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DRUG AND PROBIOTICS-BASED INTERVENTION STRATEGIES FOR THE RESTORATION OF GUT MICROBIOME DYSBIOSIS

Presentata da: Mariachiara Mengoli

Coordinatore Dottorato

Susi Pelotti

Supervisore

Patrizia Brigidi

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ABSTRACT

The gut microbiota is a highly complex and intrinsically dynamic ecosystem that undergoes significant changes throughout an individual's lifetime, influenced by various factors such as diet, lifestyle, socioeconomic status, antibiotic use and geographic location. Despite its adaptability, the microbiota's balance can be disrupted, leading to a dysbiotic state frequently associated with chronic non-communicable diseases (NCDs) such as obesity, diabetes, cardiovascular disease and cancer. Addressing this imbalance is critical, and interventions such as novel bioactive compounds and next-generation probiotics offer promising avenues for restoring microbial homeostasis.

This thesis presents two studies focusing on drug- and probiotics-based strategies aimed at re-establishing eubiosis within the gut microbiota. The first study evaluated the efficacy of four novel compounds *in vitro* using the Batch Gut Model Cultures assay, specifically targeting antibiotic-resistant *Clostridioides difficile*. Results demonstrated that certain compounds effectively reduced the target pathogen while enhancing gut microbial diversity. Moreover, the Batch Gut Model Cultures assay proved to be both time- and resource-efficient, retaining robust methodological effectiveness.

The second study investigated the impact of an *ad hoc* microbial consortium as next-generation probiotics on mitigating early weaning effects using an *in vivo* murine model. Early weaning, increasingly common in Western countries, is recognized as a contributing factor to the onset of numerous NCDs. Findings revealed that this microbial intervention reduced local and systemic inflammation while promoting beneficial bacterial taxa (e.g., *Lactobacillus*) in the murine gut. Furthermore, the study underscores the utility of this

approach as a mouse model for early weaning, addressing the current lack of research linking gut microbiota dynamics to early weaning outcomes.

In conclusion, these studies provided foundational insights into the potential of *in vitro* and *in vivo* approaches to assess the restoration of gut eubiosis. They could pave the way for future research to further explore innovative strategies to address the dysbiotic layout and its associated health challenges.

Chapter 1: INTRODUCTION

Every single living being, from cnidarians to humans, is colonized with microorganisms, with the gut housing the largest variety and quantity of these host-associated bacteria[1]. From their first discovery, in the far 1677 by Antonie van Leeuwenhoek in his famous paper "letter on the protozoa"[2], passing through the discoveries of Teodor Escherich in 1885[3], these micro-inhabitants of the gut have gained more and more interest from all the scientific community[4]. The term human microbiome refers to the collective genetic information, residing in and on our body[5] contained within the trillion-member community of the microbiota, including bacteria, viruses, and fungi[6]. In particular, bacteria are found in different niches of the human body, such as oral and skin environment, and the gastrointestinal tract. The number of microbial cells can overcome the number of human body cells: it is estimated that a human body can carry 3.9×10^{13} bacterial cells, with the large intestine hosting 10^{11} bacterial cells g^{-1} of wet stool[7], whereas the number of human cells is about $30 \pm 0.5 \times 10^{12}$

[1] Kuziel, G.A. & S. Rakoff-Nahoum; "The Gut Microbiome" in *Current Biology* 32, no. 6 (**2022**): R257–R264.

[2] Lane, N.; "The Unseen World: Reflections on Leeuwenhoek (1677) 'Concerning Little Animals'" in *Philosophical Transactions of the Royal Society B: Biological Sciences* 370, no. 1666 (**2015**): 20140344.

[3] Shulman, S.T., H.C. Friedmann, & R.H. Sims; "Theodor Escherich: The First Pediatric Infectious Diseases Physician?" in *Clinical Infectious Diseases* 45, no. 8 (**2007**): 1025–1029.

[4] Rajilić-Stojanović, M. & W.M. de Vos; "The First 1000 Cultured Species of the Human Gastrointestinal Microbiota" in *FEMS microbiology reviews* 38, no. 5 (**2014**): 996–1047.

[5] Barone, M. et al.; "Over-Feeding the Gut Microbiome: A Scoping Review on Health Implications and Therapeutic Perspectives" in *World Journal of Gastroenterology* 27, no. 41 (**2021**): 7041–7064.

[6] Shanahan, F., T.S. Ghosh, & P.W. O'Toole; "The Healthy Microbiome—What Is the Definition of a Healthy Gut Microbiome?" in *Gastroenterology* 160, no. 2, The Gut microbiome: Reaching the Promise through Discovery (**2021**): 483–494.

[7] Sender, R., S. Fuchs, & R. Milo; "Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans" in *Cell* 164, no. 3 (**2016**): 337–340.

