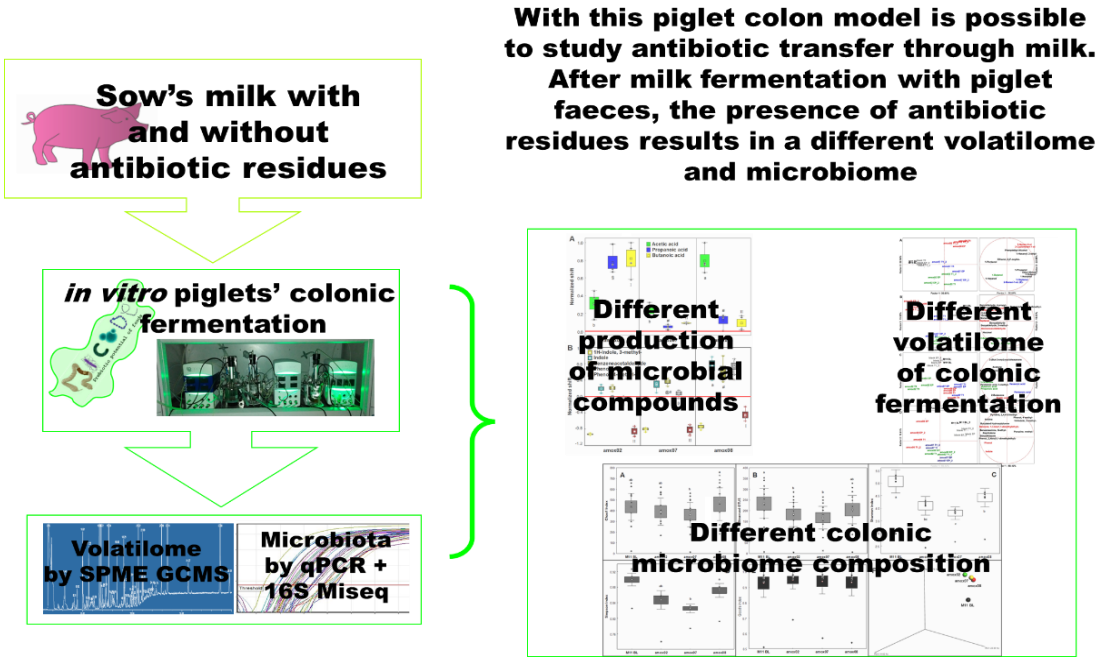

Research group presentation: *in vitro* experimentation was conducted by the reasearch group In "Microbial intestinal fermentation" of Andrea Gianotti at the University of Bologna. The group covers *in vitro* assessment of foods and food contaminants toward the gut microbiota, by using *in vitro* intestinal models either batch, for short term fermentation and substrate exposure, and complex semi-dynamic models, for long term fermentation. The group produces several protocols that range from inoculum preparation to omics outputs.

Introduction: In recent years, pigs have become a key preclinical model due to their physiological similarity to humans and ethical suitability. They are particularly useful for studying how molecules transfer into milk during lactation. Hydrophilic drugs, like amoxicillin, tend to concentrate in milk's aqueous phase. The EU project ConcePTION uses pigs to study drug safety in pregnancy and breastfeeding. Amoxicillin, widely used in medicine, has 25–31% oral bioavailability in young pigs, indicating potential effects on gut microbiota. In piglets, gut microbes, mainly inherited from the sow, are dominated by *Firmicutes*, especially *Lactobacillaceae*, which are crucial for gut development and health.

Materials and Methods: Amoxicillin was administered daily to lactating sows from week 2 to day 28. Milk samples (Amox07, Amox08, Amox02) were collected at defined timepoints post-injection, following oxytocin-induced letdown, and stored at −80 °C. Samples with varying amoxicillin levels were used in *in vitro* fermentation. Microbiota composition was assessed by 16S rDNA sequencing (MiSeq) and qPCR, while microbial metabolites were analyzed via solid-phase microextraction gas chromatography/mass spectrometry (SPME-GC/MS) to evaluate responses to milk treatments.

Results and Discussion. Fermentation of sow's milk containing amoxicillin residues produced distinct volatile profiles, with key compounds including 1-butanol, butanoic acid, indole, and phenol. While some short-chain fatty acids (e.g., acetic, propanoic, butanoic) indicated beneficial microbial activity, elevated levels of indole and phenol pointed to

potentially harmful effects. Each milk type generated a specific fermentation pattern, influenced by the concentration of antibiotic residues. Amox02 led to higher propanoic and butanoic acid production, whereas Amox08 was richer in acetic acid. However, both treatments also increased the presence of toxic aromatic metabolites. Microbial diversity was negatively affected, especially in Amox02 and Amox07, with reduced richness and evenness. Proteobacteria increased while Actinobacteria declined, while core phyla stayed mostly same. Amox08 suppressed opportunistic taxa like *Enterobacteriaceae* but also reduced beneficial groups such as *Bifidobacteriaceae*, while favoring potentially resistant families like *Enterococcaceae*. These findings suggest that antibiotic residues in milk can reshape gut microbial ecology, balancing beneficial and detrimental metabolic outcomes.

Conclusion. The integration of microbiomic and metabolomic analyses revealed shifts in microbial composition and metabolite profiles, including potential impacts on antibiotic resistance. While *in vivo* studies remain essential, this approach offers a reliable and ethical preclinical platform to investigate early-life microbial modulation and the implications of antibiotic exposure through maternal milk.

Acknowledgments/funding. The research leading to these results was conducted as part of the ConcePTION consortium. The ConcePTION project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 821520. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA.

Reference: Nissen, L., Aniballi, C., Casciano, F., Elmi, A., Ventrella, D., Zannoni, A., ... & Bacci, M. L. (2022). Maternal amoxicillin affects piglets colon microbiota: microbial ecology and metabolomics in a gut model. Applied microbiology and biotechnology, 106(22), 7595-7614.

The role of *vitro* gut models in understanding xenobiotic-microbiota dynamics

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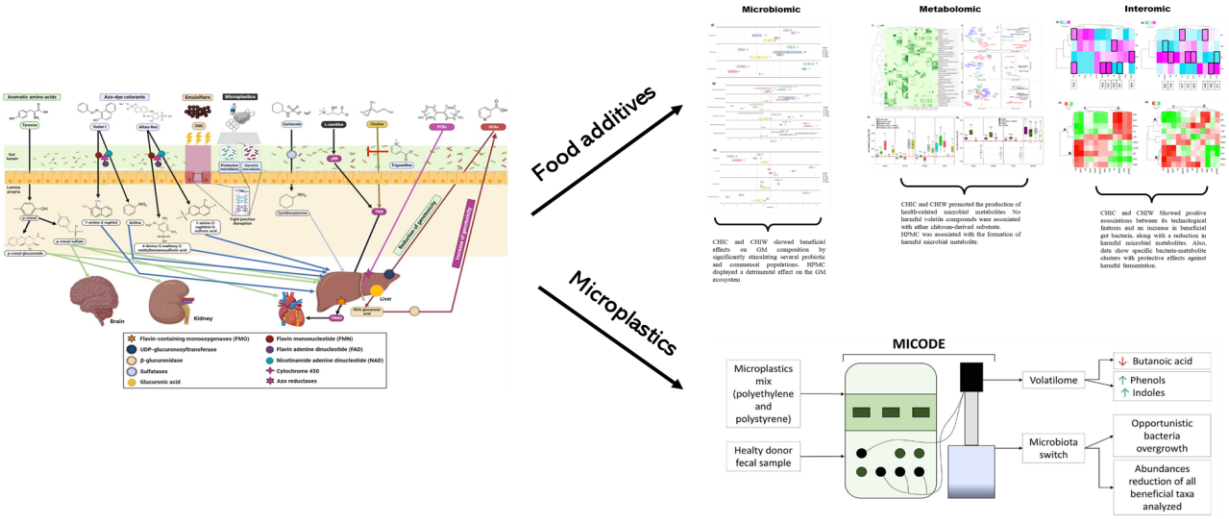

Research group presentation. *In vitro* experimentation was conducted by the Reasearch group In "Microbial intestinal fermentation" leaded by Andrea Gianotti at the University of Bologna. The group covers *in vitro* assessment of foods and food contaminants toward the gut microbiota, by using *in vitro* intestinal models either batch, for short term fermentation and substrate exposure, and complex semi-dynamic models, for long term fermentation. The group has produced several protocols that range from inoculum preparation to omics outputs.

Introduction. Xenobiotics are compounds found in an organism that are not naturally produced by it (e.g., drugs, food additives, pesticides, microplastics, pollutants, toxins). Exposure to xenobiotics occurs daily through skin contact, inhalation, or ingestion, either voluntarily (medications, supplements) or involuntarily (contaminated food or water). The gut microbiota can interact with these substances before or after they are absorbed. Orally ingested xenobiotics may be absorbed in the small intestine and processed by the liver, or pass into the colon, where they are metabolized by gut microbes. Substances absorbed elsewhere in the body can also return to the intestine via bile and again interact with microbiota.

Case study. The two case studies involve alimentary additives and microplastics. The first study investigates the effects of four food texturizers on gut microbiota composition and metabolism, including one classified as a potential xenobiotic by EFSA, using a simulated human colonic model combined with qPCR and GC-MS analyses. The second study investigates the impact of microplastics such as PE and PS, commonly found in food and beverages. Using an omics approach in an *in vitro* model of healthy human colon microbiota.

Results and Discussion. CHIW showed beneficial effects by promoting SCFA production (butyrate, propionate) and non-toxic VOCs, supporting gut-friendly microbes. In contrast, HPMC favored harmful bacteria and produced toxic compounds like skatole and p-cresol. CHIC and CHIW displayed prebiotic activity, with DD and solubility positively influencing microbial balance, unlike HPMC. About the second study an high doses of microplastics (PE, PS) disrupted gut microbiota by decreasing SCFA production and increasing toxic compounds like p-cresol. They promoted opportunistic bacteria (e.g., *E. coli, Clostridium*) and suppressed

beneficial microbes, indicating a dysbiotic shift. Even lower doses showed negative, though milder, microbial and metabolic alterations.

Conclusion. Chitosan samples (ChiW and ChiC) showed strong prebiotic properties by stimulating beneficial bacteria and promoting short-chain fatty acid production, supporting gut health. In contrast, HPMC favored harmful microbes and generated toxic metabolites like p-cresol and skatole. Separately, high doses of microplastics (PE and PS) induced dysbiosis by enriching opportunistic bacteria and suppressing beneficial taxa, shifting microbial metabolism toward harmful pathways and reducing protective SCFA production.

Acknowledgments/funding. This work was partially founded by Italian Ministry for Research.

Commonly consumed carbohydrates differentially alter *in vitro* gut microbiota of children with severe malnutrition: Potential implications for recovery strategies?

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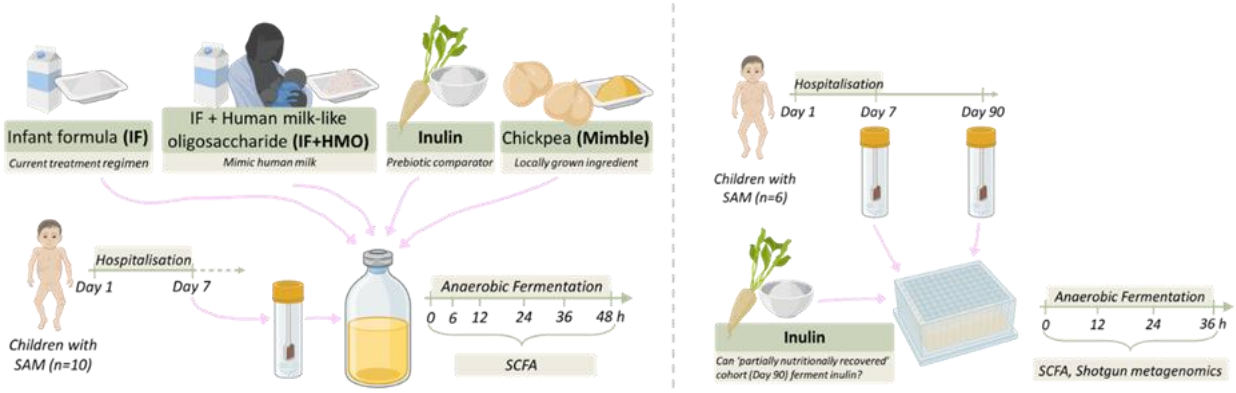

Research group presentation. The Warren Group at the Quadram Institute is a multi-disciplinary research team focused on understanding the impact of carbohydrates on the gut. The group's research spans a wide range of areas, from understanding how modifying carbohydrate quality in crop plants can impact digestion and the gut microbiome to exploring the impact of gelling carbohydrates in the treatment of irritable bowel syndrome. Our main tools are *in vitro* gut models, combined with multi-omics analyses. We work closely with clinical partners to inform the design of our studies and obtain samples.

Introduction. Malnutrition remains a serious public health concern in low- and middle-income countries. Currently used milk-based recovery strategies to treat children with severe acute malnutrition (SAM) show poor outcomes with high mortality and readmission rates. Evidence suggests that children with SAM have a disrupted gut microbiome, and strategies focusing on modifying the gut microbiome may improve overall outcomes.

Materials and Methods. Here, we first tested the fermentability of commonly consumed carbohydrates (inulin, milk oligosaccharide and chickpea feed) using an *in vitro* batch model inoculated with fecal samples from SAM children collected after 7 days of hospitalisation. In another trial, we examined the fermentability of inulin using a miniaturised *in vitro* batch system inoculated with paired fecal samples collected on day 7 and day 90 post-hospitalisation. Gut microbiota composition was analysed using 16S or shotgun sequencing, and metabolites were quantified using NMR.

Results and Discussion. Children with SAM have low alpha diversity with a high abundance of pathogenic bacteria. Chickpea-enriched feed and milk oligosaccharides promoted higher microbial diversity and SCFA production than inulin, suggesting the latter is not suitable in SAM recovery. However, after 90 days of care, the fecal alpha diversity improved and this contributed to better utilisation of inulin than at day 7 group.

Conclusion. Introducing suitable fermentable carbohydrates can support recovery from SAM by enhancing the gut microbiome. Inulin might be added later in the recovery stage, while other sources like chickpeas could be introduced earlier.

Acknowledgements/funding. We thank all the participants and trial staff who participated in the MIMBLE trials. The authors gratefully acknowledge the support of the Biotechnology and Biological Sciences Research Council (BBSRC); this research was funded by the BBSRC Institute Strategic Programme Food Microbiome and Health BB/X011054/1 and its constituent projects BBS/E/F/000PR13631 and BB/X018857/1.

Interaction between oat bran and green tea extract modulates antioxidant release and short chain fatty acid formation during colonic fermentation

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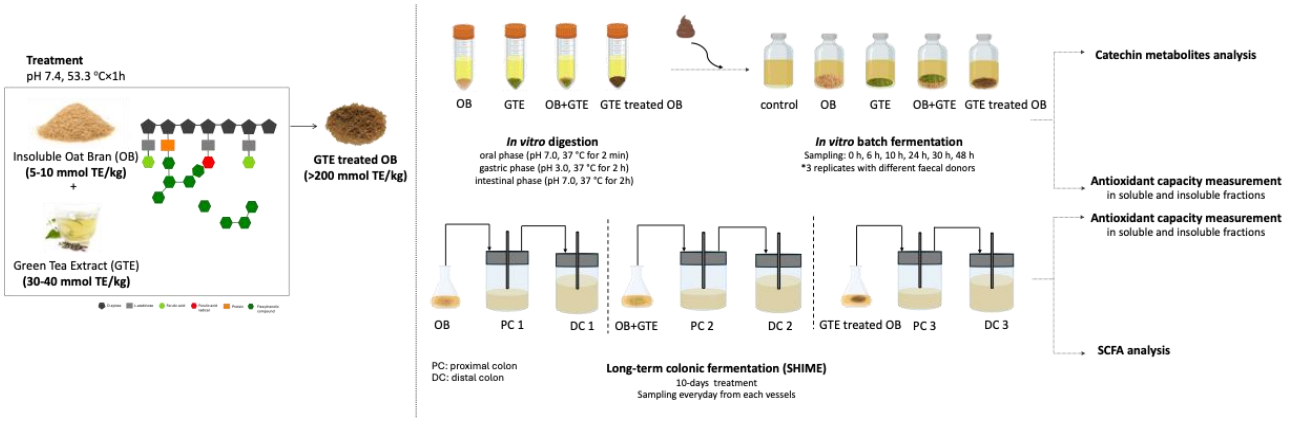

Research group presentation. FoQuS Research Group focuses on food quality and safety issues, aiming to understand the changes that occur during food processing and to design food that improves health and enhances flavor. This project was conducted in collaboration with Food Quality and Design Group at Wageningen University, which has facilities to investigate the metabolism of designed ingredients by gut microbiota.

Introduction. Dietary fiber bound antioxidants significantly benefit colon health by continuously releasing antioxidants. Cereal grains, particularly the bran fraction, are notable source of these antioxidants. However, their antioxidant capacity is limited compared to free soluble antioxidant sources. In our previous study, bound antioxidant capacity of oat bran (OB) was significantly increased to level of more than 200 mmol TE/kg by treatment with green tea extract (GTE) under optimum conditions [1].

Materials and Methods. This study aims to investigate the antioxidant release during the colonic fermentation of GTE treated OB and its effects on gut microbiota metabolites. The interaction between untreated OB and GTE (OB+GTE) during colonic fermentation was also assessed. After the samples (OB, GTE, OB+GTE, and GTE treated OB) were prepared as described in the previous study [1], they underwent *in vitro* digestion to assess their digestion behaviour. Digested samples were subjected to *in vitro* batch fermentation over 48 h using fecal matter from three donors. Samples were collected at 6h, 10h, 24h, 30h, and 48h to determine antioxidant capacity, total phenols, catechin metabolites, and short chain fatty acids (SCFA). OB, OB+GTE, and GTE treated OB were also exposed to the SHIME® model system (PRODIGEST, Belgium) to assess long-term antioxidant activity and SCFA production.

Results and Discussion. Antioxidant capacity significantly increased ($p < 0.05$) following digestion of OB or GTE treated OB, while GTE alone exhibited a slight but significant decrease ($p < 0.05$). Approximately 85% of the antioxidant capacity in GTE treated OB was transferred to the colon, where fermentation by the gut microbiota sustained a continuous and elevated antioxidant release. The released antioxidant capacity was highly correlated with pyrogallol

(r=0.693) and 3,4-dihydroxyphenyl acetic acid (r=0.625). The insoluble fraction retained significant antioxidant capacity (237.45±31.11 µmol TE) even after 48 h of fermentation. Both GTE alone and OB+GTE induced a rapid increase in antioxidant capacity within the first 24 h, followed by a significant decline thereafter. In the SHIME model, the antioxidant capacity of soluble and insoluble fractions in both the proximal and distal colon during long-term feeding was 2-6 times higher with GTE treated OB compared to OB+GTE. Additionally, GTE treated OB and OB+GTE promoted SCFA production, reaching 20.85±0.91 mM and 35.07±0.7 mM, respectively. Notably, GTE treated OB stimulated butyric acid production (3.17±0.16 mM) in the proximal colon after 10-day.

Conclusion. OB with enhanced bound antioxidant capacity through its treatment with GTE before digestion created a superior antioxidant environment in the colon both in the soluble and insoluble fraction, together with positively influencing SCFA production. These findings suggest potential benefits for colon health, such as prevention of colon cancer, which require further investigation.

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Acknowledgments/funding. This work was supported by The Scientific and Technological Research Council of Turkey in the frame of 2219-International Postdoctoral Research Fellowship Program for Turkish Citizens.

Impact of prenylated phytochemicals on the bacterial communication and host-cell-microbe interaction

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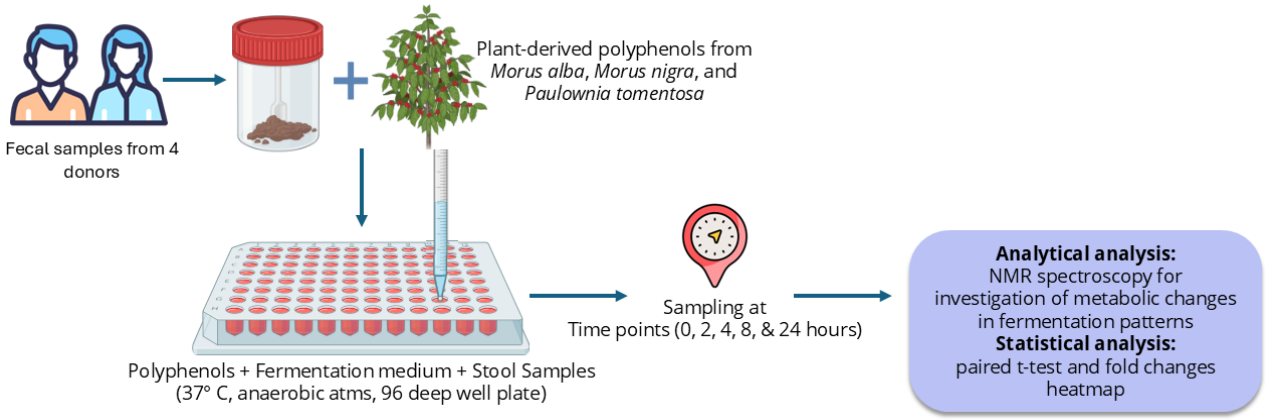

Research group presentation. The Agromics Group of the Department of Food Science, at the Czech University of Life Sciences, Prague, headed by Dr. Jaroslav Havlik collaborated on this project with the research team of Dr. Karel Smejkal at the Masaryk University, Brno.

Introduction. Plant phenolics represent a class of metabolites suppressing chronic inflammation related to cardiovascular and neurodegenerative disorders. These phytochemicals have been proven to target the gastrointestinal tract by modulating the gut microbiota activity. These changes in the gut microbiota activity further brings changes in the gut metabolome.

Materials and Methods. In this study, we evaluated the effects of six plant-derived phytochemicals on fecal fermentation and the gut metabolome using a high-throughput *in vitro* fecal batch fermentation model in a deepwell system. The system was temperature-controlled, anaerobic, and buffered as described by Tomisova et al. (2024). The tested compounds included Diplacone (DPN), Diplacol (DPL), 3-O-Me-5-Hydroxydiplacone (MHD), Broussoflavonol A (BFA), Broussoflavonol B (BFB), and Morusin (MRS). Fermentation samples containing each phytochemical at a concentration of 50 µg/mL were collected at 0, 2, 4, 8, and 24 hours. The impact on the fermentation profile was assessed using proton nuclear magnetic resonance (1H NMR) spectroscopy by comparing the area under the curve (AUC) for individual metabolites over the 24-hour period (using the trapezoidal method) between treated and control samples.

Results and Discussion. The comparison showed that BFA and BFB had marginal effect on SCFAs like acetate, butyrate and isovalerate when compared to other polyphenols, not significant after applying FDR correction. Other 4 polyphenols showed significant alterations in the gut metabolic profile. Namely, DPN, DPL, MHD and MRS significantly decreased the fermentation and utilisation of amino acids, leaving elevated levels of valine, phenylalanine, isoleucine, and leucine. DPN was positively associated with increased production of isobutyrate, pyroglutamate, and succinate, while DPL increased lactate and valerate. MHD increased maltose while MRS increased 5-aminopentanoate. These changes can be attributed to shifts in microbial activity, reflecting both a suppressive effect and stress-induced upregulation of alternative fermentation pathways.

Conclusion. These findings suggest that phytochemicals can modulate gut microbial activity, influencing both fermentative processes and alternative metabolic pathways, potentially offering novel strategies for dietary interventions targeting gut health. In conclusion, our study highlights the distinct impacts of six plant-derived phytochemicals on fecal fermentation and the gut metabolome. While BFA and BFB exhibited minimal effects on short-chain fatty acids (SCFAs) such as acetate, butyrate, and isovalerate, the other polyphenols, including DPN, DPL, MHD, and MRS, significantly altered the fermentation profile.

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The influence of sweet cherries (*Prunus avium* L.) on choline metabolism and the synthesis of trimethylamine

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Research Group Presentation: Suna is involved in WG1 of INFOGEST project. She attended the 2nd protein ring trial from Quadram Institute Bioscience (QIB), UK by performing one-to-one analyses and completed her post-doc project in the area of "*in vitro* colon models".

Introduction. Choline is an essential nutrient that is present in animal products such as meat, cheese and eggs and in some dietary supplements[1]. It has also been shown to be converted to trimethylamine (TMA) by bacteria in the human gut, and this TMA is absorbed and converted to TMA-N-oxide (TMAO) in the liver[2]. Higher circulating TMAO is associated with increased risk of cardiovascular and multiple other diseases and with inflammation and insulin resistance[3,4]. There are also several studies in rodent models and one in humans showing that increasing TMA(O) exposure causes disease, for example cardiovascular disease by increasing platelet reactivity and thrombosis[3]. We have shown that metabolism of choline in a fecal inoculated batch fermentation model of the human colon authentically replicates what happens *in vivo*[5], and we seek to use this to identify dietary components that may effectively inhibit the gut microbial conversion of choline to TMA. The aim of this study was to investigate the effect of sweet cherry on the production of TMA from choline by gut microbiota.

Materials and Methods. *In vitro* colon model was inoculated with 1% fecal inoculum from different healthy donors (n = 3) and 1g of freeze-dried cherry fruit. Fermentations were carried out under colonic conditions in a complex media with high buffering capacity that does not require external addition of acid and alkali to keep pH stable (pH 6.6-7.0, 37°C, CO_2 was added to make the vials headspace oxygen free)[6]. Samples were collected from the model at various timepoints over 30 hours. The concentrations of choline and TMA were quantified in the samples using LC-MS/MS. Differences in concentrations of metabolites were confirmed by means of Two-Way ANOVA.

Results and Discussion. Cherry showed an inhibitory effect on the metabolism of choline to TMA. In the model conditions treated with cherry, choline metabolism was slower (24h) compared with the control (20h). A significant delay was seen between these treatments (p <

0.01). TMA appeared in all models between 9-30 hours but were at significantly lower concentrations in the cherry treated vials with the significance at timepoint 20h between the control and the treatment.

Conclusion. Cherry supplementation into human colon model suppressed choline conversion to TMA, which might be a promising strategy for the reduction of TMAO levels. This was most likely because cherries contain compounds that affect choline metabolism and the production of TMA *in vitro*. For further studies, compounds extracted from cherry and the polyphenol-free matrix of cherry should be tested in-vitro to determine the active compounds contributing to the effect. Prospectively, these findings need to be confirmed in human studies.

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Study of the Isolation of fractions rich in hemicellulosic oligosaccharides in a dynamic model of the human gut microbiota

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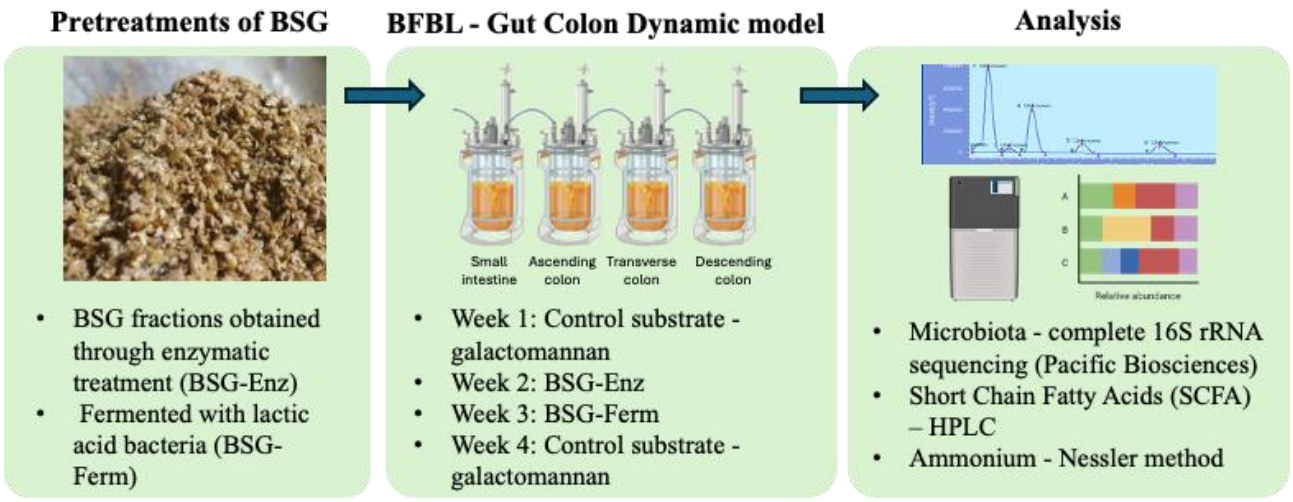

Research group presentation. MicroHealth study the ecology, functionality, and technological applications of beneficial microorganisms in relation to human health, as well as the revaluation of agri-food byproducts. The goal is to obtain ingredients that target the human intestinal microbiota (IPLA-CSIC).

Introduction. Cereal wastes are hemicellulose-rich materials that may represent an interesting source of (arabino)xylooligosaccharides (A(XOS)), compounds associated with appealing bioactive properties, including prebiotics for human use. However, economic and environmentally viable valorization strategies for hemicellulose-rich materials through (A)XOS obtainment have not yet been thoroughly explored. This work was done to investigate *in vitro* the gut microbiota modulatory capacity of a selection of extracts obtained from brewer's spent grains (BSG). To this end, a dynamic colon model of the human gut microbiota, the BFBL Gut Colon model[1], was used.

Results. The BSG substrates tested were an enzymatically modified BSG substrate (BSG-Enz) treated with anendo-xylanase (Rohalase®), and a BSG substrate fermented with lactic-acid bacteria (*Lactobacillus kimchii, Lactococcus lactis, Lactiplantibacillus pentosus* and *Pediococcus pentosaceus*) (BSG-Ferm), compared to a control substrate (galactomannan). The dynamic model is composed of three reactors that simulate the ascending (R1), transverse (R2) and descending (R3) colon and that were inoculated with the same fecal sample from a healthy adult. Stabilization of the microbiota in each phase of the study was achieved by feeding the system three times a day for one week. The experiment was run sequentially by changing the substrate each week as follows: control substrate (week1), BSG-Enz substrate (week 2), BSG-Ferm substrate (week 3) and finally returned to the control substrate (week 4).The analysis of SCFA showed a decrease in propionic and acetic concentration during the administration of the test substrates(BSG-Enz and BSG-Ferm), being more pronounced with BSG-Ferm; and an increase in butyric concentration, as compared to the basal medium. Variations in ammonium concentration were observed in the adaptation period to each substrate, with concentrations decreasing once the microbiota had stabilized with each substrate BSG-Enz induced ammonium reduction. At the microbiota level, the increase of

Bifidobacterium and *Akkermansia* with both BSG-Enzand BSG-Ferm with respect to the control substrate was remarkable.

Conclusions. The ability of the tested ingredients to modulate beneficial groups of the human intestinal microbiota is observed, supporting the possibility of using enzymatic processes (BSG-Enz) or controlled lactic acid fermentation processes (BSG-Ferm) to obtain ingredients with prebiotic properties from BSG, as a way to achieve valorization of this residue.

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Investigating the role of gut microbiota in host metal homeostasis

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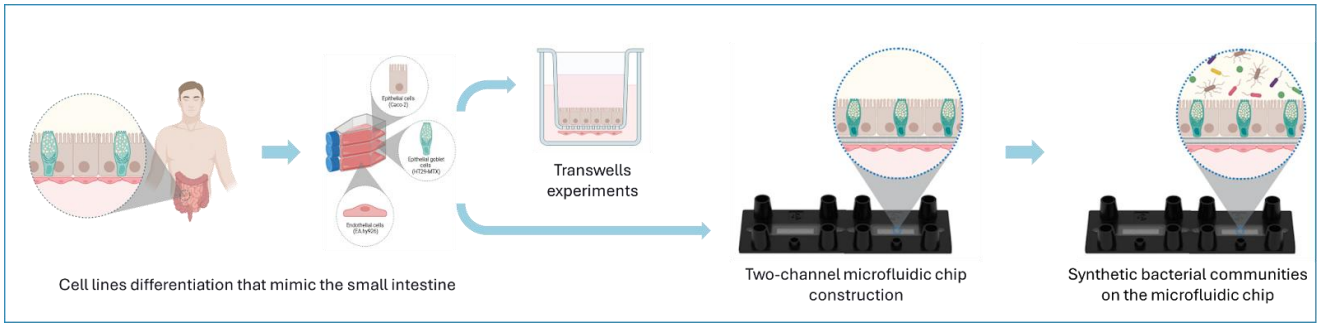

Research group presentation. In our lab, we investigate how xenobiotics, such as metals, shape the gut microbiota ecosystem using both *in vitro* systems and advanced *in vivo*–mimicking models. Our research focuses on uncovering how these compounds influence microbial composition and metabolic activity. By recreating host-like environments, we aim to better understand the mechanisms underlying host–microbiota interactions and their implications for human health and nutrition. This knowledge, coupled with insights into host-microbial interactions, will be crucial for improving human nutrition.

Introduction. Iron (Fe) is an essential micronutrient required for the survival, development, and reproduction of all organisms, including bacteria and humans. Fe operates as a co-factor for proteins, such as hemoglobin, and enzymes associated with oxygen transport [1]. Also, it is important for biological processes, such as cell growth and differentiation. In humans, dietary Fe is primarily absorbed in the duodenum and upper jejunum on the small intestine. This process is influenced by several factors, including Fe solubility, chelation with dietary components, metal-metal interactions, and the presence of inflammation or gastrointestinal diseases [2]. Conditions such as celiac disease, stomach ulcers, and inflammatory bowel disease are known to decrease Fe absorption [2]. Fe deficiency causes anemia, a disorder that affects two billion people globally, resulting in weakness and exhaustion, as well as increased morbidity and death due to impaired immunological responses [1]. The gut microbiota, which plays a key role in digestion and immune regulation, is also influenced by host's ability to absorb Fe [3] and disease conditions. While it is known that Fe availability can shape the gut microbial community, the role of the gut microbiota in regulating host Fe homeostasis remains poorly understood.

Materials and methods. We will leverage a human 3D intestine-on-chip model [4] to examine the impact of synthetic bacterial communities on host Fe absorption. This model replicates the human small intestine by integrating Caco-2 epithelial cells, EA.hy926 endothelial cells, and THP-1-derived tissue-resident macrophages. Iron uptake will be evaluated by measuring the expression of key iron transporters using qRT-PCR and immunofluorescence. In order to develop the intestine-on-chip model, we first used a Transwell model, by co-cultivating EA.hy926 and Caco-2 cell lines. The barrier integrity, cellular differentiation, and morphological features were assessed using TEER (transepithelial eletrical resistance) measurements, alkaline phosphatase (ALP) activity assays, and microscopy-based staining, respectively.

Results. The cell lines were in co-culture for 21 days, and TEER was monitored at 7, 14 and 21 days after seeding. A progressive increase in resistance was observed, with TEER values averaging 500 $\Omega \cdot cm^2$ for the co-culture at day 21—lower than the average obtained for the Caco-2 monoculture (1000 $\Omega \cdot cm^2$). ALP activity, a marker of enterocyte differentiation, was measured at day 21. Immunostaining was conducted at day 21 to assess cellular morphology and junctional protein localization. Staining for ZO-1 and Cadherin indicated the presence of tight and adherent junction proteins at cell–cell borders.

Conclusion. Our initial results confirm the suitability of the co-culture model under static conditions and will be used as a baseline for comparison with the intestine-on-chip experiments.

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intestine-on-chip model for analyzing gut mucosal immune responses. Journal of Visualized Experiments (JoVE), (207), e66603.

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Exploring the Potential of Paraprobiotic in Kefir Production: Effects on Short-Chain Fatty Acid Formation from *In vitro* Gut Fermentation

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Introduction. Recent research has shown that the positive health effects and therapeutic outcomes linked to probiotics may not strictly rely on the live state of the microorganisms (Barros et al., 2020; de Almada et al., 2018). As a result, terms like 'paraprobiotic' and 'postbiotic' have emerged to describe the beneficial roles of non-living microbial forms. Paraprobiotics refer to non-viable microbial cells or their structural components that can still exert health-supporting functions. The beneficial effects of paraprobiotics are largely attributed to their capacity to influence the composition and activity of the gut microbiota. A key functional role involves enhancing the production or stimulation of short-chain fatty acids (SCFAs), especially acetate, propionate, and butyrate. Whether paraprobiotics have bioactivity comparable to probiotics, particularly in terms of their potential to modulate the gut microbiota, is still under investigation. This study evaluated the effects of using probiotic (*Lacticaseibacillus casei* 39) and paraprobiotic (dead cells of *L. casei* 39) along with kefir culture in kefir production, focusing on short-chain fatty acid (SCFA) production after *in vitro* gut fermentation.

Materials and Method. In this study, probiotic (*Lacticaseibacillus casei* 39) and paraprobiotic kefirs (heat-inactivated *L. casei* 39), prepared as described in our previous work [1], were subjected to *in vitro* gastrointestinal digestion [2] and colonic fermentation [3]. After these processes, samples were centrifuged at 5000 rpm for 10 minutes to separate the pellet and supernatant. The pellet was used for DNA extraction, while the supernatant was further centrifuged at 15,000 rpm for 5 minutes, filtered through a 0.45 µm filter, and spiked with 1000 ppm 4-methyl valeric acid as an internal standard. The prepared samples were then directly injected into the GC system. Statistical analysis was conducted using one-way ANOVA in SPSS version 29.0 (SPSS Inc., Chicago, IL), with Duncan's multiple comparison test applied to assess differences between samples and storage times. Significance was set at $P < 0.05$.

Results and Discussion. The amounts of acetic acid, propionic acid, and butyric acid in the kefirs ranged from 145.35 to 291.49, 48.29 to 127.36, and 24.10 to 30.38 µg/mL, respectively. As a result, both paraprobiotic kefir and probiotic kefir produced similar amounts of SCFA, whereas the control kefir had lower acetic and propionic acid ($p<0.05$). These results suggest that paraprobiotics, despite being non-viable, can similarly support SCFA production during colonic fermentation as their live probiotic counterparts. The comparable levels of acetate, propionate, and butyrate produced by both probiotic and paraprobiotic kefirs indicate that inactivated cells may still exert metabolic stimulation on the gut microbiota. This highlights the potential of paraprobiotics as a safe and effective alternative to live probiotics, especially in products where viability is challenging or for vulnerable consumer groups. The lower SCFA levels in the control kefir further emphasize the functional contribution of *L. casei* 39, whether live or inactivated, in enhancing fermentation outcomes.

Conclusion. Overall, these results demonstrate that paraprobiotic kefir can effectively promote beneficial short-chain fatty acid production comparable to probiotic kefir, supporting its potential as a viable functional food ingredient where the use of live probiotics may be limited

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WG1 conclusions from the meeting presentations

The congress has clearly highlighted how the study of the gut microbiota represents one of the most complex yet promising frontiers in current biomedical research. The different contributions showcased complementary approaches, ranging from high-throughput *in vitro* models that allow the investigation of individual responses to specific dietary substrates, to advanced systems such as TIM-2 and bioengineered bioreactors, capable of more realistically simulating physiological conditions of the human colon. Particularly, the short-term batch *in vitro* approach has been covered in WG1, presenting several models, from MicroMatrix to MICODE. The presented data strongly emphasized the crucial role of diet, prebiotics, and bioactive compounds in shaping the microbial ecosystem, as well as the central function of short-chain fatty acids as mediators of metabolic and hormonal responses. At the same time, the research also pointed out persisting challenges, such as reduced fermentability under conditions of malnutrition or the difficulties in accurately monitoring fungal components of the microbiota. Moreover, bioengineering technologies emerged as powerful tools, enabling more stable, diverse, and physiologically relevant microbial cultures. In summary, this congress has provided an updated and multidisciplinary overview of gut microbiota research, underlining not only the progress achieved but also the importance of strengthening the synergy between basic, clinical, and engineering sciences. Future perspectives therefore point towards the

development of increasingly predictive and personalized models, with significant potential impact on nutrition, disease prevention, and precision medicine.

WG2: Extension to other gut compartments and host interactions

WG2 Outline and introduction

This WG is dedicated to the expansion of current *in vitro* models to include other body compartments and the implementation of *in vitro* gut models for studies on other gut compartments An important advancement is based on the extension/coupling of the gut models to encompass other digestive compartments harboring microbes (oral cavity, stomach, small intestine). The other idea is to better understand how to integrate the host compartment, at first with a mucus and epithelial barrier, but also in later stage with other cell types (such as lung, liver, brain, etc.) along several gut-organ axes. This would allow to create comprehensive and physiologically representative *in vitro* models that faithfully recapitulate *in vivo* situations.

WG2 Leaders and Team

WG2 is being lead by Clarisse Nobre (University of Minho, Braga, Portugal) and Anthony Buckley (University of Leeds, UK). Task 2.1. Connection to upper GI tract: Establish an overview of the current state-of-the art of model systems that regionalize the functionality and composition of the gut microbiota along the digestive tract, but also integrate the mucosal environment. Task 2.2. Coupling with host cells: identify *in vitro* gut models that can be (in)directly coupled to host cells (intestinal, liver, pulmonary, brain cells) or 3D cultures to provide a more accurate representation of the intestinal and extra-intestinal environments and their interactions with the host. Task 2.3. Miniaturization: analyze the potential of miniaturizing *in vitro* gut models to enable high throughput screening of dietary or diseased factors or individual fecal samples. Task 2.4. *In silico* gut models: discuss the application of *in silico* models for the exploration of human microbiomes in terms of functionality, growth kinetics, engraftment success and safety. In this context, 11 tools such as AGORA based prediction of growth kinetics, metabolic pathway prediction and screening of microbial genomes and metagenomes against publicly available databases can be further explored. Task 2.5. *In vitro-in vivo* correlations: Gathering an overview of *in vitro*/*in vivo* correlation (IVIVC) studies which are pivotal to assess the relevance and predictive capabilities of new *in vitro* gut models associated or not with cells, to accurately mimic in vivo (animal or human) systems. IVIVC will serve to bridge the gap between lab-based experiments and real-world applications.