[8]. To explore its composition in terms of taxa, it is necessary to underline that the gut microbiota (GM) is a complex intrinsically dynamic entity, which is able to change during lifetime, shaped by different factors, such as diet, lifestyle, socioeconomic status, antibiotics and geography[9, 10, 11], just to name a few.

1.1 Plasticity of human gut microbiome

1.1.1 Lifespan

The delivery is one of the main factors that can shape the microbiota. Generally speaking, babies delivered via vaginal childbirth tend to have a gut microbial ecosystem more similar to their mother's vaginal microbiota, whereas those born via C-section may have a gut microbiome more closely resembling their mother's skin microbiota[12]. At the moment of the birth, immediately after delivery, a unique microbial arrangement starts to take shape[13]. The very first colonization of the baby's gastrointestinal tract occurs by some facultative anaerobes, *i.e.* streptococci, enterococci and staphylococci. Breastfeeding seems to be crucial to the development of the early-life GM, with a dominance of *Bacteroides* and

[8] Brown, C.T. et al.; "Measurement of Bacterial Replication Rates in Microbial Communities" in *Nature Biotechnology* 34, no. 12 (**2016**): 1256–1263.

[9] Dethlefsen, L. et al.; "The Pervasive Effects of an Antibiotic on the Human Gut Microbiota, as Revealed by Deep 16S rRNA Sequencing" in *PLoS biology* 6, no. 11 (**2008**): e280.

[10] Fragiadakis, G.K. et al.; "Links between Environment, Diet, and the Hunter-Gatherer Microbiome" in *Gut Microbes* 10, no. 2 (**2019**): 216–227.

[11] Gilbert, J.A. et al.; "Current Understanding of the Human Microbiome" in *Nature Medicine* 24, no. 4 (**2018**): 392–400.

[12] Quercia, S. et al.; "From Lifetime to Evolution: Timescales of Human Gut Microbiota Adaptation" in *Frontiers in Microbiology* 5 (**2014**).

[13] Jost, T. et al.; "New Insights in Gut Microbiota Establishment in Healthy Breast Fed Neonates" in *PLOS ONE* 7, no. 8 (**2012**): e44595.

Bifidobacterium species in breastfed newborns[14, 15]. The infant GM is subjected to profound fluctuations, due to the poor microbial biodiversity, until it reaches its turning point, when the weaning begins. As solid food is introduced, the GM gradually changes, moving from a community dominated by *Bacteroides* and bifidobacteria to one dominated by Firmicutes and Bacteroidota. This shift results in a GM that resembles the adult one and is capable of metabolizing the entire complexity of the plant polysaccharides found in an adult diet. In fact, the genera *Bacteroides*, *Eubacterium*, *Ruminococcus*, *Clostridium*, *Alistipes*, *Roseburia*, and *Blautia* represent an important proportion of the entire GM, and these taxa indeed are capable of degrading fibers[16].

The composition of a "healthy microbiome" in adults could be hard to define, but it is possible to describe a stable microbial core, thanks to several cross-sectional and longitudinal studies based on 16S rRNA gene analysis, a technique widely used to explore the GM, that will be discussed deeply further. Generally speaking, the predominant bacterial phyla in the healthy adult human gut are Bacteroidota and Firmicutes, along with Verrucomicrobia, Proteobacteria and Actinobacteria[17], with *Ruminococcaceae*, *Lachnospiraceae* and *Bacteroidaceae* being the dominant families[18]. As highlighted in the work of Ruan et al. (2020), the main differences between the small and large intestine is the mucus layer, more defined in the large rather than in the small intestine, which also provides a structural

[14] Marcobal, A. & J.L. Sonnenburg; "Human Milk Oligosaccharide Consumption by Intestinal Microbiota" in *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 18 Suppl 4, no. 0 4 (**2012**): 12–15.

[15] Matamoros, S. et al.; "Development of Intestinal Microbiota in Infants and Its Impact on Health" in *Trends in Microbiology* 21, no. 4 (**2013**): 167–173.

[16] Quercia et al., "From Lifetime to Evolution."

[17] Hillman, E.T. et al.; "Microbial Ecology along the Gastrointestinal Tract" in *Microbes and Environments* 32, no. 4 (**2017**): 300–313.

[18] Vasapolli, R. et al.; "Analysis of Transcriptionally Active Bacteria Throughout the Gastrointestinal Tract of Healthy Individuals" in *Gastroenterology* 157, no. 4 (**2019**): 1081-1092.e3.

difference in the ecosystem composition[19]. This mucus layer can be divided into the inner mucus layer, which has immune effectors targeting the microbiota and physically excludes bacteria[20], and the outer mucus layer, looser and useful as a colonization site. From the lumen to the mucosa, along the colon's transverse axis, colonization takes place in relation to the mucus density and the oxygen gradient. So, the colonic lumen is mainly characterized by microorganisms that prioritize dietary starches and nutrients, while the inner mucus layer is populated by mucin-user bacteria, such as *Akkermania*, *Ruminococcus* and some *Bacteroides* species[21, 22, 23, 24]. Moreover, Shetty et al. defined *Faecalibacterium prausnitzii*, *Oscillospira guillermondii* and *Ruminococcus obeum* as the top three taxa shared by all adults[25].

With aging, the relationship between GM and host progressively becomes compromised. Immunosenescence, age-related modifications such as the alteration of taste and smell, of mastication and gastrointestinal motility and more in general an alteration of lifestyle and diet result in a condition called “inflammaging”[26]. Inflammaging is a self-sustained pro-

[19] Ruan, W. et al.; “Healthy Human Gastrointestinal Microbiome: Composition and Function After a Decade of Exploration” in *Digestive Diseases and Sciences* 65, no. 3 (**2020**): 695–705.

[20] Johansson, M.E.V., J.M.H. Larsson, & G.C. Hansson; “The Two Mucus Layers of Colon Are Organized by the MUC2 Mucin, Whereas the Outer Layer Is a Legislator of Host-Microbial Interactions” in *Proceedings of the National Academy of Sciences of the United States of America* 108 Suppl 1, no. Suppl 1 (**2011**): 4659–4665.

[21] Berry, D. et al.; “Host-Compound Foraging by Intestinal Microbiota Revealed by Single-Cell Stable Isotope Probing” in *Proceedings of the National Academy of Sciences* 110, no. 12 (**2013**): 4720–4725.

[22] Crost, E.H. et al.; “The Mucin-Degradation Strategy of Ruminococcus Gnavus: The Importance of Intramolecular Trans-Sialidases” in *Gut Microbes* 7, no. 4 (**2016**): 302–312.

[23] Tropini, C. et al.; “The Gut Microbiome: Connecting Spatial Organization to Function” in *Cell Host & Microbe* 21, no. 4 (**2017**): 433–442.

[24] Yasuda, K. et al.; “Biogeography of the Intestinal Mucosal and Lumenal Microbiome in the Rhesus Macaque” in *Cell Host & Microbe* 17, no. 3 (**2015**): 385–391.

[25] Shetty, S.A. et al.; “Intestinal Microbiome Landscaping: Insight in Community Assemblage and Implications for Microbial Modulation Strategies” in *FEMS microbiology reviews* 41, no. 2 (**2017**): 182–199.

[26] Biagi, E. et al.; “Ageing and Gut Microbes: Perspectives for Health Maintenance and Longevity” in *Pharmacological Research* 69, no. 1 (**2013**): 11–20.

inflammatory loop and can lead to dysbiosis in gut microbiota, with lower α-diversity compared to the adult one, pathogen overgrowth and the support of pathobionts' bloom (*Enterobacteriaceae*) to the detriment of immunomodulatory groups (*i.e. Clostridium* cluster IV and XIVa and *Bifidobacterium*)[27, 28, 29, 30]. This GM configuration, displaying restricted stability and extreme variability, is detrimental for host health and contributes to the onset and progression of different diseases.

1.1.2 Diet

The diet has a strong impact on GM. Different diets, and so different micro- and macro-nutrients, could interact with microorganism both directly and indirectly and be processed and modified by the GM in different ways.

The composition of the human GM is highly sensitive to fluctuations in dietary intake and the physiological condition of the digestive system[31, 32] with detectable shifts occurring within just 24 hours[33]. Remarkably, diets consisting in a low amount of carbohydrates, e.g. ketogenic diets, could have a strong impact on the GM and on the metabolites produced at

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[27] Biagi, E. et al.; "Through Ageing, and Beyond: Gut Microbiota and Inflammatory Status in Seniors and Centenarians" in *PLOS ONE* 5, no. 5 (**2010**): e10667.

[28] Franceschi, C. et al.; "Inflamm-Aging. An Evolutionary Perspective on Immunosenescence" in *Annals of the New York Academy of Sciences* 908 (**2000**): 244–254.

[29] Franceschi, C. et al.; "The Network and the Remodeling Theories of Aging: Historical Background and New Perspectives" in *Experimental Gerontology* 35, no. 6–7 (**2000**): 879–896.

[30] Grignolio, A. et al.; "Towards a Liquid Self: How Time, Geography, and Life Experiences Reshape the Biological Identity" in *Frontiers in Immunology* 5 (**2014**): 153.

[31] Qin, N. et al.; "Fish Oil Extracted from Coregonus Peled Improves Obese Phenotype and Changes Gut Microbiota in a High-Fat Diet-Induced Mouse Model of Recurrent Obesity" in *Food & Function* 11, no. 7 (**2020**): 6158–6169.