WG2 Meeting Introduction and speakers

Working Group 2 aims to expand the application of *in vitro* gut models beyond the colon by integrating microbiota-host interactions across various gastrointestinal compartments and target organs. This includes exploring models that connect upper gastrointestinal regions (oral cavity, stomach, small intestine) with the colon, integrating host cells (intestinal, hepatic, pulmonary, neural), and incorporating *in silico* tools for predictive modelling. WG2 also addresses the miniaturization of models to allow for high-throughput screening and focuses on evaluating the *in vitro–in vivo* correlation (IVIVC) of these systems. This integrated approach will foster the development of physiologically representative models that can better mimic host–microbiome dynamics in both health and disease. At the 1st INFOGUT Annual Meeting, keynote speaker Dr. Pranjul Shah (University of Luxembourg) presented HuMiX (human–microbial crosstalk), a microfluidics-based model that enables the co-culture of human and microbial cells under conditions that closely mimic the human gastrointestinal interface. This innovative system bridges the gap between traditional cell culture methods and the complex *in vivo* environment of the human gut. In addition, the WG2 session featured two complementary *in vitro* models designed to study the small intestinal microbiota—a crucial yet understudied component of human health due to the invasive nature of sampling from this region. The first study, presented by Karen Delbaere (Ghent University, Belgium), described a comprehensive approach using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The second study, presented by Auriane Bron (University of Clermont Auvergne, France), focused specifically on the ileum through the Mucosal Artificial Ileum (M-ARILE**)** model. Furthermore, eight additional models were presented in poster format, showcasing some of the most advanced systems representing the upper gastrointestinal region. Several of these models incorporate miniaturization, microbiota–host and mucus interactions and emphasize the evaluation of *in vitro–in vivo* correlations. Collectively, they contribute to the refinement and physiological relevance of gastrointestinal simulations and align closely with the objectives outlined in WG2.

WG2 ORAL PRESENTATIONS

Advancing Digestive Physiology and Microbiome Research with the microGUT System: A Microfluidic Platform for Studying Human Intestinal Function

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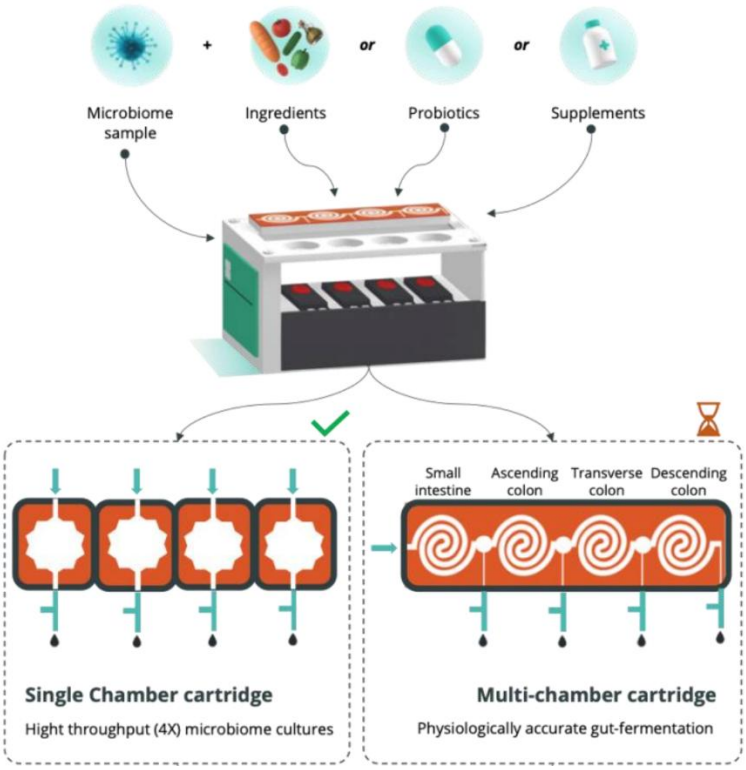
Abstract. The human gastrointestinal (GI) tract is a highly dynamic environment where physical forces, chemical gradients, and nutrient flow orchestrate the complex processes of digestion and absorption. Traditional *in vitro* and *in vivo* models often lack the precision and controllability needed to replicate these dynamic conditions. To address this gap, we present the microGUT system — an advanced microfluidic platform that recreates key physiological features of the human intestine, including peristaltic-like flow, physiologically relevant pH and oxygen gradients, dynamic nutrient delivery, programmable food transit-time, and automated sampling for real-time analysis. This versatile system enables high-resolution monitoring of digestive processes and nutrient transport under precisely controlled conditions. In addition, we are integrating the co-culture capabilities of the HuMiX system into the microGUT platform, creating an unprecedented, fully automated gut-on-chip system for the simultaneous study of digestion and host-microbiome interactions. This enhancement will enable complex, multi-dimensional investigations of how microbial communities influence digestive dynamics and host responses within a controllable microenvironment. In this talk, we will detail the engineering and validation of the microGUT platform and highlight its application in modeling human digestion via the commercially available iMATS platform. We will showcase how the system enables in-depth studies of nutrient breakdown, absorption dynamics, and the impact of dietary components and microbiome composition on digestive efficiency. With its capacity for continuous monitoring, automated sampling, and integrated co-culture capabilities, the iMATS platform bridges the gap between reductionist models and complex *in vivo* systems, paving the way for advancements in nutrition science, microbiome research, and the development of digestion-targeted therapeutics.



In this talk, we will detail the engineering and validation of the microGUT platform and highlight its application in modeling human digestion via the commercially available iMATS platform. We will showcase how the system enables in-depth studies of nutrient breakdown, absorption dynamics, and the impact of dietary components and microbiome composition on digestive efficiency. With its capacity for continuous monitoring, automated sampling, and integrated co-culture capabilities, the iMATS platform bridges the gap between reductionist models and complex *in vivo* systems, paving the way for advancements in nutrition science, microbiome research, and the development of digestion-targeted therapeutics.

Development of a small intestinal microbial community in the M-SHIME model

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Research group presentation. The Center for Microbial Ecology and Technology (CMET), part of the Faculty of Bioscience Engineering at Ghent University, is specialized in the study and application of mixed microbial cultures or communities. Within CMET, the research group Host-Microbe interaction led by Prof. Tom Van de Wiele, aims at designing *in vitro* enabling technology platforms to study the dynamics of human-derived microbiota. In particular, the lab has extensive experience in applying the *in vitro* Simulator of the Human Intestinal Microbial Ecology (SHIME) model to study the human gastrointestinal ecology, with recent developments on the exploration of the mucosal microbiome, the small intestine microbiome and several gastrointestinal disease states. Simulation of the microbiome from other body sites such as oral cavity, upper respiratory tract and skin have also been successfully conducted.
Introduction The small intestinal microbiota is crucial for human health yet remains largely unexplored due to sampling limitations. This study aimed to establish the small intestinal microbiota in the Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) using human saliva and fecal microbiota.
Material and Methods. A saliva community was established in an oral reactor and transferred each meal throughout the *in vitro* model consisting of separate oral, stomach, proximal small intestine, terminal ileum and colon compartments. Additionally, a fecal-derived proximal colon community was inoculated once into the terminal ileum compartment. This was repeated for five healthy individuals.
Results and Discussion. Results showed distinct microbial and metabolic patterns across compartments, indicating preliminary alignment with trends reported in *in vivo* studies. Notably, the oral reactor appears to facilitate the establishment of key small intestinal taxa.
Conclusion. This *in vitro* approach provides a valuable tool for studying small intestinal microbiota, increasing insights into its dynamics, function and therapeutic potential to improve human intestinal health.
Acknowledgments/funding. This work was supported by Ghent University (Microbes4Immunity—BOFGOA2022000803), FWO-FNRS (Homistasis FWOEOS202200603, KD was funded by FWO scholarship (1SE1721N), LM was supported by EU Marie Sklodowska-Curie Individual fellowship (project 101067622), K.D.P. was funded by Ghent University (BOF/GOA 01G03122).

Small is not large intestine: how to integrate these distinct features in a new *in vitro* model of the human ileal microbiome?

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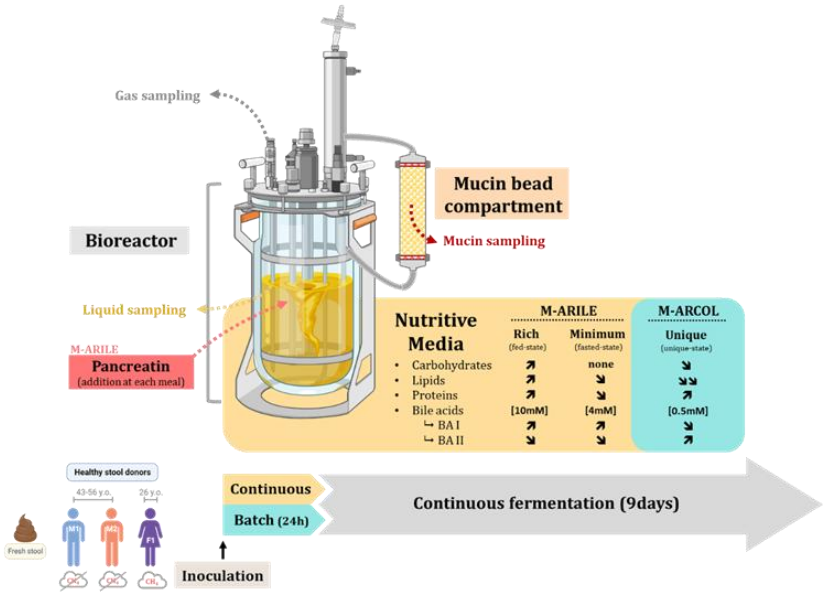

Introduction. The small intestine is the main site of food digestion and nutrient absorption in humans. The small intestinal microbiota certainly plays a key role in host health, but until now it was largely understudied due to sampling invasiveness, especially in healthy volunteers. Due to ethical, technical and cost reasons, *in vitro* dynamic models of the human small intestine appear as a great alternative to *in vivo* studies. However, there was yet no available *in vitro* system simulating the ileal compartment and its associated microbiota, that has been fully developed and validated based on *in vivo* data in humans.

Materials and Methods. In this context, the Mucosal Artificial Colon (M-ARCOL) was adapted to simulate the nutritional, physicochemical and microbial conditions found in a healthy

human ileum, leading to the Mucosal Artificial Ileum (M-ARILE). A wide literature review was performed to set-up this new *in vitro* model according to pH, transit time, digestive secretions and nutrient availabilities described under fed and fasted states in the human ileum. In order to validate the newly developed model, *in vitro* fermentations were performed during 9 days in both conditions (M-ARILE and M-ARCOL), when bioreactors were inoculated with the same stool sample (n=3 biological replicates, using fecal samples from three healthy adult volunteers). Gut microbiota composition (lumen and mucus-associated microbiota) and metabolic activities (gas and short-chain fatty acids -SCFA-) were daily monitored and compared to *in vivo* data in humans from the literature.

Results and Discussion. Total SCFA, acetate, propionate and butyrate concentrations as well as CO_2 and CH_4 proportions were significantly lower in the ileal model compared to the colonic one, while O_2 was enriched in the M-ARILE. Microbiota activity measured within the ileal model was mostly in line with data from the literature[I-IV]. Total bacteria levels as well as bacterial diversity (observed ASVs) were also decreased under ileal compared to colonic conditions. Some bacterial populations were enriched in the M-ARILE compared to the M-ARCOL, such as *Clostridiaceae*, *Veillonellaceae* and *Enterobacteriaceae* families, in accordance with *in vivo* data[V-XIII].

Conclusion. This new ileal model provides a powerful platform for mechanistic studies on the human small intestinal microbiome. M-ARILE will be helpful to decipher the role, at the individual level, of ileal microbes in human nutrition, drug metabolism and interactions with enteric pathogens targeting the small intestine, also considering the impact of nutritional status.

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Table 1. Validation of the M-ARILE model through *in vitro-in vivo* comparisons regarding microbial activity and bacterial composition. *In vitro* results in the M-ARILE were compared to *in vivo* data in the ileum of healthy human adults (based on *in vivo* analyses of aspirate or mucosal biopsies), regarding SCFA and gas production and most abundant families. ✓*: in vitro results are fully in accordance with in vivo data,* ×*: in vitro and in vivo data are not in accordance.*

			M-ARILE	*In vivo* (ileum)	*In vivo-in vitro* correlation
Microbiota activity	SCFA production[I, II] (mM)	Total SCFA	19.1	11.7-81.6	✓
		Acetate	14.5	7.9-64.6	✓
		Propionate	3.8	1.5-3.3	✓
		Butyrate	0.9	2.3-13.7	×
	Gas proportion[III, IV] (%)	CH_4	0	0	✓
		CO_2	50.3	6-17.5	×
		O_2	3.7	5-41	✓
		H_2	9.3	0.3-3.5	×
Microbiota load	Total bacteria[V]		10^8	10^7-10^8	✓
Microbiota composition[VI-XIII] (Family)	*Streptococcaceae*	Luminal & Mucosal	-	+	×
	Veillonellaceae		+	+	✓
	Clostridiaceae		+	+	✓
	Lactobacillaceae		-	+	×
	Enterobacteriaceae		+	+	✓
	Bacteroidaceae		+	+	✓
	Bifidobacteriaceae		+	+	✓

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Project Gut$_2$Brain: First insights in exploring the microbiota-gut-brain axis in obesity

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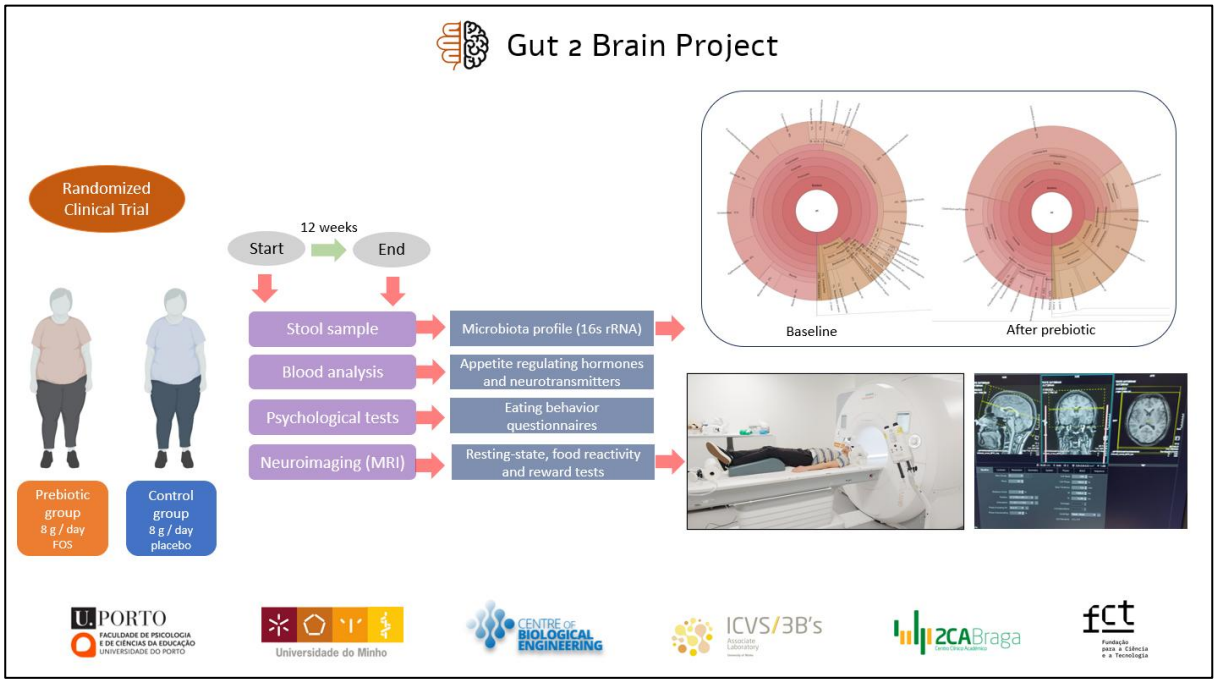

Research group presentation. We are a multidisciplinary network from the University of Minho (CIPsi, CEB, and ICVS/3B's), the University of Porto, and the Academic Clinical Center of Braga Hospital, combining complementary expertise in psychology, bioengineering, neuroscience, and medicine. We aim to generate knowledge on the microbiota–gut–brain axis (MGBA) both *in vivo* and *in vitro*.

Work Introduction. Obesity is associated with inflammation, microbiota imbalance, and altered neurotransmitters[1,2]. Gut bacteria release metabolites that influence the brain through the microbiota–gut–brain axis (MGBA), though its molecular mechanisms remain poorly understood. This project aim is to advance knowledge on: i) microbiota-host *in vitro* models; ii) the role of microbiota and its modulation through prebiotics can influence obesity development; and iii) the influence of the MGBA on psycho-behavioural and emotional aspects, as well as brain function, in the context of obesity.

Materials and Methods. A randomized clinical trial was conducted with individuals with obesity and binge eating disorder (BED), who received a prebiotic/placebo for 3 months. fMRI scans, psychological questionnaires, stool and blood samples were collected at the beginning and end of the study. Fecal samples were also used for *in vitro* fermentation.

Results and Discussion. Prebiotic supplementation led to an increase in beneficial bacteria such as *Bifidobacterium* and *Ruminococcus*, and a decrease in *Faecalibacterium* and *Escherichia/Shigella*. Questionnaire results indicated improved control over eating and reduced snacking. *In vitro*, the prebiotic also enhanced the abundance of *Bifidobacterium*, *Ligilactobacillus*, and *Enterococcus*, along with increased production of short-chain fatty acids (SCFAs), particularly propionate.

Conclusion. Although this study is still ongoing, early preliminary results—both *in vivo* and *in vitro*—are promising, suggesting that modulation of the MGBA with a prebiotic can help restore gut microbiota balance and improve control over food cravings.

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WG2 POSTER PRESENTATIONS

Influence of resistant starch on human gut microbiota and immune response using *in vitro* complementary approaches

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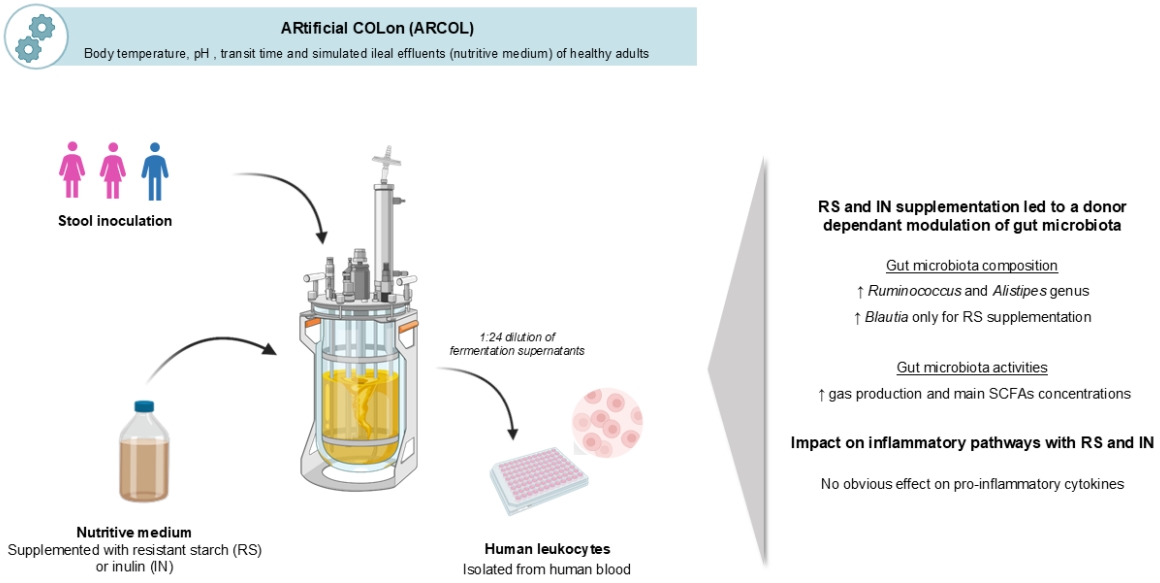

Research group presentation. MEDIS is a french joint research unit (Université Clermont Auvergne-INRAe, Clermont-Ferrand) aiming to study the role of gut microbiota in human and animal nutrition and health. For over 30 years now, MEDIS has developed a well-known expertise in *in vitro* gut simulation in both human and monogastric animals, with an original platform gathering complex and dynamic *in vitro* models of the oral, gastric, small and large intestinal compartments.

Introduction Resistant starch is a prebiotic insoluble fiber mainly found in cooked and cooled starchy food. RS can be provided by High Amylose Wheat (HAW) for which gut microbiota modulation has been documented in humans, but without being linked to immune responses

so far. The aim of this study was to evaluate the anti-inflammatory properties of RS from HAW, compared with inulin, a soluble prebiotic fiber, as mediated by gut metabolites, by using complementary *in vitro* human colon model and leukocytes isolated from human blood.

Materials and methods. An original approach combining the human ARtificial COLon (ARCOL) model and leukocytes isolated from human blood was used. Three ARCOL bioreactors were run in parallel; one used as a control and two daily supplemented with 15 g/L of RS or inulin (n=3 donors). Microbial activities were evaluated through gas and main short chain fatty acid (SCFA) measurement. Bacterial composition was assessed by 16S Metabarcoding. Supernatants from ARCOL fermentations were diluted and incubated with human leukocytes to measure pro-inflammatory cytokines after cell stimulation with lipopolysaccharides.

Results Supplementation with RS or inulin led to significant increases in main SCFAs concentrations (mainly acetate and butyrate) and gas production, with donor-dependent effects on profiles. The impact of RS and inulin on microbiota composition was also different depending on the donors. However, both prebiotics led to an increase in *Ruminococcus* abundance. Interestingly, *Blautia* was more prevalent with all donors only when fermentative media was supplemented with RS. Supernatants from ARCOL bioreactors led to a decrease in some pro-inflammatory cytokines by human leukocytes. However, no significant difference was observed between the control and treated (both RS and inulin) conditions.