[32] Turnbaugh, P.J. et al.; "The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice" in *Science translational medicine* 1, no. 6 (**2009**): 6ra14.

[33] David, L.A. et al.; "Diet Rapidly and Reproducibly Alters the Human Gut Microbiome" in *Nature* 505, no. 7484 (**2014**): 559–563.

a GM level. Ketogenic diets are characterized by carbohydrates reduction (usually to less than 50 g/day) and a relative increase in the proportions of protein and fat[34]. This diet exploits the concept of ketosis, a condition in which the body is able to use fat and transform it into ketone bodies such as acetoacetate, β-hydroxybutyric acid and acetone through a process called ketogenesis. Actually, insulin activates key enzymes involved in energy storage pathways, primarily from carbohydrates. When carbohydrate intake is low or absent, reduced insulin levels result in decreased lipogenesis and fat storage. After a few days of fasting or a drastic reduction in carbohydrate consumption, glucose reserves are insufficient to sustain normal fat oxidation via the Krebs cycle or to provide glucose to the central nervous system (CNS), which relies on glucose for energy[35]. For that, the CNS must use alternative energy sources such as the ketone bodies. In fact, low-carbohydrate diets lead to an overproduction of acetyl-CoA, which triggers the synthesis of ketone bodies in the liver's mitochondrial matrix[36]. There is strong evidence on the efficacy of these diets for the

[34] Veech, R.L.; "The Therapeutic Implications of Ketone Bodies: The Effects of Ketone Bodies in Pathological Conditions: Ketosis, Ketogenic Diet, Redox States, Insulin Resistance, and Mitochondrial Metabolism" in *Prostaglandins, Leukotrienes, and Essential Fatty Acids* 70, no. 3 (**2004**): 309–319.

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[36] Fukao, T., G.D. Lopaschuk, & G.A. Mitchell; "Pathways and Control of Ketone Body Metabolism: On the Fringe of Lipid Biochemistry" in *Prostaglandins, Leukotrienes, and Essential Fatty Acids* 70, no. 3 (**2004**): 243–251.

treatment of epilepsy[37, 38, 39], cardiovascular disease[40, 41, 42], and type 2 diabetes (T2D)[43, 44]. This kind of diets could have a strong impact not only on the GM *per se* but more specifically on the so-called microbiota-gut-brain axis (MGB axis). The MGB axis represents a bidirectional communication pathway between the CNS and the enteric nervous system, linking emotional and cognitive processes in the brain with peripheral gastrointestinal functions[45]. So, ketogenic diets may change the pool of metabolites and messengers produced by the GM. In general, ketogenic diets decreases the GM biodiversity, altering the proportion of specific microbial taxa (e.g., *Akkermansia* and *Parabacteroides*)[46, 47, 48] and affecting the levels of microbially derived metabolites such as short chain fatty acids

[37] Muthaffar, O.Y. et al.; "Short-Term Effectiveness and Side Effects of Ketogenic Diet for Drug-Resistant Epilepsy in Children with Genetic Epilepsy Syndromes" in *Frontiers in Neurology* 15 (**2024**).

[38] Poorshiri, B. et al.; "The Efficacy Comparison of Classic Ketogenic Diet and Modified Atkins Diet in Children with Refractory Epilepsy: A Clinical Trial" in *Acta Neurologica Belgica* 121, no. 2 (**2021**): 483–487.

[39] D'Andrea Meira, I. et al.; "Ketogenic Diet and Epilepsy: What We Know So Far" in *Frontiers in Neuroscience* 13 (**2019**).

[40] Volek, J.S. et al.; "Carbohydrate Restriction Has a More Favorable Impact on the Metabolic Syndrome than a Low Fat Diet" in *Lipids* 44, no. 4 (**2009**): 297–309.

[41] Volek, J.S., M.J. Sharman, & C.E. Forsythe; "Modification of Lipoproteins by Very Low-Carbohydrate Diets" in *The Journal of Nutrition* 135, no. 6 (**2005**): 1339–1342.

[42] Sharman, M.J. et al.; "A Ketogenic Diet Favorably Affects Serum Biomarkers for Cardiovascular Disease in Normal-Weight Men" in *The Journal of Nutrition* 132, no. 7 (**2002**): 1879–1885.

[43] Nielsen, J.V. & E.A. Joensson; "Low-Carbohydrate Diet in Type 2 Diabetes: Stable Improvement of Bodyweight and Glycemic Control during 44 Months Follow-Up" in *Nutrition & Metabolism* 5 (**2008**): 14.

[44] Yancy, W.S. et al.; "A Low-Carbohydrate, Ketogenic Diet to Treat Type 2 Diabetes" in *Nutrition & Metabolism* 2 (**2005**): 34.

[45] Carabotti, M. et al.; "The Gut-Brain Axis: Interactions between Enteric Microbiota, Central and Enteric Nervous Systems" in *Annals of Gastroenterology : Quarterly Publication of the Hellenic Society of Gastroenterology* 28, no. 2 (**2015**): 203.

[46] Gubert, C. et al.; "Exercise, Diet and Stress as Modulators of Gut Microbiota: Implications for Neurodegenerative Diseases" in *Neurobiology of Disease* 134 (**2020**): 104621.

[47] Paoli, A. et al.; "Ketogenic Diet and Microbiota: Friends or Enemies?" in *Genes* 10, no. 7 (**2019**): 534.

[48] Olson, C.A. et al.; "The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet" in *Cell* 174, no. 2 (**2018**): 497.

(SCFAs), lactate and H_2S[49]; the latter causes damage to the intestinal mucosal barrier that appeared impaired after these dietary treatment[50, 51]. Furthermore, these diets can increase the relative abundances of Bacteroidota and *Bacteroides*, while a decrease of Firmicutes, Actinobacteria and Proteobacteria has been specifically reported in children treated with ketogenic diets to ameliorates epilepsy symptoms[52, 53].

Similarly, diets high in carbohydrates, particularly ultra-processed sugars, and fats can induce alterations in the composition of the gut microbial ecosystem and also impact metabolic traits, including insulin resistance, dyslipidemia, and oxidative stress, leading to a plethora of Non-Communicable Diseases (NCDs) such as obesity and T2D. In particular, the high-fat diet (HFD) is examined below, with its gut-level metabolic effects and contribution to NCDs explained and illustrated in **Figure 1**.

[49] Paoli et al., "Ketogenic Diet and Microbiota."

[50] Gubert et al., "Exercise, Diet and Stress as Modulators of Gut Microbiota."

[51] Paoli et al., "Ketogenic Diet and Microbiota."

[52] Xie, G. et al.; "Ketogenic Diet Poses a Significant Effect on Imbalanced Gut Microbiota in Infants with Refractory Epilepsy" in *World Journal of Gastroenterology* 23, no. 33 (**2017**): 6164–6171.

[53] Zhang, Y. et al.; "Altered Gut Microbiome Composition in Children with Refractory Epilepsy after Ketogenic Diet" in *Epilepsy Research* 145 (**2018**): 163–168.