Discussion and conclusion. RS and inulin supplementation induced donor-dependent modulation of microbiota composition and metabolic activities. However, in our study, gut metabolites produced after fiber supplementation did not exhibit any effect on immune pathways. In a next step, it would be of great interest to investigate if the two prebiotics have more marked effects when simulating *in vitro* diseased situations associated with microbiota perturbations, such as obesity.

Acknowledgments/funding. This study was funded by the project Plan France Relance 2020 “Resistant starch” and Limagrain company.

The simgi® Simulator and COST INFOGUT: Towards Harmonized complex *In vitro* Colon Models

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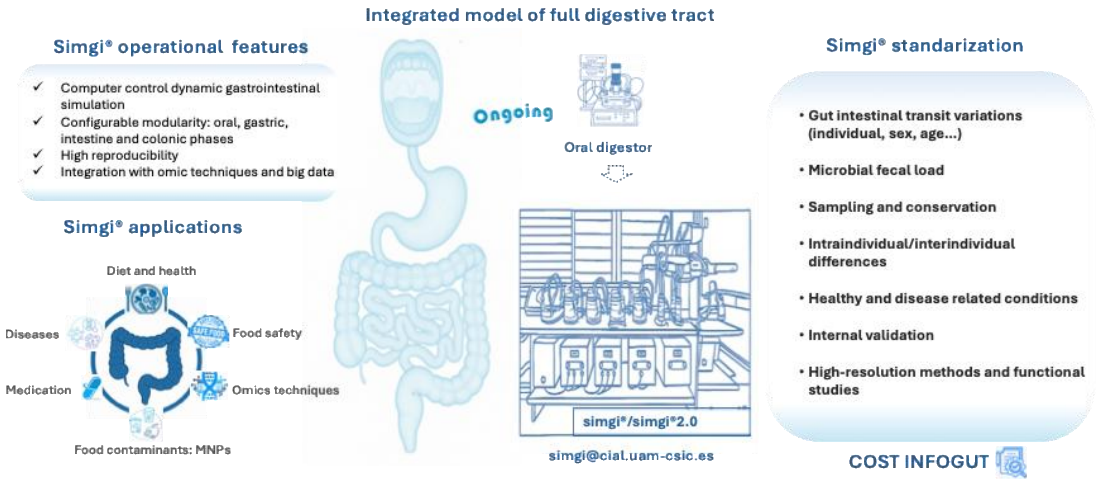

Research group presentation. The **simgi®** platform is offered as a scientific-technical service of the Spanish National Research Council (CSIC), located at the Institute of Food Science Research (CIAL). The Microbiome, Nutrition and Health Group leads and provides support to the simgi®-metagenomics Platform; https://www.cial.uam-csic.es/investigacion/departamentos/departamento-de-biotecnologia-y-microbiologia-de-alimentos/grupo-de-microbioma-alimentacion-y-salud-bea/. The simgi® dynamic gastrointestinal simulator is a computer-controlled model of the human gut that enables both continuous and individual simulations. It comprises five compartments representing the stomach, small intestine, and the three main colonic regions, —ascending, transverse, and descending colon— while reproducing its complex physiology, biochemistry, and microbiota. Currently, oral digestion, including oral microbiota and mouth environment, has been integrated to mimic the full digestive tract. At the colonic level, simgi® has shown metabolic consistency with *in vivo* data**,** highlighting its potential. The platform's protocols can be tailored to simulate specific populations and disease conditions**,** in both short- and long-term studies. Experimentation in the simgi® can be easily combined with omic approaches and big data. With more than 10 years of experience, simgi® has continuously evolved, to support customized simulations in food-health, contaminants, pathogens, antimicrobial resistance, fecal transplants and drug research, reinforcing the importance of standarized protocols and guidelines within COST INFOGUT.

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Acknowledgments/funding. The authors gratefully acknowledge funding from the Spanish Ministry of Science (grants EQC2019-005402-P and PID2023-148419OB-I00), and the EU (PlasticsFatE and PRIMA InnSol4Med projects).

Development of an innovative human gastric and small intestinal model simulating differential gastric emptying of real-size food particles and ileal microbiota

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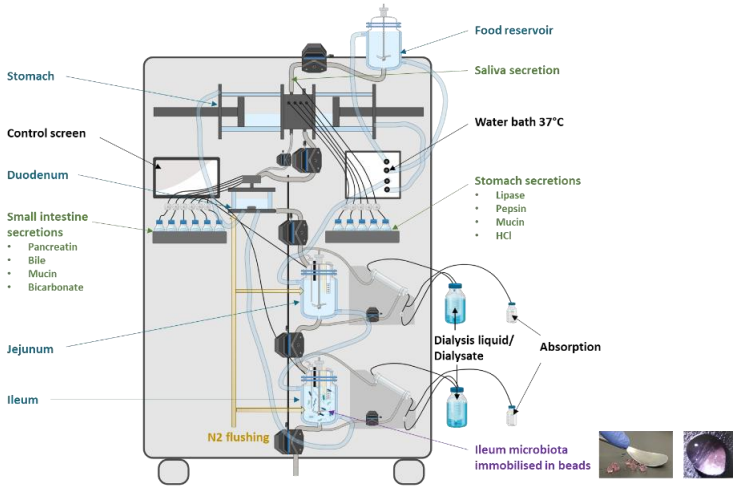

Schematic representation of the Engineered Stomach and Small Intestinal Model (ESIN)

Research group presentation. MEDIS is a french joint research unit (Université Clermont Auvergne-INRAe, Clermont-Ferrand) aiming to study the role of gut microbiota in human and animal nutrition and health. For over 30 years now, MEDIS has developed a well-known expertise in *in vitro* gut simulation in both human and monogastric animals, with an original

platform gathering complex and dynamic *in vitro* models of the oral, gastric, small and large intestinal compartments.

Introduction Numerous studies have highlighted the key role of food structure in digestibility and nutrient bioaccessibility. In line with the European 3Rs rules, a relevant alternative to *in vivo* assays to perform mechanistic studies is the use of *in vitro* models. However, up to now, there is no validated model of the full upper human gastrointestinal tract combining multi-compartmentation, ability to digest real-size food particles, absorption of nutrients and maintenance of a resident microbiota. To fill this gap, we are currently developing a new gastric and small intestinal system, the Engineered Stomach and Small Intestine (ESIN)[I-II].

Materials and Methods. ESIN is a dynamic and multi-compartmental system reproducing the stomach, duodenum, jejunum and ileum, set-up with *in vivo* data from the literature collected from healthy adult humans. Temperature is maintained at 37°C and kinetics of pH are reproduced in all compartments using hydrochloric acid (stomach) or sodium bicarbonate (small intestine). A food reservoir and the innovative structure of the gastric compartment enable realistic meal ingestion, digestion of real-size food particles and differential gastric emptying of liquids and solids. Oral, gastric, biliary and pancreatic secretions are introduced with physiological inputs. Progressive reduction of oxygen along the gastro-intestinal tract is possible thanks to nitrogen flushing. A dialysis system allows to reproduce passive absorption of nutrients, drugs and water. Lastly, resident microbiota will be introduced into the ileal compartment using an immobilization process to avoid microbial wash-out during the digestive process.

Results and Discussion. The first validation experiments of the ESIN model, set up to simulate a fasted state (ingestion of a glass of water), demonstrate that the stomach and ileal emptying closely replicate *in vivo* conditions[II-III]. Paracetamol bioaccessibility was also measured in the jejunal and ileal dialysis fluids and *in vitro* results compared to *in vivo* data in humans, showing good correlations[II,IV,V]. Preliminary experiments were performed using a real meal, showing that ESIN allows differential emptying of liquids and solids up to a size of 8 mm. An immobilization protocol of small intestine bacteria (*Lactobacillus* spp. as model strains) using gellan-xanthan gum beads is currently tested to evaluate its ability to protect and maintain the bacteria into the *in vitro* ileal environment[VI].

Conclusion. This new ESIN model will help to move towards a better understanding of the role of food structure and ileal microbes in human nutrition and health, e.g. in relation to food matrix-microorganism interactions and impact of intestinal microbiota on macronutrient digestibility or drug bioaccessibility.

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Acknowledgments/funding. This equipment was cofunded by the European Union as part of the European regional development fund (FEDER ESIN). The study was founded by Clermont Auvergne Université, Ghent University and Prodigest through a PhD grant to C. Beltramo, and the LabCom MIMET-iv (ANR-22-LCV1-003-01, 2023-2027).

MiTooth: A Scalable, Automated Platform for Clinically Relevant Modelling of Sub-gingival Biofilms

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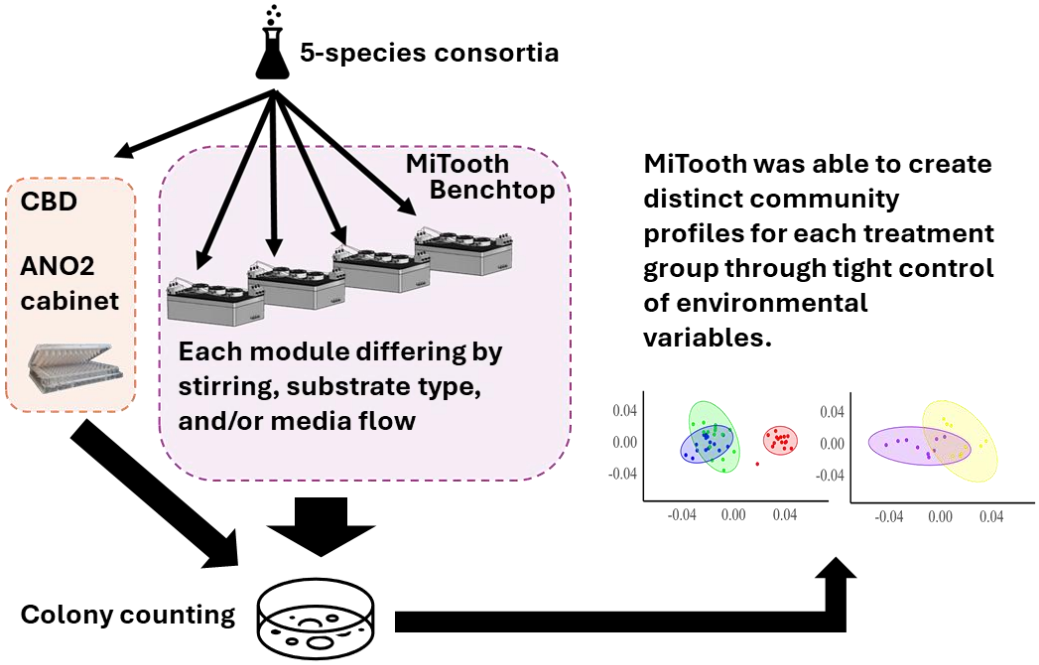

Research group presentation. Led by Dr Anthony Buckley, Professor Nik Kapur and Professor Peter Culmer, supported by post-doctoral fellows, PhD students, and colleagues from the Faculty of Medicine and Health, University of Leeds - this multidisciplinary group applies mechanical engineering and mechatronic expertise to create high fidelity *in vitro* platforms for microbiome science. Building on the success of their flagship achievement, the MiGut triple stage colonic model, they have ventured into further ecologies of clinical significance, notably the subgingival oral environment with MiTooth.

Introduction. *In vitro* modelling of oral biofilms often strikes a compromise between clinical relevance and throughput. The "MiTooth" platform overcomes these constraints by offering a scalable solution, specifically engineered to replicate the sub-gingival environment, and offers an automated hands-free solution to anaerobic biofilm cultivation and testing with minimalist laboratory outlay. A modular design and interconnectivity with laboratory apparatus enables adaptability for diverse experimental designs.
Materials and Methods. MiTooth was assessed using a validated five-species consortium intended to represent a periodontal disease state. Twelve reactors were inoculated from a single common inoculum, and biofilms were cultivated for two weeks under varying conditions of substrate type, media flow, and stirring, before harvesting and enumeration by colony counting.
Results and Discussion. MiTooth successfully supported the cultivation of defined-species biofilms over a two-week period, demonstrating precise control of experimental parameters, as evidenced by distinct community compositions among the different sample groups. These biofilm communities displayed a highly conserved rank order in species abundance across all tested environments. Gram-positive early colonisers exhibited the lowest abundance, whereas Gram-negative species were relatively dominant, consistent with typical disease progression. Additionally, community evenness increased in response to greater environmental complexity.
Conclusions. MiTooth is a reliable, economical, and versatile platform for oral biofilm research. Its modest initial laboratory investment, low benchtop footprint, and minimal consumption of energy and consumables make it suitable for widespread adoption. It retains the enhanced clinical relevance associated with continuous-fed bioreactor models, whilst providing improved capacity for biological replicates. Thus, MiTooth constitutes a useful instrument for high-throughput exploration of biofilm dynamics in oral microbiology.

MICRO-B: a versatile bioreactor for the gut microbiota *in vitro* culture

Costanza Daddi[1,2], Marco Calvigioni[3], Irene Chiesa[1,2], Amedeo Franco Bonatti[1,2], Gabriele Maria Fortunato[1,2], Sushila Maharjan[4], Carmelo De Maria[1,2], Emilia Ghelardi[3], Yu Shrike Zhang[4], And Giovanni Vozzi[1,2]

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Work Introduction. MICRO-B is a dynamic bioreactor for the *in vitro* culture of the gut microbiota and the investigation of its interaction with the host. MICRO-B reproduces the gut

microenvironment *in vitro*, applies an *in vivo*-like gut peristaltic stimulation to the cultured sample and potentially will allow the *in vitro* co-culture of the human gut microbiota (HGM) and eukaryotic cells. MICRO-B, together with an ad hoc developed scaffold (Biagini F. et al., Sci. Rep., 2020), was exploited to perform human fecal microbiota and Caco-2 cell cultures, separately.

Results and Conclusions. The bioreactor ensured the development of a homogeneous biofilm, and the peristaltic stimulation promoted biofilm formation, metabolic activity and proliferation over time. Caco-2 experiments assessed a high cell viability in all culture conditions, the formation of microvilli-like structures and the expression of typical intestinal surface proteins both in short- and long-term cultures. Collectively, MICRO-B represents a versatile platform for advancing our understanding of gut physiology and developing more physiologically relevant *in vitro* models that will boost the investigation of the HGM-host interplay.

Complex long-term triculture intestinal barrier model for evaluating micronanoplastic's hazard within the gut in different health states

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Work Introduction. Developing realistic *in vitro* models is essential in scientific contexts where human studies are unfeasible and to reduce animal research. It is increasingly important to simulate health conditions, such as diseases, infections, eating disorders, etc. In this context, micro- and nanoplastics (MNPs), are a public health concern as they accumulate in food along the production and consumption chain.

Results and Conclusions. Two independent chronic simulations in the Dynamic Gastrointestinal Simulator (simgi®) were carried out with different fecal microbiota donors, mimicking arbitrary healthy and dysbiotic states. After digestion and colonic fermentation, polyethylene terephthalate (PET) MPs modulated the microbiota´s secretome, affecting the integrity, interleuquin production and metabolic activity of a complex Caco-2/HT-19-MTX-E12/Raji B barrier model. Notably, these effects depend on the gut simulation step and health state. Our results suggest that microbiome-host interactions are influenced not only by the modulating agent, but also by the host's health status.

WG2 conclusions from the meeting presentations

Some of the most relevant dynamic upper gastrointestinal models developed by the scientific community were presented at the 1st InfoGut Annual Meeting, along with models simulating microbiota–host interactions. Among the bioreactors showcased were MiTooth, a scalable, automated platform for clinically relevant modelling of subgingival biofilms; the SHIME (Simulator of the Human Intestinal Microbial Ecosystem), incorporating human saliva and fecal microbiota, combined with *in vivo* data and epithelial models; the ARCOL (Artificial Human Colon) model, complemented with leukocytes isolated from human blood; the M-ARILE (Mucosal Artificial Ileum) model, representing the luminal and mucosal microenvironments of the ileum; the ESIN (Engineered Stomach and Small Intestine), which simulates differential gastric emptying of real-food-size particles and includes ileal microbiota; the Simgi® simulator, complemented with oral digestion; MICRO-B, a versatile bioreactor for microbiota–host crosstalk investigations; the microGUT system, a microfluidic platform for studying human intestinal function; and the Gut2Brain platform, designed to explore the microbiota–host–brain axis in obesity by integrating *in vivo* and *in vitro* data. Together, these models represent a significant advancement in the ability to simulate and study complex gastrointestinal and microbiota–host interactions under physiologically relevant conditions.

WG3 Extension to diseased situations

WG3 Outline and introduction

This Working group (WG) aims to extend the potential of *in vitro* gut models for the study of human and animal diseases by considering not only the dysbiotic microbiota (provided by fecal or oral samples) but also the full range of parameters shaping the gut diseased environment. The group investigates model adaptations for gastrointestinal diseases including inflammatory bowel diseases -IBD, irritable bowel syndrome (IBS) and colorectal cancer (Task 3.1), metabolic and non-communicable disorders such as obesity, diabetes, coeliac disease (Task 3.2), and enteric infections, evaluating microbial dynamics and host–pathogen interactions (Task 3.3). WG3 seeks to investigate the use of *in vitro* systems to evaluate the effects of dietary xenobiotics and environmental pollutants on gut microbiota composition and function, as well as the microbial metabolism of such compounds (Task 3.4). Finally, WG3 assesses both conventional and innovative microbiota-targeted therapeutic strategies—including pharmaceutical formulations, prebiotics, probiotics, postbiotics, live biotherapeutic products, and fecal microbiota transplantation—for their potential to restore gut homeostasis in disease contexts (Task 3.5) (Figure 1).

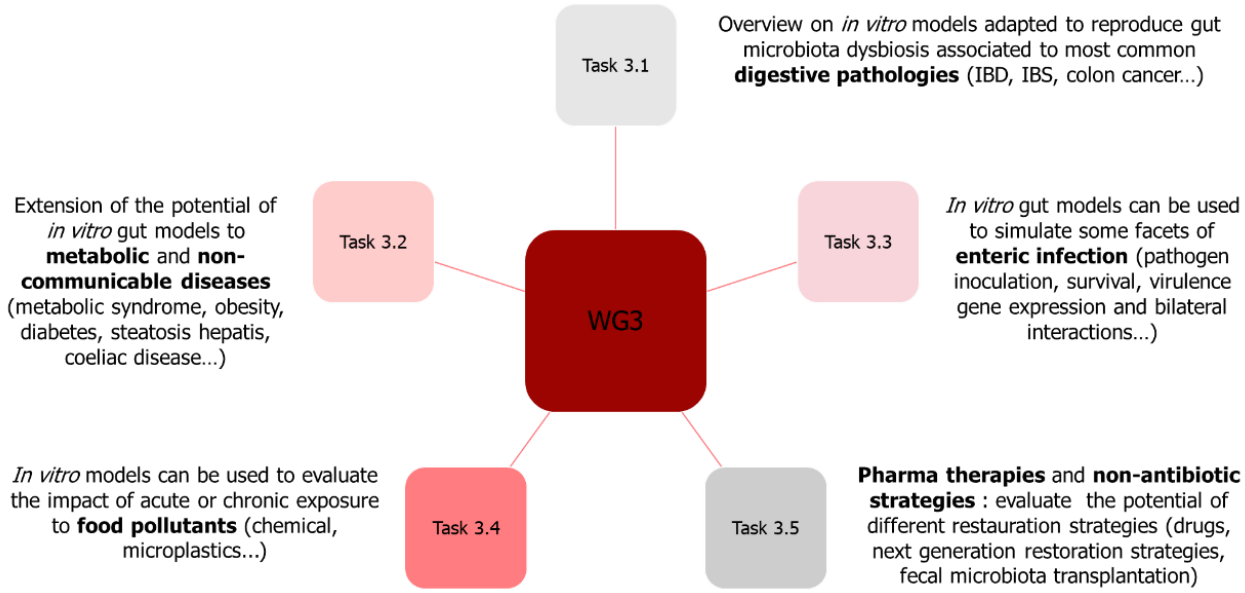

Figure 1. Activities covered by Working Group 3

WG3 Leaders and Team

The group is led by two WG leaders Lucie Etienne-Mesmin (Université Clermont Auvergne, France) and Lidia Tomás-Cobos (Ainia, Spain) who oversee the overall coordination and strategic direction. Each of the five tasks is managed by a team of dedicated young task leaders from different countries (France, Spain, UK, Belgium, Portugal, Turkey, Italy, Sweden) ensuring focused expertise and efficient progress across all work areas (Figure 2).