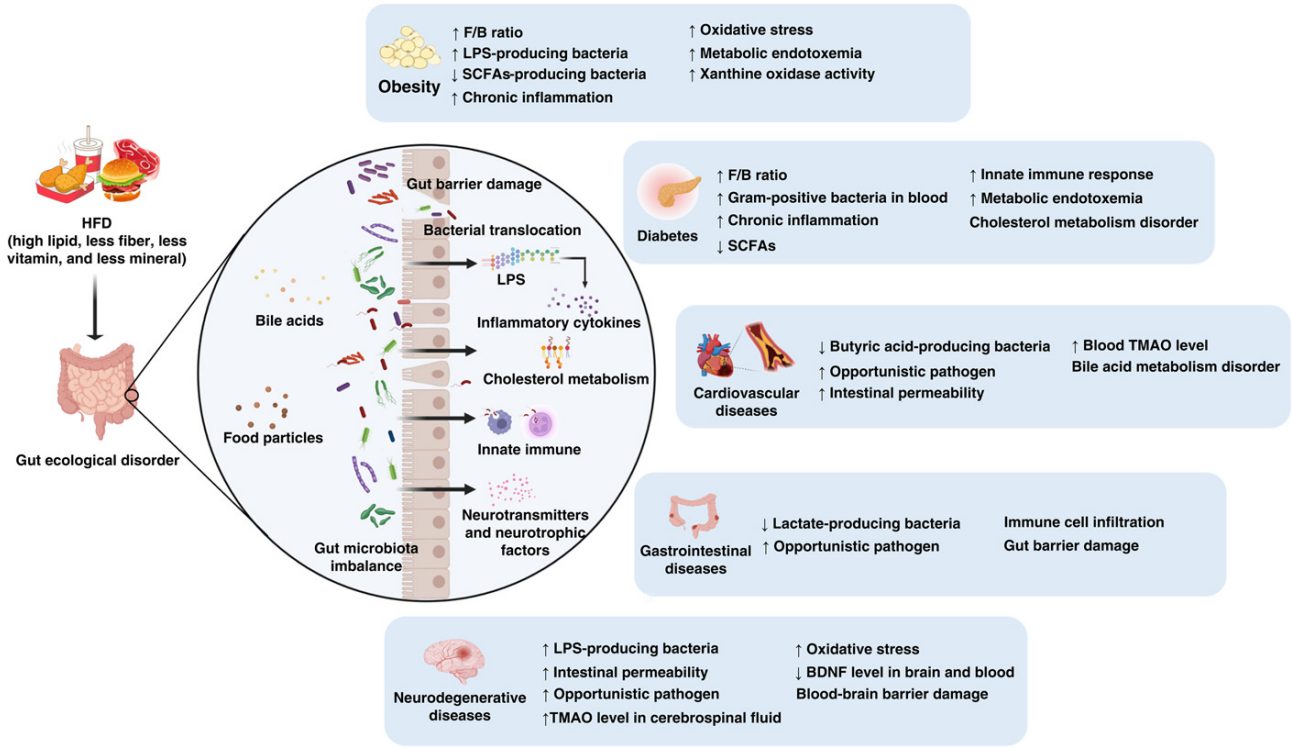

Figure 1. Roles of gut microbiota in chronic diseases induced by high-fat diet. HFD, high-fat diet; BDNF, brain-derived neurotrophic factor; F/B ratio, ratio of Firmicutes to Bacteroidota; HFD, high-fat diet; LPS, lipopolysaccharide; SCFAs, short-chain fatty acids; TMAO, trimethylamine N-oxide[54].

Generally, HFD has been shown to significantly decrease the diversity of the GM[55], leading to a reduction in the abundance of bacteria essential for preserving the gut mucosal barrier and an increase in bacteria that compromise its integrity[56]. This microbial shift is featured by a reduced relative abundance of Bacteroidota and an increased prevalence of Firmicutes[57].

[54] Chen, J. et al.; "New Insights into the Mechanisms of High-Fat Diet Mediated Gut Microbiota in Chronic Diseases" in *iMeta* 2, no. 1 (**2023**): e69.

[55] Wan, Y. et al.; "Effects of Dietary Fat on Gut Microbiota and Faecal Metabolites, and Their Relationship with Cardiometabolic Risk Factors: A 6-Month Randomised Controlled-Feeding Trial" in *Gut* 68, no. 8 (**2019**): 1417–1429.

[56] Monk, J.M. et al.; "Navy Bean Supplemented High-Fat Diet Improves Intestinal Health, Epithelial Barrier Integrity and Critical Aspects of the Obese Inflammatory Phenotype" in *The Journal of Nutritional Biochemistry* 70 (**2019**): 91–104.

[57] An, J. et al.; "The Source of the Fat Significantly Affects the Results of High-Fat Diet Intervention" in *Scientific Reports* 12, no. 1 (**2022**): 4315.

Furthermore, HFD has been linked to elevated levels of lipopolysaccharide correlating with higher Actinomycetes levels, accompanied by a decline in bifidobacteria. The conversion of excess sulfate to hydrogen sulfide further weakens the gut barrier and triggers inflammatory responses[58].

Another example that goes in the opposite direction for its positive health effects to HFD is the Mediterranean diet (MD), that also highlights the plasticity of the GM. Most research indicates that adherence to the MD is linked to enhanced GM biodiversity, reflected in a higher number of bacterial species, which is generally considered beneficial for health[59]. For instance, while the Western diets and HFDs, as previously seen, are typically associated with elevated levels of *Bacteroides*, the MD correlates with the presence of the genus *Prevotella* and increased levels of *F. prausnitzii*[60, 61]. In a study involving patients with metabolic syndrome, the MD was shown to reduce dysbiosis and increase populations of the *Bifidobacterium* genus[62, 63]. However, not all studies confirm the influence of the MD on GM. For example, one study reported no significant differences in GM composition after six months of either an MD or a Western-type diet[64]. This suggests that, at least over the short term, achieving substantial and lasting changes in GM through dietary interventions may be challenging[65].

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[58] Chen, S. et al.; "Decreased Expression of Cystathionine β-Synthase Exacerbates Intestinal Barrier Injury in Ulcerative Colitis" in *Journal of Crohn's and Colitis* 13, no. 8 (**2019**): 1067–1080.

[59] Merra, G. et al.; "Influence of Mediterranean Diet on Human Gut Microbiota" in *Nutrients* 13, no. 1 (**2021**): 7.

[60] Jin, Q. et al.; "Metabolomics and Microbiomes as Potential Tools to Evaluate the Effects of the Mediterranean Diet" in *Nutrients* 11, no. 1 (**2019**): 207.

[61] Merra et al., "Influence of Mediterranean Diet on Human Gut Microbiota."

[62] Haro, C. et al.; "The Gut Microbial Community in Metabolic Syndrome Patients Is Modified by Diet" in *The Journal of Nutritional Biochemistry* 27 (**2016**): 27–31.

[63] Merra et al., "Influence of Mediterranean Diet on Human Gut Microbiota."

[64] Djuric, Z. et al.; "Colonic Mucosal Bacteria Are Associated with Inter-Individual Variability in Serum Carotenoid Concentrations" in *Journal of the Academy of Nutrition and Dietetics* 118, no. 4 (**2018**): 606-616.e3.

[65] Merra et al., "Influence of Mediterranean Diet on Human Gut Microbiota."

1.1.2.1 GM metabolites

GM metabolites and GM-derived bioactive compounds serve as the link between the gut microorganisms and the overall health of the human holobiont. The GM could be considered as a biological reactor that produces a wide range of molecular substances through the anaerobic fermentation of dietary elements or the transformation of endogenous compounds produced by the host or other members of the community[66]. Although these signals are produced locally, they have the capability to impact organs outside the gut, establishing a system-level connection between the microbiota and the immune, endocrine, metabolic, and nervous systems. A wide spectrum of microbial molecules, generated within the colon, are absorbed by colonocytes and released into the bloodstream, eventually making their way to the liver through the portal vein[67]. Once in the liver, these molecules are then released systematically via the peripheral venous system, sometimes undergoing further modification by the host[68].

Carbohydrates constitute among the host's most significant energy sources in adulthood. However, the majority of plant polysaccharides and complex carbohydrates are indigestible by human enzymes. Instead, a number of microbes that live in the gut niche can break down carbohydrates such cellulose, xylans, resistant starch, and inulin to produce energy for the development of microorganisms and host cells. The end products of this microbial fermentation, such as SCFAs, primarily acetate, propionate, and butyrate, can be advantageous for gut health as local (butyrate) and peripheral (acetate and propionate)

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[66] Turroni, S. et al.; "Microbiota-Host Transgenomic Metabolism, Bioactive Molecules from the Inside" in *Journal of Medicinal Chemistry* 61, no. 1 (**2018**): 47–61.

[67] Cummings, J.H. et al.; "Short Chain Fatty Acids in Human Large Intestine, Portal, Hepatic and Venous Blood" in *Gut* 28, no. 10 (**1987**): 1221–1227.

[68] Candela, M. et al.; "Dynamic Efficiency of the Human Intestinal Microbiota" in *Critical Reviews in Microbiology* 41, no. 2 (**2015**): 165–171.

energy sources, vasodilators, modulators of inflammation, and regulators of wound healing and gut motility[69, 70] as well as gene expression modulators[71]. For instance, a small number of glycoside hydrolases and polysaccharide lyases, often known as carbohydrate-active enzymes, or CAZymes, are encoded by the human genome. As a result, undigested glycans such fructo-oligosaccharides (FOS), pectin, lignin, resistant starch, and inulin enter the large intestine. The microbiome is estimated to encode tens of thousands of CAZymes, in contrast to humans. Bacteria, including members of the *Bacteroides*, *Bifidobacterium* and *Ruminococcus* genera, the so-called "primary degraders", are able to degrade these glycans[72, 73]. This primary degradation of glycans releases glucose and, combined with fermentation by secondary degraders, results in a complex cross-feeding metabolic network with the production of lactate, succinate, butyrate, acetate, formate and propionate. For instance, in the human gut, methanogens, acetogens, and sulfate-reducing bacteria consume hydrogen gas, which is produced when fermentation occurs[74].

SCFAs play a key role in all major physiological processes that affect the human host, including immunological response, metabolism control, and CNS activity, triggering, at a molecular level, G-protein-coupled receptors that are known to be "metabolite sensing"[75].

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[69] Bäckhed, F. et al.; "Dynamics and Stabilization of the Human Gut Microbiome during the First Year of Life" in *Cell Host & Microbe* 17, no. 5 (**2015**): 690–703.

[70] Bergman, E.N.; "Energy Contributions of Volatile Fatty Acids from the Gastrointestinal Tract in Various Species" in *Physiological Reviews* 70, no. 2 (**1990**): 567–590.

[71] Davie, J.R.; "Inhibition of Histone Deacetylase Activity by Butyrate" in *The Journal of Nutrition* 133, no. 7 Suppl (**2003**): 2485S-2493S.

[72] Eilam, O. et al.; "Glycan Degradation (GlyDeR) Analysis Predicts Mammalian Gut Microbiota Abundance and Host Diet-Specific Adaptations" in *mBio* 5, no. 4 (**2014**): e01526-14.

[73] Cantarel, B.L., V. Lombard, & B. Henrissat; "Complex Carbohydrate Utilization by the Healthy Human Microbiome" in *PLOS ONE* 7, no. 6 (**2012**): e28742.

[74] Fischbach, M.A. & J.L. Sonnenburg; "Eating for Two: How Metabolism Establishes Interspecies Interactions in the Gut" in *Cell Host & Microbe* 10, no. 4 (**2011**): 336–347.

[75] Thorburn, A.N., L. Macia, & C.R. Mackay; "Diet, Metabolites, and 'Western-Lifestyle' Inflammatory Diseases" in *Immunity* 40, no. 6 (**2014**): 833–842.