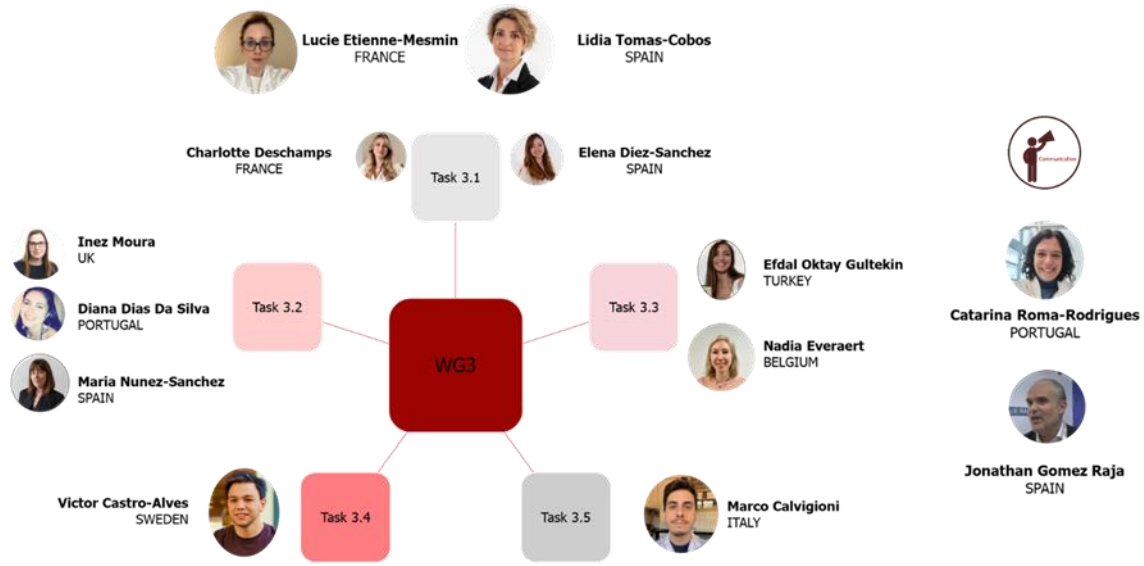

Figure 2. Team for Working Group 3

WG3 Meeting Introduction and speakers

The WG3 session highlights innovative applications of *in vitro* gut models to study and investigate microbiota-related mechanisms across diverse gastrointestinal conditions. Ludovica Marinelli (Ghent University, Belgium) presents an *in vitro* approach to identify microbial and cellular signatures in Short Bowel Syndrome. Charlotte Deschamps (Université Clermont Auvergne, France) explores how patient-derived stool samples are used to model IBS-D microbiota in the *in vitro* Mucosal Artificial COLon (M-ARCOL) system. Elena Diez (ANIA, Spain) discusses advanced experimental models designed to reproduce intestinal dysbiosis (ColonSim, MinigutSim and NanogutSim). Susanna Delgado (CSIC, Spain) introduces a colonic *in vitro* infant model to investigate the interaction between allergenic cow's milk proteins and patient-specific microbiota. Finally, Ines Moura (University of Leeds, UK) demonstrates how the MiGut model can define microbial ecologies under varying fecal concentrations. The poster session offers an opportunity to explore a diverse range of cutting-edge research on gut microbiota and *in vitro* modeling across multiple disciplines.

WG3 ORAL PRESENTATIONS

Development of an *in vitro* approach to identify microbial signatures of Short Bowel Syndrome

Ludovica Marinelli[1], Karen Delbaere[1*], Pieter De Clercq[1], Tim Vanuytsel[2,3], Tom Van de Wiele[1]*

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Research group presentation. The Center for Microbial Ecology and Technology (CMET), part of the Faculty of Bioscience Engineering at Ghent University, is specialized in the study and application of mixed microbial cultures or communities. Within CMET, the research group Host-Microbe interaction led by Prof. Tom Van de Wiele, aims at designing *in vitro* enabling technology platforms to study the dynamics of human-derived microbiota. In particular, the lab has extensive experience in applying the *in vitro* Simulator of the Human Intestinal Microbial Ecology (SHIME) model to study the human gastrointestinal ecology, with recent developments on the exploration of the mucosal microbiome, the small intestine microbiome and several gastrointestinal disease states. Simulation of the microbiome from other body sites such as oral cavity, upper respiratory tract and skin have also been successfully conducted.
Introduction. Short bowel syndrome (SBS) is a severe condition characterized by reduced bowel length, impairing digestion, nutrients absorption, and altered microbial ecology. Upon surgical resection, spontaneous morphological changes in the intestinal mucosa occur in half of the patients, resulting in improved absorption. However, microbial alterations associated to both resection and spontaneous adaptation, are poorly described. By using an adapted *in vitro* Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME), we aim to describe alterations in microbial ecology and host-bacteria interaction in pre- and post-resection.
Material and Methods. A SHIME model was developed to include oral, small intestinal and colon compartments, inoculated with samples from five healthy donors and then applied to simulate an SBS-type resection. Bacterial composition and metabolic activity pre- and post-resection were quantified. This model was repeated with for five donors. In parallel, seven SBS patients were recruited and the fecal bacterial composition analyzed.
Results and Discussion. Preliminary observations indicated alterations in both bacterial composition and metabolic activity, induced by the simulated *in vitro* resection. Some patterns observed post-resection are consistend with findings from literature on *in vivo* SBS. The comparison of *in vitro* and SBS patients' data to identify SBS signatures is ongoing.
Conclusions. By combining the optimized *in vitro* SHIME model with patients' data, this approach may provide insights into microbial ecology in SBS and support future investigations into host-microbiota interactions. Further work is ongoing to validate and expand these findings.

Acknowledgments/funding. This work was supported by Ghent University (Microbes4Immunity—BOFGOA2022000803), FWO-FNRS (Homistasis FWOEOS202200603, LM was supported by EU Marie Sklodowska-Curie Individual fellowship (project 101067622), KD was funded by FWO scholarship (1SE1721N), PDC was funded by FWO scholarship (1S51925N).

From patient stool to mechanistic insights: modelling IBS-D gut microbiota in M-ARCOL

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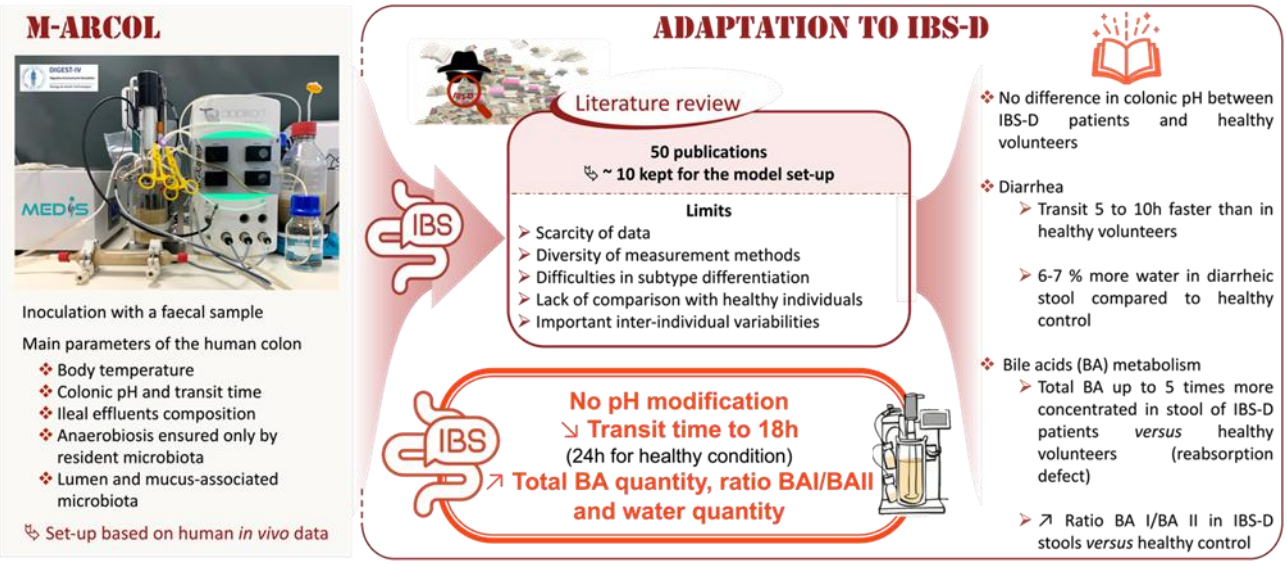

Research group presentation. UMR 454 MEDIS UCA-INRAE (Clermont-Ferrand, France) aims to study the role of the intestinal microbiota in human and animal health. The lab has extensive expertise in *in vitro* digestion and fermentation, with a dedicated platform including static batch models and dynamic systems reproducing the stomach and small intestine (TIM-1 and ESIN v2), the ileum (M-ARILE), the large intestine (M-ARCOL), or the entire gastrointestinal tract (M-SHIME), that can be combined with epithelial cell culture (Caco-2 TC7, HT-29 MTX). These models have been optimized over 25 years and adapted to both human and animal (pig and dog) situations, to various age groups (infants, adults and elderly), physiological states, food matrices (from water to complete meals), and pathologies (such as obesity and IBS).

Introduction. Irritable bowel syndrome (IBS) is one of the most prevalent chronic functional disorders of the human gastrointestinal tract. Characterized by recurrent abdominal pain,

intestinal discomfort and bowel transit disturbance, this condition can be highly debilitating. Over the past decades, studies have revealed alterations in the gut microbiota, highlighting its potential role in the onset and progression of the disease. *In vitro* human gut models offer a valuable alternative to *in vivo* approaches for preclinical investigations of IBS. However, no existing *in vitro* system accurately replicates the colonic ecosystem of IBS patients. In this context, we aimed to develop a new *in vitro* model of the human colon adapted to diarrhoea-predominant IBS (IBS-D), based on the Mucosal Artificial Colon (M-ARCOL), previously set-up under healthy conditions. Here, we report the application of this new model through a cross-comparison of gut microbiota structure and activity in fermentations conducted under either healthy or IBS-D conditions, using stools from IBS-D patients or healthy volunteers.

Material and Methods. Following an extensive literature review, we designed a modified version of the M-ARCOL incorporating the specific physicochemical and nutritional parameters of IBS-D. In particular, transit time was reduced while total bile acid concentration, the ratio of primary to secondary bile acid and water content were increased. To validate the system and explore its potential for mechanistic studies, *in vitro* fermentations were performed using bioreactors inoculated with fecal samples from either healthy individuals (n = 4) or IBS-D patients (n = 4), under healthy or IBS-D conditions.

Results and discussion. When the IBS-D parameters were applied to M-ARCOL inoculated with IBS-D stools, the system preserved key microbial features characteristic of the disease *in vivo*, thereby validating the new system. Specifically, compared to the healthy condition, the IBS-D model showed reduced bacterial diversity, decreased abundances of *Rikenellaceae* and *Prevotellaceae*, and increased levels of *Proteobacteria* and *Akkermansiaceae*. Notably, applying IBS-D conditions to healthy stools did not induce an IBS-D-like dysbiosis, and conversely, applying healthy conditions to IBS-D stools was insufficient to restore microbial balance.

Conclusion. This validated IBS-D colonic model represents a robust and flexible *in vitro* platform to investigate gut microbial dynamics in the absence of host factors. It enables the study of both luminal and mucosal contributions to IBS-D-associated dysbiosis. Moreover, it offers a promising tool for testing dietary or microbiota-targeted interventions aimed at the personalized restoration of intestinal eubiosis.

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Acknowledgments/funding. This study was performed in the frame of the PSPC RESTORBIOME (2019–2023). This project was supported by the Program Investissement Avenir operated by BpiFrance, General Mills/Yoplait and Greencell industrial partners, Clermont Auvergne Metropole and Région Auvergne Rhône Alpes.

Advanced *in vitro* experimental models to reproduce intestinal dysbiosis

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Research group presentation. The Preclinical *In vitro* Studies Department at AINIA specializes in simulating the complete gastrointestinal digestion and evaluating the biological activity of functional ingredients representing the host interactions and other tissue or organ targets. Its research focuses on developing advanced protocols and dynamic *in vitro* systems to assess bioaccessibility, gut microbiota modulation, and colonic fermentation. The department also uses monoculture and co-culture cell models that replicate target organs, allowing for the *in vitro* analysis of physiological processes through biomarker-based pathway evaluation to assess the biological activity of functional ingredients.

Introduction. The growing interest in the role of the gut microbiota in human health has highlighted the need for robust *in vitro* models capable of simulating intestinal dysbiosis under physiologically relevant conditions. In this context, three complementary colonic fermentation systems have been developed at different operational scales. These tools integrate fermentation approaches, allowing the simulation of complex microbial and metabolic dynamics associated with imbalances in the intestinal ecosystem. By enabling the controlled evaluation, these models represent a valuable platform for designing novel preventive or therapeutic strategies aimed at restoring microbiota balance and promoting overall health.

Materials and Methods. Three *in vitro* colonic fermentation systems at different scales to simulate intestinal dysbiosis under controlled conditions. The NanogutSim, (working volume 5-20 ml) enables high-throughput screening under static conditions. The MinigutSim, (working volume 125 ml), allows controlled fermentation with enhanced sampling capabilities. Finally, the ColonSim, (working volume 1-1.5L) is a dynamic model integrating gastrointestinal digestion and colonic fermentation (ascendent, transversal and descendent colon) with continuous feeding, suitable for long-term and preclinical validation studies.

To develop new *in vitro* tools capable of simulating intestinal dysbiosis at different scales, the incorporation of an antibiotic during the colonic fermentation simulation is used.

Results and Discussion. To simulate intestinal dysbiosis under controlled laboratory conditions, three different *in vitro* colonic fermentation models at different operational scales—NanogutSim, MinigutSim, and ColonSim—were developed. Each system was designed to address specific experimental needs, ranging from rapid screening to long-term, repeated-dose studies. Below is a comparative analysis of their operational characteristics and experimental capabilities.

NanogutSim: High-Throughput Static Screening Platform

The NanogutSim model was optimized for rapid, parallel testing of multiple experimental conditions and combinations. This system is particularly suited for preliminary screening

studies, where the goal is to evaluate the short-term effects of single-dose interventions on microbial communities.

- Parallel Testing: Capable of running several test conditions simultaneously, enhancing throughput and efficiency.
- Static Configuration: Designed for colonic fermentation.
- Single-Dose Administration: Designed for one-time exposure to test compounds.
- Short-Term Duration: Experiments typically span 24 to 48 hours, allowing for quick turnaround.
- Application: Ideal for initial hypothesis testing, compound selection, and feasibility assessments.

MinigutSim: Intermediate-Scale Static System

The MinigutSim model was designed as a static colonic fermentation system at an intermediate volume, offering improved environmental control and sampling capacity compared to smaller-scale systems. While maintaining a single-dose, short-term format, its larger volume enables more representative microbial activity and supports more detailed microbial and metabolic analyses.

- Increased Volume: Enables better control of fermentation parameters and allows for more frequent and diverse sampling.
- Static Configuration: Designed for colonic fermentation.
- Single-Dose Administration: Maintains a simplified dosing strategy for short-term exposure studies.
- Moderate Duration: Experiments typically last between 48 and 96 hours, allowing for the observation of medium-term microbial responses.
- Multiple Endpoints: Supports comprehensive analysis, including microbial composition, metabolite production, and functional outputs.

ColonSim: Long-Term, Repeated-Dose Model

The ColonSim system was developed for more advanced studies requiring repeated administration of test compounds over extended periods. This model closely mimics chronic exposure scenarios and is ideal for evaluating sustained microbial and metabolic changes. It also simulates the previous gastrointestinal digestion (stomach and small intestine).

- Limited Parallel Testing: Supports a maximum of two simultaneous conditions in parallel.
- Repeated-Dose Administration: Allows for daily or periodic dosing, simulating real-world intake patterns.
- Extended Duration: Experiments typically last 20 to 30 days, enabling the observation of long-term microbial dynamics and host-microbe interactions.
- Multiple Endpoints: Comprehensive data collection includes microbial shifts, metabolite production, and functional biomarkers.
- Application: Best suited for chronic intervention studies, validation of therapeutic strategies, and modelling persistent dysbiosis.

Figure 1. Image of the Colonic fermentation models at different operation scales designed.

This work presents only the results of the dysbiosis model using the ColonSim system, in which, in addition to the use of antibiotics, a treatment is applied to restore the microbiota. The results showed that antibiotic addition on day 7 induced clear dysbiosis, with significant reductions in beneficial bacteria such as Bifidobacteria and Lactobacillus. By day 14, microbial levels were at their lowest. Following treatment on day 21, partial recovery was observed, especially in Bifidobacteria and LAB, though not all populations returned to baseline levels.

Conclusion This work presents the development of three *in vitro* colonic fermentation systems at different scales, each tailored to specific experimental needs—from rapid screening to long-term, dynamic studies. Together, these platforms offer a flexible and scalable approach for investigating gut microbiota responses and evaluating interventions under controlled conditions, contributing valuable tools for advancing intestinal health research.

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In vitro infant colonic model to study interactions between allergenic cow's milk proteins and the patient's microbiota

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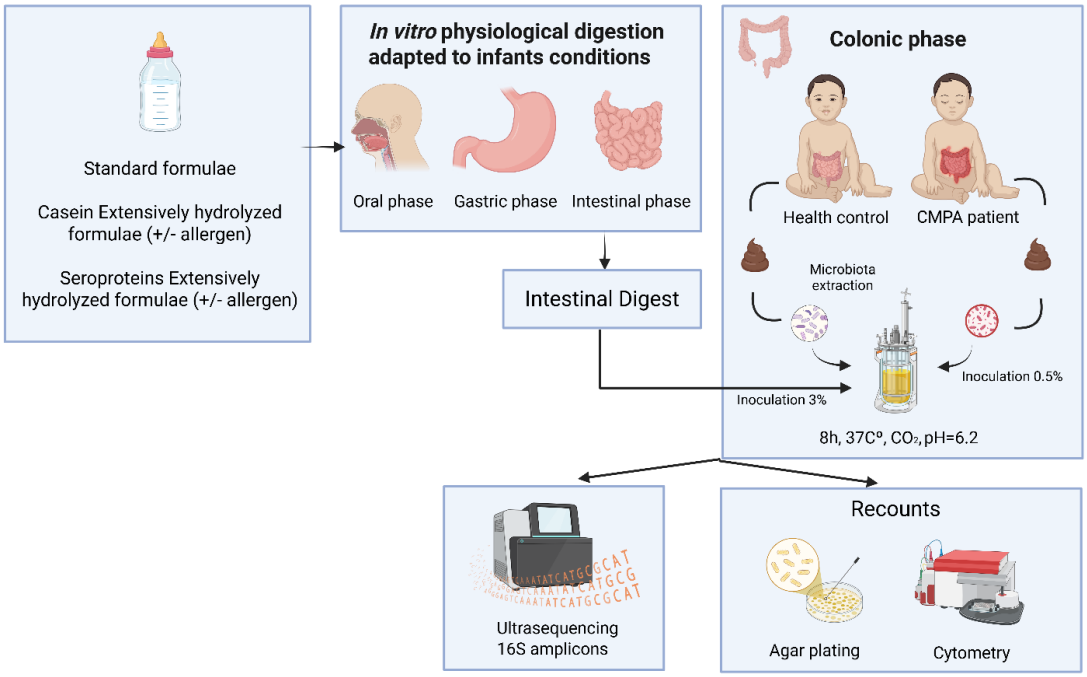

Research group presentation. The MicroHealth Group (Functionality and Ecology of Beneficial Microbes MicroHealth) has several research lines aiming to study i) interactions of intestinal microorganisms with gastrointestinal and technological conditions and ii) dysbiosis associated with different physiological and pathological conditions (food allergies amog others) by focusing on microbial disease markers, strategies to modulate the microbiota and development of adapted functional foods (https://www.ipla.csic.es/en/functionality-and-ecology-of-beneficial-microbes-microhealth/).

Introduction. The importance of the gut microbiota in food allergy in early life has been highlighted. In the first years of life, one of the foods most likely to cause food allergy is milk. Cow's milk protein allergy (CMPA) can be non-IgE mediated. These forms of allergy usually present with gastrointestinal symptoms and are difficult to diagnose due to the lack of analytical methods. *In vitro* models that mimic the infant digestive environment and these pathophysiological allergic conditions are very valuable.

Materials and Methods. In this study, we have used *in vitro* digestion simulations according to INFOGEST protocols but adapted to infant conditions, followed by colonic fermentation in

mini-bioreactors to test interactions between the fecal microbial component of CMPA patients and controls of the same age (~ 5 months old). We use frozen inoculum (Hevia et al., 2015) at 0.5% and basal media previously proven to support the growth of inhabitants of the infant gut (Arboleya et al., 2013). The intestinal digest of different formulae; extensive hydrolyzed formulae from caseins (cEHF) or seroproteins (sEHF) with the addition or not of intact allergenic cow's milk proteins (CMPs) as allergens were added to the bioreactors at 3%. Fermentation elapsed for 8 hours in anaerobic conditions at pH 6.2. Recounts were made at time 0 and after 8 h by agar plating and flow cytometry. Finally, at t8 the profile of the microbial communities was evaluated by ultrasequencing of 16S amplicons.

Results and Discussion. Numbers of microorganisms were shown to be low at t0, around 10^3 in controls and less in patients, 10^2. However, after 8h of colonic fermentation numbers increased to 2logaritimic units, reaching 10^5 in fecal fermentation from controls infants and 10^4 in patients. Analysis of the microbial populations revealed that members of the *Enterobacteriaceae* family dominate the batch fermentations when inoculated with fecal microbiota from infants with CMPA. In particular, enterobacteria populations were higher when intact CMPs were intentionally added to hydrolyzed infant formulas. Differences were also observed depeding on the type of hydrolyzed formulae, with *Clostridiaceae* members growing when use cEHF but not in sEHF (Figure 1).

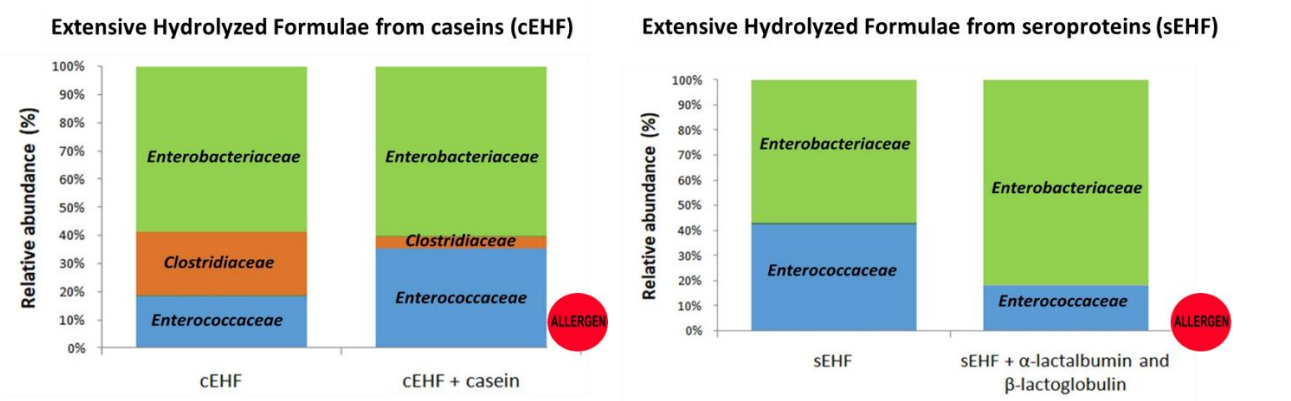

Figure 1. Relative abundance of members of microbial families after growing in the colonic model with distinct types of hydrolyzed formulae and the addition or not of different CMP.

Conclusion. Although some studies have used *in vitro* digestion simulations according to INFOGEST protocols in infants, the microbial component is usually not considered. This study presents the first attempt to incorporate the infant microbiota into an *in vitro* colonic model of CMPA after the digestion. We observed that the main factors modifying the colonic microbial profile were the type of microbiota (from patients or controls) and the type of digested formula. To a lesser extent, the addition of intact proteins appears to promote the growth of certain microorganisms. Further research is needed to corroborate these findings and to validate and standardize the model.

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WG3 POSTER PRESENTATIONS

Using a miniature gut model (MiGut) to define microbiota ecologies using different fecal slurry concentrations

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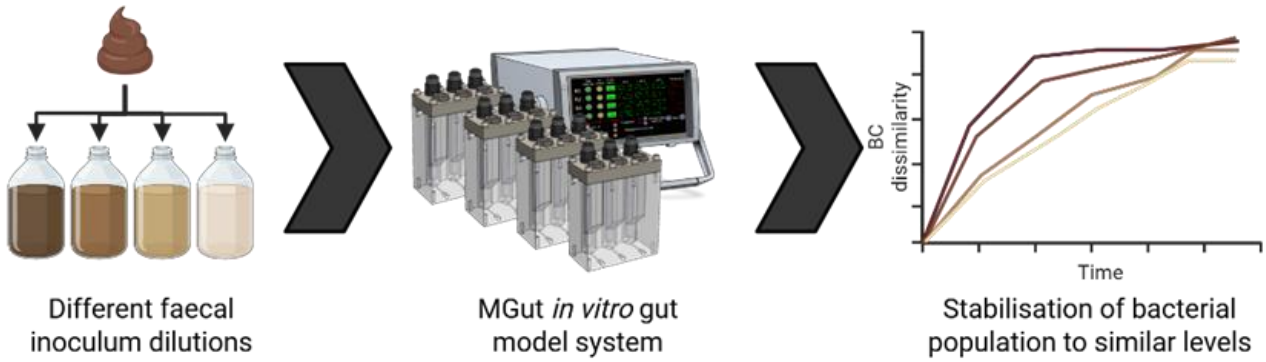

Research group presentation. MiGut was developed by a multidisciplinary team of engineers, microbiologists and food experts of the University of Leeds. WDB is a research fellow in engineering that developed MiGut during his PhD and leads a commercial unit at Leeds looking into technology applications. AB has expertise in nutrition impact on the human gut microbiome and bioreactor experiments. PC brings mechatronics and sensing expertise to MiGut. MW is a clinician expert on gastrointestinal diseases that offers clinical insight. NK has experience in designing controlled fluidic environments for biochemical applications and works closely with industry. IBM has a background studying gastrointestinal diseases and the

impact of antibiotics on the human microbiome, having developed the methods for quantifying samples in MiGut.

Introduction. The human gut microbiome plays a crucial role in food digestion and host immunity. *In vitro* models of the human colon offer a reliable and ethical alternative to *in vivo* testing (1). *In vitro* models can be inoculated with fecal samples to ensure the microbiome is accurately recaptured. However, the large sample requirements of such models can pose practical constraints to complex studies. Here we used a high-throughput, small size model of the human colon, MiGut, to determine whether more dilute fecal inocula can be used, reducing the sample size requirements and facilitating future studies where sample volume is critical.

Materials and Methods. Two independent experiments were performed, each consisting of eight MiGut models (2). Each model consists of three anaerobic chambers maintained at 37°C and continuously fed with a complex nutrient media, replicating the physiological conditions and pH of the proximal (pH 5.5±0.1) medial (6.25±0.1) and distal (6.75±0.1) colon. Each set of models was inoculated in duplicate with a fecal sample from a healthy donor, using four different slurry dilutions (10%, 5%, 2.5%, and 1.25% w/v). All models were run continuously for 20 days with samples collected thrice weekly for analysis via quantitative PCR (qPCR) (3) and 16S rRNA sequencing (2).

Results and Discussion. Bray-Curtis (BC) dissimilarity plots comparing each model to its fecal inoculum (Figure 1, top) throughout the experiment, were used to assess overall population dynamics as previously described for MiGut (2) and other *in vitro* gut model systems (4).

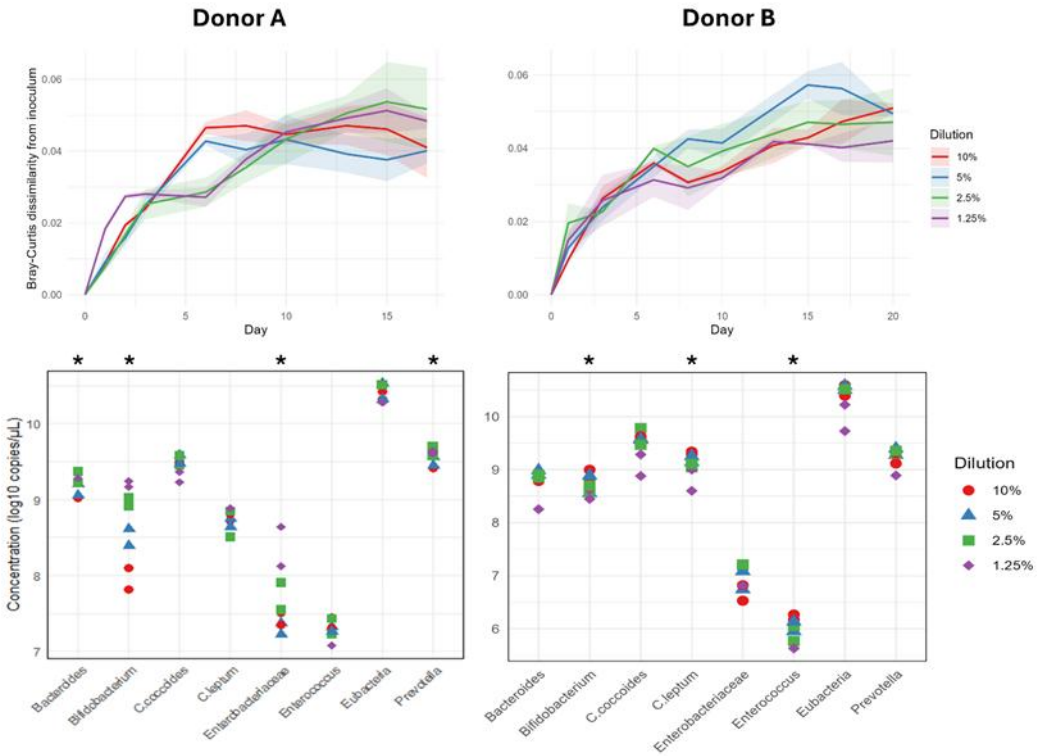

Figure 1. Top: Bray-Curtis dissimilarity from the original fecal inoculum over time for Donor A (left) and Donor B (right) across different fecal dilutions. Lines represent the mean of biological replicates, with shaded areas indicating standard error. Bottom: Linear regression analysis of bacterial populations

quantified by qPCR (log_{10} copies/µL) at experimental day 3. Asterisks (*) indicate bacterial populations showing significant correlations between concentration and dilution level ($p < 0.05$, linear regression analysis).

Although some differences were evident in the early days of the study (e.g. Donor A, day 6), the populations in all reactors stabilized by the end of the experimental period, shown by the plateauing of the plots. Linear regression analysis of qPCR data at day 3 (Figure 1, bottom) revealed that some populations were significantly correlated with dilution: more dilute slurries favored the growth of *Bifidobacterium* spp. and Enterobacteriaceae in donor A, while *Enterococcus* spp. and *C. leptum* were more prevalent in concentrated slurries for donor B. These initial differences are likely due to different growth rates between populations, whereby some groups had an initial competitive advantage before a stable microbial community is established (5). These differences appear to be donor-specific and transient, having resolved as populations stabilised, with no populations being significantly correlated with dilution by the end of the study. 16S rRNA sequencing analysis confirmed convergence to similar communities as both prevalent (e.g. Bifidobacteriaceae and Lachnospiraceae) and low abundant (e.g. Acidaminococcaceae and Coriobacteriaceae) bacterial families were recaptured at similar abundance levels, resulting in consistent compositions and Shannon diversity indices.

Conclusion. MiGut models successfully recaptured the gut microbial composition of the healthy microbiota using reduced fecal slurry concentrations. While initial population dynamics varied between dilutions, all models converged to a similar profile from day 10 onwards, recapturing the donor microbial communities. Unlike batch culture systems which exhibit blooms of some populations (6), the extended run time of MiGut and strict environment control allow for a stable equilibrium to be established *in vitro* even when using low inoculum concentrations. Reducing the donor material allows repeat testing with donors of interest and the ability to use clinical samples, such as those from clinical trials or patients, where sample quantity is often a limiting factor. Future work should also evaluate the metabolic profile of the microbiota to ensure that functional output is retained at reduced concentrations.

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Investigation of the Effects of Type 2 Diabetes Mellitus-Associated Genetic and Inflammatory Markers on Intestinal Microbiota and Metabolites

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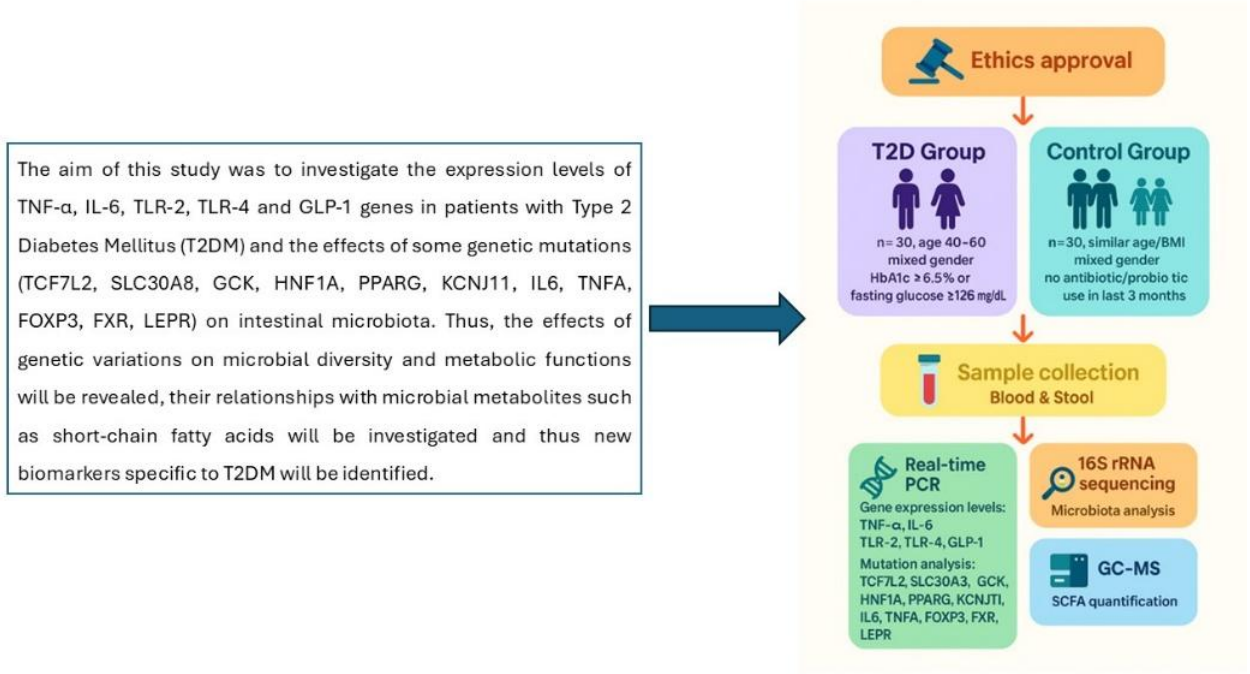

Research group presentation. This study included 30 Type 2 Diabetes patients (HbA1c ≥ 6.5% or fasting glucose ≥ 126 mg/dL) and 30 healthy controls, all aged 40-60 with similar BMIs, recruited from Mersin Tarsus State Hospital. A G*Power analysis indicated 60 participants were sufficient for the two-group comparison. Exclusion criteria for both groups included antibiotic/probiotic use within the last 3 months and a history of chronic inflammatory disease.

Work Introduction. Type 2 Diabetes Mellitus (T2DM) is a growing chronic metabolic disease characterized by insulin resistance and β-cell dysfunction [1]. Its development is influenced by genetic, lifestyle, immunological, and microbial factors [2]. Proinflammatory cytokines (e.g., TNF-α, IL-6) and Toll-like receptors (e.g., TLR-2, TLR-4) disrupt insulin signaling via inflammation, impacting metabolic balance [3]. Genetic variations in genes like TCF7L2, SLC30A8, and PPARG are key to T2DM, though their effects on gut microbiota remain unclear [4]. The gut microbiota is vital for glucose metabolism and immune response, with metabolites like short-chain fatty acids (SCFAs) and bile acids directly affecting host physiology [5].

Investigating the link between T2DM gene expression profiles, microbial diversity, and metabolites will enhance understanding of the disease's pathophysiology and aid in developing personalized treatments [6].

Materials and Methods. Peripheral blood and stool samples obtained from participants will be evaluated by Real-Time PCR for TNF-α, IL-6, TLR-2, TLR-4, GLP-1 gene expression levels and mutation analysis of 11 target genes ((TCF7L2, SLC30A8, GCK, HNF1A, PPARG, KCNJ11, IL6, TNFA, FOXP3, FXR, LEPR)); microbiota analysis will be performed by 16S rRNA sequencing and SCFA (acetate, propionate, butyrate) quantification by GC-MS method. The data obtained will be evaluated by statistical analyses to explain the role of genetic and microbial interactions in the pathophysiology of Type 2 Diabetes.

Conclusion. Type 2 Diabetes Mellitus (T2DM) is a widespread chronic metabolic disease characterized by insulin resistance and β-cell dysfunction. Its development is influenced by genetic, lifestyle, immunology, and microbial factors. Inflammatory markers like TNF-α, IL-6, TLR-2, and TLR-4 disrupt insulin signaling, impacting metabolic balance. Genetic variations in genes such as TCF7L2, SLC30A8, GCK, HNF1A, PPARG, and KCNJ11 contribute to T2DM, though their specific effects on gut microbiota are not fully known. The gut microbiota plays a crucial role in glucose metabolism and immune response, with metabolites like short-chain fatty acids (SCFAs) and bile acids directly affecting host physiology. Examining the relationship between T2DM-specific gene expression, microbial diversity, and metabolic products will enhance our understanding of the disease and aid in developing personalized treatments.

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The use of patient-derived preclinical models for the investigation of colorectal cancer biomarkers

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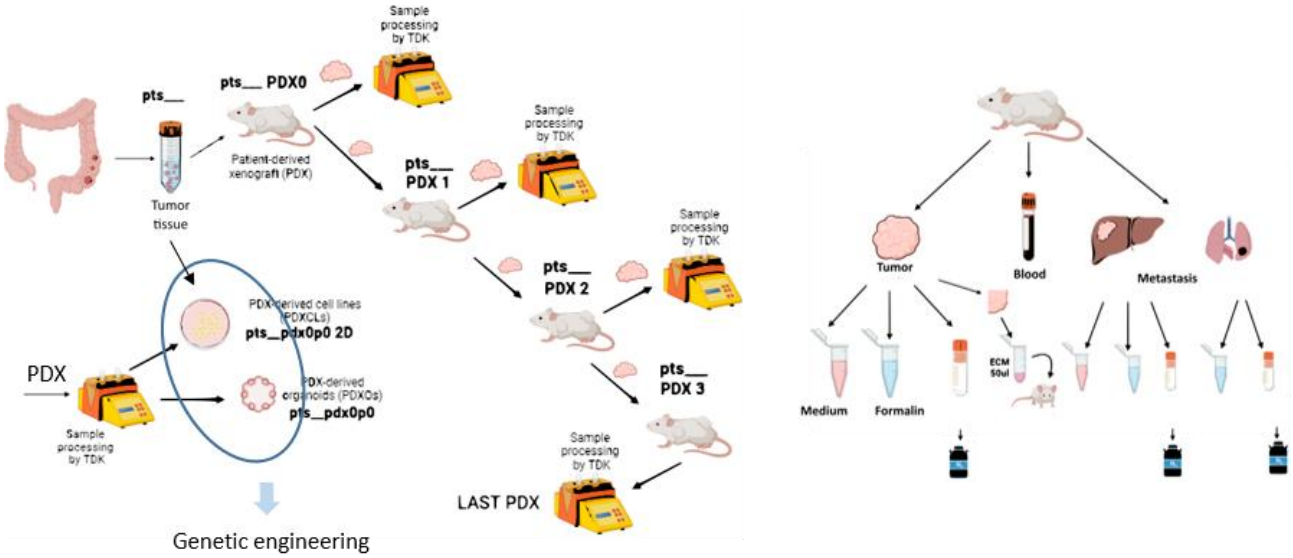

Research group presentation. We focus on the mechanisms of metastasis and chemoresistance in colorectal cancer (CRC), searching for predictive and prognostic markers, including the application of liquid biopsy techniques. Thanks to cooperation with clinics, we employ models such as organoids and patient-derived xenografts. Recently, we study the role of aldehyde dehydrogenases in CRC and investigate their epigenetic regulation. Genetic modification of tumour cells and the CRISPR-Cas9 approach represent essential tools in our research.

Work Introduction. Colorectal cancer is the second leading cause of cancer-related deaths worldwide [1]. Patient-derived models play an essential role in understanding tumour biology, identifying reliable biomarkers, and developing new treatment options. Recent tools enable the precise engineering of patient-derived preclinical models, allowing for the study of the impact of overexpression/attenuation/knock out of particular gene(s) on the traits of tumour cells.

Materials and Methods. We have processed more than 750 vital human colorectal cancer specimens, including liver metastases. Pieces (approx. 2 mm^3) of tumour tissue were subcutaneously administered to NSG mice in the presence of extracellular matrix under inhalation anaesthesia to induce patient-derived xenografts. Simultaneously, organoids and

adherent xenolines were prepared. *In vitro* models were also prepared directly from the vital CRC specimens. Subsequently, retroviral vectors or the CRISPR-Cas9 approach have been used to edit the expression of genes involved in tumorigenesis or metastasis.

Results and Discussion. We established a collection of patient-derived epithelial cell lines, cancer-associated fibroblasts, organoids, and patient-derived xenografts. One cell line exhibited significant overexpression of aldehyde dehydrogenase 1A1 (ALDH1A1), a marker of cancer stem cells in many malignancies. We employed the CRISPR-Cas9 approach to attenuate the ALDH1A1 gene. Attenuation led to changes in the cell line's characteristics towards an aggressive phenotype.

Conclusion. Patient-derived cancer models represent a valuable model for studying of mechanisms of tumour biology and metastasis.

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Acknowledgments/Funding. The work was supported by the Slovak Research and Development Agency under contract APVV-21-0296, by the COST action CA23110 – INFOGUT, and by VEGA 2/0170/22. Illustrations were created by BioRender.com.

An electrospun gelatin membrane-based 3D *in vitro* model of the gut microbiota to study infectious diseases and pathologies related to intestinal mucus alterations

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Research group presentation. This interdisciplinary consortium of clinical microbiologists and bioengineers, all from the University of Pisa, Italy, was born from the desire to develop a groundbreaking *in vitro* model for culturing the human gut microbiota, untangling through it the countless roles of the gut microbiota in human health.
Work Introduction. *In vitro* models represent important tools to study human diseases *ex vivo*, including infectious diseases and pathologies associated with mucus layer abnormalities. Here, we introduce a novel microplate *in vitro* model of the gut microbiota based on the use of electrospun gelatin membranes to explore *in vitro* gut microbiota-related diseases.
Materials and Methods. After an initial validation of the model [1], a mucin coating was overlaid on the membranes to reproduce the mucosal environment found in the intestine and select mucus-adhering microbes [2]. Then, *Bacillus cereus*, a common spore-forming pathogen responsible for food poisoning in mammals, has been added in the model to investigate its impact on the gut microbiota composition [3]. Real-time quantitative PCRs and 16S rRNA gene sequencing were performed to characterize microbial composition.
Results and Discussion Mucin-coated scaffolds harbored microbial consortia enriched in mucus-adhering bacteria, including *Akkermansia*, *Bifidobacterium*, *Faecalibacterium*, and *Lactobacillus*, thus effectively selecting a mucosa-associated microbiota starting from fecal samples. These findings highlight the importance of mucins in shaping intestinal populations, even in artificial systems. The presence of *B. cereus* determined significant modifications of the intestinal communities. Notably, abundances of *Pseudomonadota* (particularly *Escherichia coli*), *Akkermansia*, and *Lactobacillus* were reduced, while levels of *Bifidobacterium*, *Clostridium*, and *Mitsuokella* increased, suggesting a potential role of such alterations in microbiota composition in *B. cereus* overall pathogenicity.
Conclusion. The model is very promising, versatile, and easy-to-manage and can be useful for studying the effect of enteropathogens, as well as drugs, pro/prebiotics, and other compounds, on the gut microbiota and diseases derived from alterations of the mucus layer by modulating the thickness of the mucus coating.

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Microbial eukaryotes and gut health: underappreciated interactions?

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Work Introduction. The role of bacteria in the intestinal microbiome is well established, with numerous studies linking microbiome composition to health and disease. Although it is well known that the intestinal microbiome includes archaea, viruses, bacteriophages, and eukaryotes, research has overwhelmingly focused on bacteria.
Results and Discussion. Our data indicate that microbial eukaryotes - organisms with much larger and more complex genomes than bacteria - significantly influence bacterial microbiome composition and are correlated with human health. For example, the widespread protist Blastocystis (estimated to be present in one billion people globally) is strongly associated with a healthy gut microbiome rather than dysbiosis. However, other widespread but understudied microbial eukaryotes, such as *Entamoeba dispar*, *Entamoeba coli*, *Endolimax nana*, and *Iodamoeba bütschlii*, may also play important roles. Our ongoing metagenomic studies of healthy and diseased cohorts aim to further clarify their role in gut health and disease.
Conclusion. A key next step is to develop *in vitro* gut models to investigate how these eukaryotes interact with bacteria, influence gut homeostasis, and ultimately affect human health.

WG3 Conclusion from the Meeting presentations

Together, these presentations showcase the breadth and innovation of current *in vitro* gut model research, spanning disease-specific microbiota signatures, mechanistic insights across a spectrum of diseases and highlight the use of such models as advanced platforms for studying host-microbiota interactions. The WG3 session clearly demonstrates the growing relevance and diversity of *in vitro* gut models in the study of microbiota-related mechanisms underlying disease. The studies presented reflect a strong emphasis on modeling dysbiosis in different physiological and pathological contexts, including short bowel syndrome, IBS-D, food allergy, and early-life microbiota disturbances. Across all contributions, there is a consistent drive to develop realistic, disease-adapted *in vitro* platforms that integrate host and microbial factors with high physiological relevance. These models enable the controlled investigation of host–microbiota interactions, support the identification of disease-specific microbial and cellular signatures, and offer valuable insights for the evaluation of therapeutic strategies targeting the gut ecosystem. Collectively, the work presented reinforces WG3's commitment to advancing predictive, ethical, and mechanistic *in vitro* approaches for the understanding and modulation of dysbiosis across a broad spectrum of human and animal diseases.

WG4 Data science and data management

WG4 Outline and introduction

The goal of this working group is to advance data science and data management in the context of gut microbiome research in *in vitro* models. WG4 aims to identify, organize, and catalogue open-access microbiome data sources, analytical methods, and bioinformatics tools relevant to gut multi-omics. In addition, it will evaluate the current state of *in silico* approaches used to model gut function, with a particular focus on the microbiome.

The main tasks of WG4 are:

- Task 4.1: Create FAIR catalogues of gut omics data and bioinformatics tools. Identify and organize relevant omics data repositories and open studies related to the gut microbiome, ensuring that they follow FAIR principles (Findable, Accessible, Interoperable, Reusable).
- Task 4.2: Evaluate *in silico* approaches for gut modelling. Collect, assess, and benchmark commonly used artificial intelligence (AI), machine learning (ML), and bioinformatics tools applied to gut microbiome analysis.
- Task 4.3: Develop training materials. Design and produce educational resources for gut data analysis, to be used in training activities by WG5.

WG4 Leaders and Team

Thomas C. A. Hitch (leader), University Hospital of RWTH Aachen, Aachen, Germany

Dr. Hitch is a post-doctoral researcher in the Functional Microbiome Research Group at the University Hospital of RWTH Aachen. His research focuses on the characterization, and naming, of novel bacteria from the gastrointestinal tract. In addition to the cultivation and taxonomic assignment of bacteria, his research develops methods to create synthetic communities (SynComs) that represent complex microbiota, and their potential application for treating health conditions.

Enrique Carrillo de Santa Pau (co-leader), IMDEA Nutrition, UAM+CSIC, Madrid, Spain

Dr. Carrillo is the head of the Computational Biology Group at the IMDEA Nutrition Institute and co-founder of Microsei Biotech SL, a spin-off company aimed at transforming microbiome research into innovative health solutions. His research focuses on advancing precision nutrition through the development of computational, molecular, and translational tools to characterize human variability in response to diet, with the ultimate goal of building actionable models for chronic disease prevention and healthy aging.

Anna Mascellani Bergo (Task 4.1 leader), Czech University of Life Sciences Prague, Prague, Czech Republic. Dr. Mascellani Bergo is a researcher at the Department of Food Science at the Czech University of Life Sciences Prague, with a strong focus on NMR-based metabolomics approaches. Her research focuses on understanding metabolic variability and host–microbiome interactions in both human and ecological contexts, with the goal of identifying mechanisms relevant to health, nutrition, and environmental sustainability.
Petra Polakovičová (Task 4.2 leader), Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic. Petra Polakovicova is a junior bioinformatician at the Institute for Clinical and Experimental Medicine (IKEM) in the Laboratory of Pathophysiology of Metabolism. Their group is dedicated to clinical research on the human gut microbiome and its potential role in the development of non-communicable diseases. They also study the effects of diet on the composition and function of the gut microbiome. By integrating various omics, especially metagenomics, metabolomics, and proteomics, they aim to understand this complex ecosystem and identify potential biomarkers that contribute to health and disease.
Leo Lahti (Task 4.3 leader), University of Turku, Finland. Prof. Lahti is group leader at the Department of Computing, University of Turku, Finland. His research focuses on computational microbiome analysis, in particular on population cohort studies. He is a member of the Bioconductor Community Advisory board, a global research network for computational life sciences.
Miguel Rocha (Task 4.3 co-leader), Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal.

WG4 Meeting Introduction and speakers

Within the framework of WG4 of the INFOGUT COST Action (CA23110), three key initiatives were highlighted during the 1st INFOGUT Annual Meeting, held in Bologna from 26 to 28 May 2025, all aimed at advancing data science and data management in gut microbiome research using *in vitro* colon models.
The first initiative, presented by Anna Mascellani Bergo (Czech University of Life Sciences Prague), describes a journal club focused on multi-omics strategies for *in vitro* colon models. This activity contributes to Task 4.2 (evaluation of *in silico* gut modelling approaches) through monthly meetings where selected publications are discussed. The goal is to identify accessible methods and tools for multi-omics microbiome integration, followed by an analysis of their application by current studies. The journal club also maintains structured note-taking and literature review practices, with the potential to lead to a future review publication.
The second initiative, led by Petra Polakovičová (Institute for Clinical and Experimental Medicine, Prague), focuses on the collaborative benchmarking of bioinformatics tools for microbiome data analysis. This effort aims to systematically evaluate pipelines for 16S rRNA and whole metagenome sequencing (Illumina, ONT, PacBio), starting with gold-standard datasets such as simulated reads and synthetic communities, and expanding to real *in vitro* samples. The benchmarking process covers all stages of microbiome data processing and downstream analysis, with the goal of offering a reproducible framework to support robust microbiome research.
In addition, Prof. Nicola Segata (University of Trento) delivered an invited lecture on standardized data and tools for large-scale, reproducible human microbiome research, providing key insights into the importance of harmonization and best practices in microbiome data analysis at scale. His contribution emphasized the relevance of building shared resources and pipelines to improve reproducibility and comparability across studies.

WG4 ORAL PRESENTATIONS

Journal club: Multi-omics in *in vitro* colon models

Mascellani Bergo Anna

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Abstract. Within the "WG4: Data Science and Data Management", Task 4.2 aims to evaluate *in silico* approaches for gut modelling. A journal club has been established with monthly meetings, during which three publications are selected for discussion. The current discussion theme, which may also lead to a future review publication, focuses on multi-omics approaches for *in vitro* models. Our goal is to explore accessible methods, software, and tools that facilitate multi-omics microbiome integration and to examine how studies have applied multi-omics strategies. To support this effort, literature searches are conducted to identify relevant methods, and structured note-taking have been established during journal club meetings.
Funding: This work was supported by the "COST Action CA23110 "International networking on *in vitro* colon models simulating gut microbiota mediated interactions (*INFOGUT*).

Robust Benchmarking of Microbiome Bioinformatics Tools: A Collaborative Effort in INFOGUT WG4

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Abstract. The accuracy and reliability of microbiome research depend heavily on bioinformatics pipelines, yet comprehensive and robust benchmarking on a broader scale is lacking. As part of COST Action INFOGUT (CA23110) Working Group 4, this initiative aims to systematically review and benchmark bioinformatics tools for microbiome sequencing data processing and downstream analysis. By providing a structured evaluation, this effort will serve as a valuable resource for microbiome researchers, helping to guide the selection of robust and reproducible bioinformatics pipelines. This initiative focuses on benchmarking bioinformatics tools for 16S rRNA gene sequencing and whole metagenome sequencing across Illumina, ONT, and PacBio platforms. The first phase involves reviewing existing benchmarking studies and defining key evaluation criteria. The second phase will assess tools for processing raw sequencing data into analyzable taxonomic profiles, starting with gold-standard datasets, including simulated reads and synthetic communities. Benchmarking will

then be extended to real microbiome datasets, including *in vitro* models. Following the third phase—interpretation and reporting of results—the same approach will be applied to benchmarking downstream analysis methods, ensuring a comprehensive evaluation across all stages of microbiome data analysis.

Funding. COST Action CA23110 "International networking on *in vitro* colon models simulating gut microbiota mediated interactions (INFOGUT).

WG4 POSTER PRESENTATIONS

Biome-specific genome catalogues reveal functional potential of shallow sequencing

Matti O. Ruuskanen[a], Alejandra Escobar-Zepeda[b*], Martin Beracochea[b], Jennifer Lu[b], Dattatray Mongad[a], Lorna Richardson[b], Robert D. Finn[b], Leo Lahti[a]*

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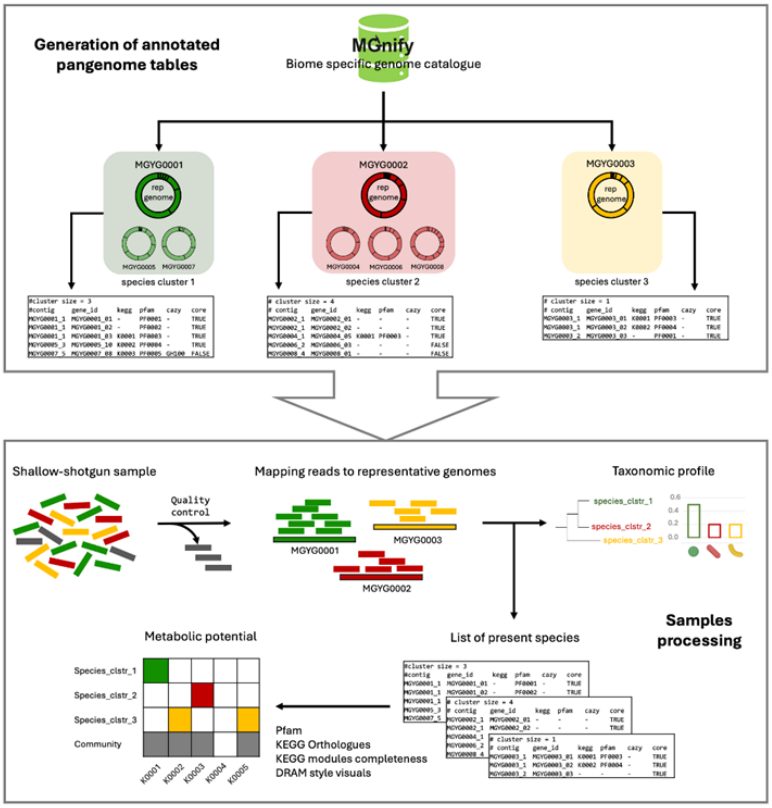

Abstract. The use of 16S rRNA metabarcoding for functional prediction is limited by several biases. Shallow shotgun sequencing is a cost-effective and taxonomically high-resolution alternative to 16S rRNA metabarcoding, but the low sequencing depth limits functional

inference. Our BioSIFTR tool maps shallow shotgun sequencing reads against single-biome databases and extrapolates into their precalculated functional profiles. We used three datasets from red junglefowl, mice, and human gut, containing matched deep shotgun metagenomic, and 16S rRNA metabarcoding data for taxonomic and functional benchmarking. An additional human gut deep shotgun sequencing data set was subsampled to 1 million reads and analyzed with BioSIFTR to attempt replicating previously obtained results. BioSIFTR taxonomic and functional profiles closely agree with the results of the full deep sequencing data in all biomes. We also replicated differences in the human gut microbiome between high and low trimethylamine N-oxide producing participants, using only < 2 % of the original deep sequencing data. The BioSIFTR tool is a powerful approach, which approximates the functional information of a deep-sequenced metagenome while using only a fraction of the data. Shallow shotgun sequencing combined with BioSIFTR could be a stand-in replacement for 16S rRNA metabarcoding with an increased taxonomic and functional resolution, and lower bias. **Funding.** This project was supported by the European Union's Horizon 2020 research and innovation programme, the "FindingPheno" project [952914], the COST Action project, "International networking on *in vitro* colon models simulating gut microbiota mediated interactions (INFOGUT)" [CA23110], the Research Council of Finland [338818 to M.O.R.], and the Finnish Cultural Foundation [M.O.R.].

Bioinformatics tools for functional microbiome data analysis

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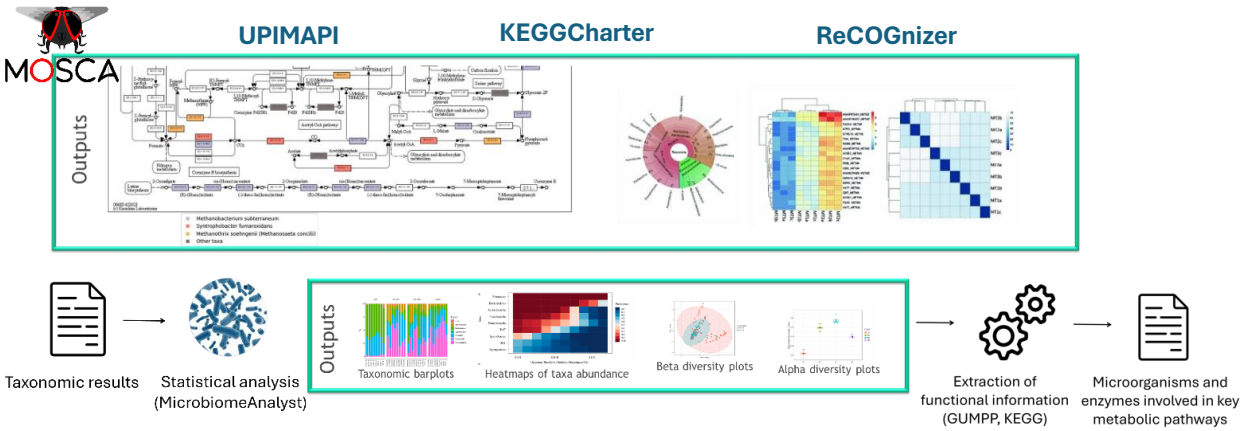

Research group presentation. We are a research group based at the Centre of Biological Engineering (CEB) at the University of Minho, in Braga, Portugal. Our work is rooted in the development of functional foods and the evaluation of their impact on the microbiota-gut-brain axis, combining *in vivo* and *in vitro* methodologies. We have been developing bioinformatics in-

house tools for the analysis of microbial communities and multi-omics datasets. Our goal is to provide robust and scalable resources that allow deeper biological insight into microbiome dynamics and their modulation.

Work Introduction. Bioinformatics tools are crucial for unravelling the intricate microbial relationships and functions within the gut microbiota, where important knowledge gaps still remain. In this work we present a set of bioinformatics tools designed to support high-resolution taxonomic profiling (based in 16S rRNA sequencing data), functional annotation, microbial network reconstruction, and integrative analysis of metagenomics, metatranscriptomics, and metaproteomics data.

Materials and Methods. Several command-line bioinformatics tools meta-omics data analysis were developed. MOSCA a fully automated framework was built with a Python-based workflow manager that specifies different rules for running distinct tasks of a workflow. These rules follow a standardized declaration format, selecting inputs, outputs, commands (calls to command-line tools and Python or R scripts). The input consists of DNA sequencing data (metagenomics, metatranscriptomics) and mass spectra (metaproteomics). More than 30 tools are included in MOSCA performing all steps of analysis (preprocessing, assembly, annotation, differential expression, mapping in metabolic pathways). UPIMAPI, reCOGnizer, and KEGGCHARTER are integrated in MOSCA. Another pipeline was developed in Python to infer the functional potential of microorganisms based on taxonomic data, including statistical analysis using the MicrobiomeAnalyst. The input consists of taxonomic profiles of the microbiota and a list of enzymes associated with selected metabolic pathways.

Results and Discussion. The integration of the MOSCA Pipeline enabled comprehensive analysis of metagenomics, metatranscriptomics, and metaproteomics datasets, providing high-resolution annotation, functional characterization, and graphical visualization of metabolic pathways, including protein expression levels. By leveraging taxonomic data, another pipeline extracts functional information from the most relevant microbial taxa, enabling the establishment of links between microbial identity and functional roles within the microbiome.

Conclusion. Together, these tools supported the interpretation of complex microbial functions and helped reveal coordinated responses at the gene and protein levels, advancing our understanding of microbiome-driven metabolic interactions.

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Funding. This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of UIDB/04469 unit and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems, LA/P/0029/2020.

Comparing Bioinformatics Pipelines for Analyzing Short-Read 16S rRNA Amplicon Data

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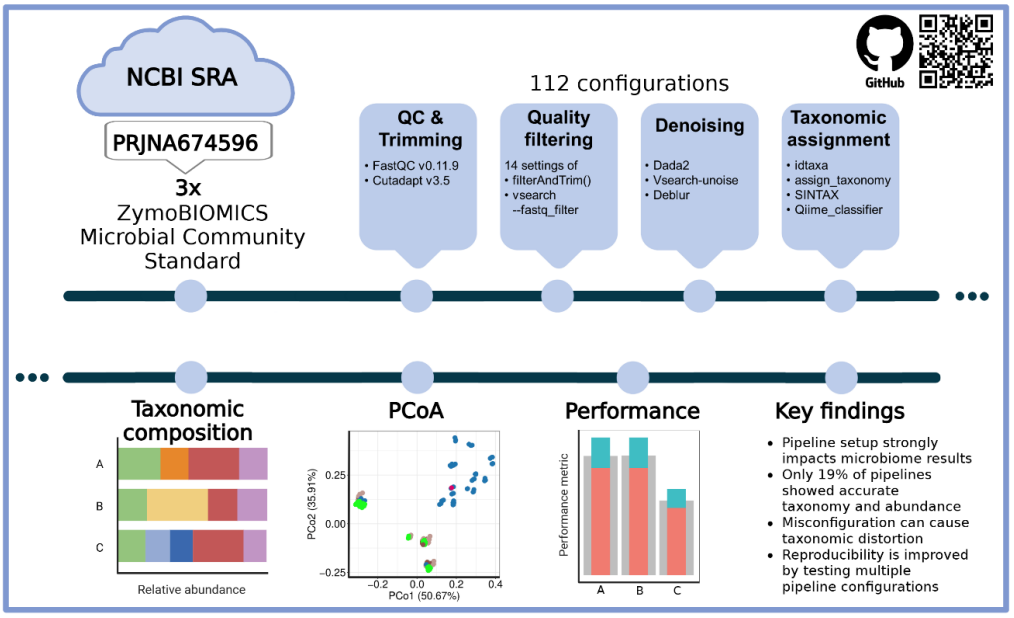

Abstract. Microbiome research has expanded significantly in recent years, driven by advances in next-generation sequencing. However, despite the increasing availability of sequencing data, there is still no consensus on standardized bioinformatics workflows, leading to variability in analytical outcomes. In this study, we evaluated 112 different bioinformatics pipeline configurations for processing 16S rRNA (V3-V4) amplicon sequencing data. Using the publicly available data of ZymoBIOMICS Microbial Community DNA as a reference standard, we assessed the accuracy and consistency of taxonomic profiles generated by each pipeline. Our results highlight the substantial variability introduced by different analysis strategies and emphasize the importance of developing validated and reproducible pipelines for microbiome data processing.
Funding. MH CR – conceptual development of research organization ("Institute for Clinical and Experimental medicine - IKEM, IN 00023001"), COST Action CA23110 "International networking on *in vitro* colon models simulating gut microbiota mediated interactions (INFOGUT).

The mutual interaction of ferulic acid and the gut microbiota

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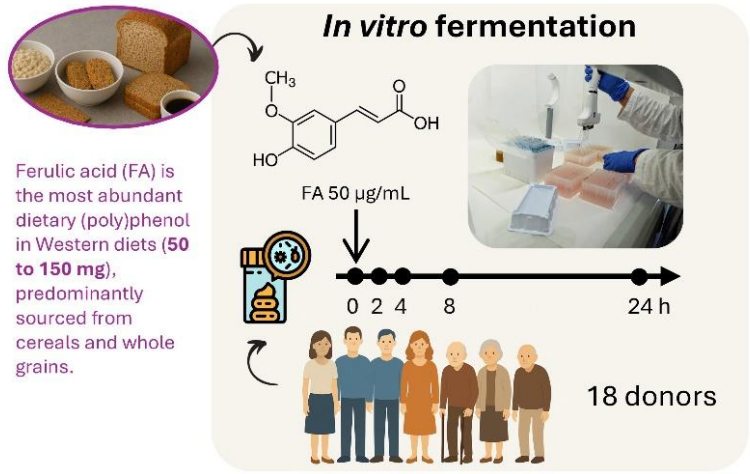

Abstract. Interindividual variability in the metabolism of dietary polyphenols, such as ferulic acid (FA), may influence health outcomes. This study aimed to identify distinct FA metabotypes using *in vitro* fecal fermentation of FA with samples from 18 healthy donors, followed by ^{1}H NMR metabolite profiling and 16S rDNA microbiome analysis. Data from both platforms were processed independently and integrated using data fusion techniques. Donors were classified based on the timing and extent of FA degradation and production of microbial catabolites. Substantial variability was observed, with distinct patterns in the formation of key metabolites, including 3-(3-hydroxyphenyl)propionic acid and dihydroferulic acid. Metabotypes differed in fecal metabolite profiles and microbial activity. For example, early FA degraders showed higher valerate and lower amino acid levels, along with a reduced abundance of *Turicibacter sanguinis*. These findings support the existence of reproducible FA metabotypes and suggest that gut microbiota composition plays a key role in shaping individual metabolic responses.
Funding. This work was supported by the Grant Agency of the Czech Republic (GACR 23–04655S), METROFOOD-CZ research infrastructure project (MEYS Grants LM2018100 and "COST Action CA23110 “International networking on *in vitro* colon models simulating gut microbiota mediated interactions (*INFOGUT*).

Network science methodologies to characterize gut microbial ecosystems in human health

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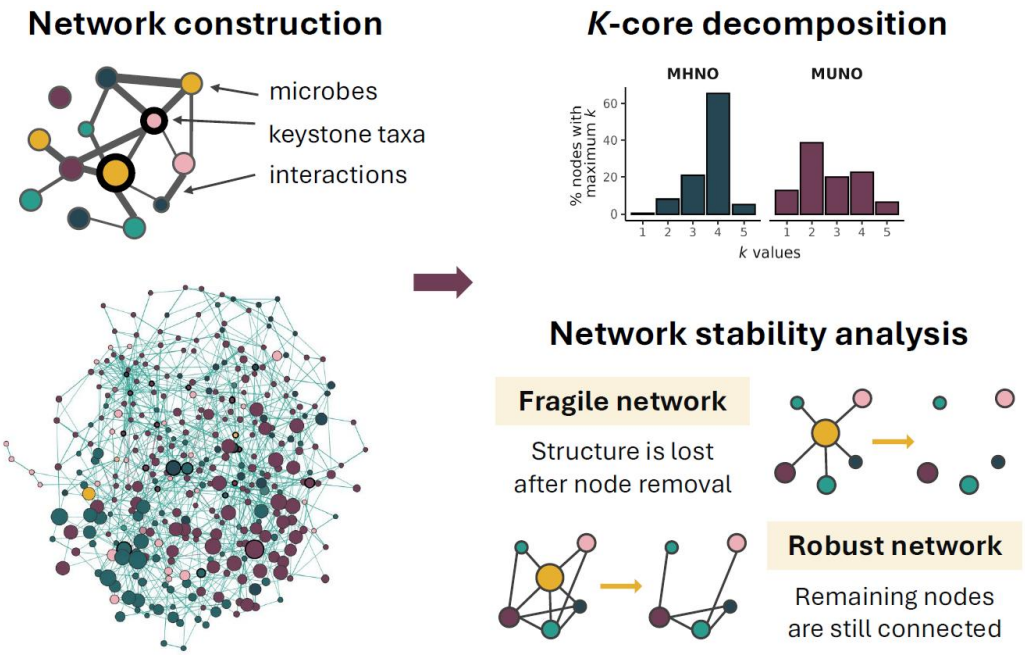

Abstract. Network science can be a useful tool to examine the ecological behaviors of microbial communities, providing results that are useful not only for biomarker identification but also from a community perspective. Computationally inferred interactions have been experimentally validated in several settings, from the lung and skin myco- and microbiomes to a variety of environmental microbiomes, demonstrating that co-occurrence networks can be used to obtain biologically relevant information. In the human microbiome field, co-occurrence networks are being increasingly used to look for patterns in a variety of settings, describing microbial community features that may be associated with host health or disease states. These studies show the promise that network-based methods hold for the study of human microbial communities.

We used co-occurrence networks to study how obesity and metabolic disorders shape the human gut microbiome. We built co-occurrence networks from metabolically healthy non-obese (MHNO), metabolically healthy obese (MHO), metabolically unhealthy non-obese (MUNO) and metabolically unhealthy obese (MUO) populations.

Network topology revealed that microbial communities in MHNO and MHO individuals remained connected, whereas those in MUNO and MUO showed isolated microbes and lower edge densities. Key network parameters, including degree, shortest path length, and betweenness, highlighted disrupted connectivity and altered ecological roles in metabolically unhealthy states. Furthermore, k-core decomposition and stability analyses showed that networks from unhealthy phenotypes were more fragile, with faster disintegration upon node removal. These findings underscore the value of network science in capturing structural features beyond taxonomic abundance, offering insights into microbial community resilience and potential therapeutic targets.

Funding. Funded by Comunidad de Madrid, TEC-2024/BIO-167 -CD3DTech-CM- (ORDEN 5696/2024, B.O.C.M.Núm.307 26/12/2024). PID2023-150146OA-I00. FPU2022-04053. Cost Action CA23110 (INFOGUT), AI4FOOD-CM (Y2020/TCS-6654), COST Action CA18131, RED2022- 134934-T, IJC2019-042188-I and MSCA (101105645). CIVP21A7025 funded by Ramón Areces Foundation.

Link preprint. https://www.biorxiv.org/content/10.1101/2025.04.25.650405v1.full

WG4 Conclusion from the Meeting presentations

During the meeting, the progress of various WG4 activities was presented and discussed. Notably, the ongoing journal club focused on multi-omic integration methods in *in vitro* colon models was highlighted as a valuable platform for knowledge exchange and capacity building within the group.

The benchmarking task has been divided into two complementary efforts. The standardization subtask is currently conducting a systematic review to identify and prioritize publicly available datasets suitable for standardization. Meanwhile, the benchmarking subtask is collecting and comparing the analytical pipelines used by WG members, covering steps from raw data processing to taxonomic assignment, with the aim of identifying best practices and potential harmonization strategies.

Throughout the discussions, the value of expanding the group to include more bioinformaticians was emphasized. Greater bioinformatics involvement would enrich the group's expertise and enhance the quality and impact of the ongoing activities

WG5 Regulatory, education, technology transfer, trainings and dissemination

WG5 Outline and introduction

As INFOGUT aims to become a business incubator for technology related to reference *in vitro* gut models and related protocols, this requires an efficient and permanent information flow throughout the scientific community and between academia and industries, also taking into consideration the guidance of regulatory bodies involved. Moreover, the development capacity of INFOGUT lies in the opportunity for academic laboratories and industrial partners to develop an educational program. In line, the WG activities are organized around these three main tasks: 5.1. Translational studies of food/feed, ingredients, supplements, drug therapies; 5.2. Educational tools directed towards young researchers but also for consumers; 5.3. Transfer to industries and training to professionals.

WG5 Leaders and Team

WG Leaders: Pilar Acedo (UCL, UK) and Laure-Alix Clerbaux (UCLouvain, Belgium). 5.1. Task leaders: Georgiana Gilca from University of Medicine and Pharmacy of Iasi, Romania and Samira Prado from Orebro University, Sweden). 5.2. Task leaders: Eliana Ibrahimi from University of Tirana, Albany. 5.3. Task leaders: Mathilde Guerville, Lactalis and Ivana Gondolfi, Parmalat).

WG5 Meeting Introduction and speakers

Konstantinos Paraskevopoulos (European Food Safety Agency, EFSA) presented on integrating microbiome data for food and feed risk analysis. Celine Druart (Pharmabiotic Research Institute, PRI) addressed bridging science and regulation through preclinical models essential for microbiome-derived products evaluation. Georgiana Gilca (University of Medicine and Pharmacy of Iasi, Romania) focused on implementing translational research in fecal microbiota transplantation through building translational teams. Dr Susanne Bremer-Hoffmann and Dr Alessia Bogni from the Joint Research Centre (JRC) presented their work on

integrating New Approach Methodologies (NAMs) into nanomaterial risk assessment through a proposed qualification framework.

WG5 ORAL PRESENTATIONS

Bridging Science and Regulation: Why Preclinical Models Are Essential for Microbiome-Derived Product Evaluation

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Abstract. As microbiome-derived or microbiome-targeting innovations advance toward regulatory assessment, the need for robust, relevant preclinical models is becoming increasingly critical. This presentation addressed the scientific and regulatory rationale for developing New Approach Methodologies (NAMs), particularly *in vitro* and other non-animal systems, to support the regulatory evaluation of microbiome-based products. In the context of microbiome innovation, *in vitro* colon models simulating gut microbiota mediated interactions is of course a NAMs of high relevance and working towards their regulatory acceptance, in addition to their scientific validation, is needed from the earliest stages of model development.
Regulatory Landscape and Challenges. Microbiome-based innovations may fall under various regulatory classifications, such as medicinal products, food supplements, foods for special medical purposes (FSMPs), or cosmetics. These classifications are governed by distinct EU legislative texts, each imposing specific requirements, standards, and constraints for market access. While demonstrating product quality and safety is a fundamental prerequisite for all regulatory pathways, doing so in the context of microbiome interventions remains particularly challenging. This is largely due to the inherent complexity of host–microbiome interactions and the current lack of standardized evaluation methodologies.
Key Arguments Supporting the development, validation and use of NAMs:

1. Ethical and Legal Imperatives – The 3Rs Principle:
   In Europe, the directive 2010/63/EU mandates the replacement, reduction, and refinement (3Rs) of animal used for scientific purposes. In the US, the Food and Drug Administration (FDA) has recently announced a plan to phase out animal testing requirement in drug development, such as monoclinal antibody. That's clearly a signal from the regulatory agencies to the willingness to favour the use of NAMs in product evaluation when it is scientifically relevant.
2. Scientific Limitations of Animal Models:
   Animal models are not always a good predictor of the human situation. The EURL ECVAM survey report (JRC) on Establishing the scientific validity of complex *in vitro* models mentions « *Animal studies have been the dominant means to increase scientific knowledge on human biology and diseases. However, in recent years, evidence has emerged that animal models may be a poor predictor of the human situation, particularly in the areas of safety pharmacology and toxicology* » [1]. This is especially true in the

context of microbiome research and microbiome innovation development due to the « holobiont concept ». Indeed, microbiome-host interactions are specific, due to interspecies differences in gut anatomy, diet, immune responses, and microbiota composition [2].

Several EMA and ICH guidelines now explicitly acknowledge that traditional toxicological testing may be inappropriate for biotechnology-derived products. Regulators emphasize the use of relevant models and caution against studies in non-relevant species, which may yield misleading results.

3. Regulatory agencies started to cite *in vitro* GI models in their guidelines:

   A very concrete example is the mention by EFSA in the latest version of the "*Guidance on the scientific requirements for an application for authorization of a novel food*" of models such as M-ARCOL and SHIME as *in vitro* models mimicking the human gut and its microbiota dynamics to identify relevant novel food-derived metabolites which could be of safety concern [3].

   Criteria for Regulatory Acceptance of NAMs: A key concept for the regulatory acceptance of NAMs is the "fit-for-purpose". Indeed, for regulatory acceptance, a NAM must be validated for its intended purpose, not broadly.

To be considered acceptable by regulatory agencies, NAMs must fulfil the following conditions [4]: i) Availability of defined test methodology, including standard protocols with clearly defined /scientifically sound endpoints; ii) Context of Use, includes a description of the circumstances under which the testing approach is applicable; iii) Relevance, where relevance describes the relationship of the test method to the effect of interest and whether it is meaningful and useful for a particular purpose (context of use). It is the extent to which the test correctly measures or predicts the biological effect of interest; iv) Reliability and Robustness, a measure of the extent that a test method can be performed reproducibly over time when using the same protocol.

Conclusion. Advancing microbiome-based innovations requires a shift toward fit-for-purpose preclinical strategies. NAMs offer an opportunity to improve translational relevance while aligning with ethical standards and evolving regulatory expectations. Regulatory acceptance is contingent on scientific validity, appropriate context of use, and transparent documentation, elements that must be integrated from the earliest stages of model development.

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Fostering Implementation of Translational Research in Fecal Microbiota Transplantation- A Focus on Building Translational Teams

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Introduction. There is currently a gap between murine models of gut microbiota and clinical applications, due to several physiological and environmental differences, but also due to suboptimal collaboration between basic scientists and clinicians focusing on this research area, especially in Eastern European countries. Our aim is to create the frame to building bridges between the expertise of basic scientists and clinicians involved in gut microbiota research, and particularly in fecal microbiota transplantation.

Methods. The steps for achieving the translational research teams with members from various institutions include the creation of a gut microbiota research platform via interinstitutional support. One step for implementation is organizing an onsite/online event for meet and greet of dedicated researchers from both preclinical and clinical sides, involving: gastroenterologists, (hemato)oncologists, psychiatrists, bioinformaticians, molecular biologists, psychologists, dietitians. The initial meeting should include 5 minutes presentation of the scientific expertise from each involved researcher, followed by translational team assembly under the guidance of a senior supervisor from the involved institutions. This is followed by a training session of each team with the support of senior researchers with regard to: methodology, research ethics, application for funding. One senior mentor for each team is to be assigned to coordinate the research project.

Outcome measurements. Creating at least 3 translational research teams, based on complementary research expertise and focusing on different areas of FMT applications; delivering at least one feasible research proposal per team; involvement of at least 2 industry partners willing to offer initial financial support for one research proposal; organize at least one event including researchers and industry partners for presentation of the proposed research projects and establish the most feasible research project to begin implementation.

Background. The understanding of the role of the human gut microbiota in health and disease has significantly evolved in the past decade [1]. However, the intricate mechanisms maintaining the balance of the complex ecosystem of microorganisms are still sub-optimally understood, therefore limiting the therapeutic applications in the current daily clinical practice [2]. Fecal microbiota transplantation (FMT) represents an important therapeutic option with confirmed results in restoring the microbial ecosystem in *Clostridioides difficile* infection and further non-infectious indications have shown promising results [3,4,5]. There is currently a gap between murine models of gut microbiota and clinical applications, due to several physiological and environmental differences [6]. In the pursuit to improve the current understanding of the underlying mechanisms and optimize the current applications of FMT, our goal is to improve collaboration between basic scientists and clinicians focusing on this research area. Furthermore, there is a need to better understand regional particular patterns of microbiota dynamics in both health and disease [7]. The suboptimal framework of collaboration between preclinical and clinical research and lack of coagulated research teams, especially in Eastern European countries, is a drawback in the development of microbiota research and expanding FMT applications. Our aim is to create the frame to building bridges between the expertise of basic scientists and clinicians involved in gut microbiota research, and particularly in FMT.

Methods. The steps for achieving the establishment of translational research teams with members from various institutions include inclusion of a gut microbiota research platform via interinstitutional support. One step for implementation is organizing an onsite/online event (onsite if only local and no travel funds needed, or online if international) for meet and greet of

dedicated researchers from both preclinical and clinical sides, involving: gastroenterologists, (hemato)oncologists, psychiatrists, bioinformaticians, molecular biologists, psychologists, dietitians. The initial onsite/online meeting should include 5 minutes presentation of the scientific expertise from each involved researcher, followed by translational team assembly under the guidance of a senior supervisor from the involved institutions. Each team will include at least 2 researchers with preclinical background and at least 2 clinicians. The participation of early career specialists will be encouraged. This step of team assembly is followed by a training session of each team with the support of senior researchers with regard to: methodology, research ethics, application for funding. One senior mentor for each team is to be assigned to coordinate the research project. The senior mentors will be assigned on a voluntary basis, taking into consideration prior expertise in coordinating research projects in similar research area. All teams will then work on a self-established timetable to shape their research projects proposals, within a preestablished time frame.

A second onsite/online meeting will be scheduled, aiming to present the proposed projects. A jury including industry partners and invited senior researchers outside the project mentors will evaluate the quality of the proposed research projects by each team. The objective evaluation will include aspects regarding originality of the proposed research, the feasibility of the proposals, the length and rhythm of the timetable of the activities within the project, the level and the optimization of budget use, the extent of using the team's expertise, final practical utility of the estimated results, both scientifically and clinically.

Outcome measurement. The proposed framework will measure the following outcomes: creating at least 3 translational research teams, based on complementary research expertise and focusing on different areas of FMT applications; delivering at least one feasible research proposal per team; involve at least 2 universities and/or associated research entities; involve at least 50% young researchers/clinicians (≤35 years old); involvement of at least 2 industry partners willing to offer initial financial support for at least one research proposal ; organize at least one event including researchers and industry partners for presentation of the proposed research projects and establish the most feasible research project to begin implementation.

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Integrating New Approach Methodologies (NAMs) into Nanomaterial Risk Assessment: A Proposed Qualification Framework for NAMs

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Introduction. The European Union is dedicated to promoting New Approach Methodologies (NAMs), including for chemical risk assessment (RA) frameworks [1]. These innovative, non-animal testing methods aim to improve the hazard identification and characterisation of chemicals, including nanomaterials, by integrating advancements in toxicology with regulatory standards. The European Food Safety Authority (EFSA) is also working to integrate New Approach Methodologies (NAMs) into its operations [2]. EFSAs guidance documents for assessing the risk of nanomaterials and other small particles in the food chain feature NAM-based methods in the first tiers of the RA, offering options for possibly avoiding nano-specific *in vivo* testing [3,4]. They focus on initial key effect critical health endpoints like genotoxicity, inflammation, and oxidative stress, and for analyzing if and how these particles are taken up and move through the intestinal barrier.

However, the availability of validated and regulatory-accepted NAMs for nanomaterials remains limited. Nevertheless, a significant number of *in vitro*, *in silico*, and in chemico methods have been developed in recent years, often supported by public funding [5]. The evaluation of these NAMs in pertinent dossiers is complex and time-consuming, frequently lacking adequate documentation. Additionally, the absence of harmonization in the evaluation process leads to a lack of transparency. To address this, EFSA is planning the implementation of a qualification system that would support the use of non-guideline NAMs.

NAMs4NANO project. The development of a proposal for such a qualification system is a key objective of the EFSA-funded NAMS4NANO project (2023-2027). Coordinated by the German Federal Institute for Risk Assessment (BfR) and the Italian National Institute of Health (ISS), the project brings together a consortium of 10 partners with the goal of advancing the understanding and application of NAMs for the risk assessment of nanomaterials in the food and feed chain. The project is addressing open questions in risk assessment of real-world materials arising from the evaluation of food additives, nutrient sources, novel foods, food contact materials, feed additives and pesticides. The European Commission's Joint Research Centre is supporting the consortium in the effort of developing the qualification system and contributes to several case studies investigating the performance of NAMs in testing strategies.

A qualification system for NAMs used in the food and feed sector. The first proposal for a systematic approach that can facilitate the integration of non-guideline methods in the food and feed sector was published by EFSA in 2024 [6]. The qualification system proposes a process that is designed to ensure that NAMs are relevant, reliable and fit for use in a specifically defined context-of-use. The system is structured around a stepwise process involving multiple stages, including the submission of a letter of intent, development of a

qualification plan, preliminary qualification advice, and a formal qualification opinion. These stages incorporate consultation, review against established criteria, and public engagement to ensure transparency and robustness.The proposal also outlines a number of potential criteria to be fulfilled which are aligned for instance with the OECD GD211 [7]. Key components of the qualification criteria include categories such as general information (developer, version updates), method description (biological model, assay details, controls), relevance (context of use, biological relevance), and reliability (variability, transferability).

Application of the qualification criteria to the Triculture model. The EFSA-funded NAMs4NANO project aims to assess promising methods by analyzing them against established criteria for relevance and reliability. One such promising *in vitro* method for assessing nanomaterials is based on a triculture of enterocytes, goblet cells, and M cells [8]. This method is currently being proposed for assessing the uptake and translocation of nanomaterials across the small intestinal barrier and identifying potential barrier impairment [6, 8-9]. Since the method has been optimized and utilized in several EU projects, including the EFSA-funded NANOCELLUP project [9], it is well-documented. Standard operating procedures, along with information related to the cell source, culture conditions, and specific readouts for assessing toxicological endpoints, are available. The biological relevance of the triculture method is demonstrated by its ability to mimic physiological barriers. The assessment of transepithelial resistance, along with imaging of components of the intestinal epithelium such as the mucus layer and tight junctions, as well as the ability to internalize and translocate nanomaterials, supports the method's biological relevance for nanomaterial hazard identification. The model's performance in terms of variability within a laboratory as well as its transferability to an independent laboratory, indicates the consistency and robustness of the method. Nevertheless, knowledge gaps remain, including data interpretation procedure, the definition of acceptance criteria as well as the definition of appropriate controls, and will be addressed in the course of NAMS4NANO.

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WG5 Conclusion from the Meeting presentations

Regulatory uptake, education, and technology transfer are critical to the successful implementation of *in vitro* colon models in Europe. Engagement with relevant stakeholders has already begun and will remain essential throughout the course of the project.

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