Several cell types in the body, including adipocytes, immune cells, endocrine cells, and colonocytes, express these receptors[76]. Actually, the development of adaptive immunity coincides with the acquisition of GM, most likely as a result of the necessity to preserve control over the wide variety of microbial cells that live in the gut[77]. SCFAs act on both innate and adaptive components of the immune system, locally and systematically, and are powerful anti-inflammatory agents[78, 79, 80, 81]. SCFAs could contribute to the correct functionality of the host nervous system, with direct and indirect action on several components of the neuronal apparatus, such as enteric nervous system, afferent nerves of the peripheral nervous system and the CNS[82]. Particularly, butyrate constitutes the primary energy source for human colonocytes, promoting apoptosis in colon cancer cells, and stimulates intestinal gluconeogenesis, which benefits glucose and energy homeostasis[83]. It is also crucial for epithelial cells to undergo β-oxidation, a process that maintains oxygen balance and creates a hypoxic environment that prevents GM dysbiosis[84]. In contrast, propionate is transported to the liver, where it influences gluconeogenesis and appetite

[76] Cani, P.D. & C. Knauf; "How Gut Microbes Talk to Organs: The Role of Endocrine and Nervous Routes" in *Molecular Metabolism* 5, no. 9 (**2016**): 743–752.

[77] Belkaid, Y. & T.W. Hand; "Role of the Microbiota in Immunity and Inflammation" in *Cell* 157, no. 1 (**2014**): 121–141.

[78] Honda, K. & D.R. Littman; "The Microbiota in Adaptive Immune Homeostasis and Disease" in *Nature* 535, no. 7610 (**2016**): 75–84.

[79] Koh, A. et al.; "From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites" in *Cell* 165, no. 6 (**2016**): 1332–1345.

[80] Rooks, M.G. & W.S. Garrett; "Gut Microbiota, Metabolites and Host Immunity" in *Nature Reviews. Immunology* 16, no. 6 (**2016**): 341–352.

[81] Thaiss, C.A. et al.; "The Microbiome and Innate Immunity" in *Nature* 535, no. 7610 (**2016**): 65–74.

[82] Cani and Knauf, "How Gut Microbes Talk to Organs."

[83] De Vadder, F. et al.; "Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits" in *Cell* 156, no. 1–2 (**2014**): 84–96.

[84] Byndloss, M.X. et al.; "Microbiota-Activated PPAR-γ Signaling Inhibits Dysbiotic Enterobacteriaceae Expansion" in *Science (New York, N.Y.)* 357, no. 6351 (**2017**): 570–575.

regulation through its interaction with fatty acid receptors[85]. Acetate, the most abundant SCFA, is important for the growth of other bacteria and is utilized in peripheral tissues for cholesterol metabolism and lipogenesis. It may also contribute to appetite regulation at the central level[86]. Higher synthesis of SCFAs has been linked to lower diet-induced obesity and improved insulin resistance. In mice, butyrate and propionate seem to regulate gut hormones and decrease hunger and food intake, but not acetate[87, 88]. Examples of major butyrate-producing genera of the human gut are *Roseburia*[89] and *Ruminococcus*[90]. *Roseburia intestinalis* could maintain the gut barrier function through upregulation of the tight-junction proteins[91]. Supplementation of *R. intestinalis* and *Roseburia hominis* could ameliorate alcoholic fatty liver disease in mice[92], while *Ruminococcus bromii* is a keystone species for resistant starch degradation in the human colon[93].

Beyond SCFAs, other crucial GM–derived small molecules are vitamins[94]. Some gut microorganisms in fact produce riboflavin, menaquinone, cobalamin and folate, better

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[85] De Vadder et al., "Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits."

[86] Frost, G. et al.; "The Short-Chain Fatty Acid Acetate Reduces Appetite via a Central Homeostatic Mechanism" in *Nature Communications* 5, no. 1 (**2014**): 3611.

[87] Lin, H.V. et al.; "Butyrate and Propionate Protect against Diet-Induced Obesity and Regulate Gut Hormones via Free Fatty Acid Receptor 3-Independent Mechanisms" ed. Brennan in *PLoS ONE* 7, no. 4 (**2012**): e35240.

[88] Zhao, L. et al.; "Gut Bacteria Selectively Promoted by Dietary Fibers Alleviate Type 2 Diabetes" in *Science (New York, N.Y.)* 359, no. 6380 (**2018**): 1151–1156.

[89] Duncan, S.H. et al.; "Roseburia Intestinalis Sp. Nov., a Novel Saccharolytic, Butyrate-Producing Bacterium from Human Faeces." in *International Journal of Systematic and Evolutionary Microbiology* 52, no. 5 (**2002**): 1615–1620.

[90] Ze, X. et al.; "Ruminococcus Bromii Is a Keystone Species for the Degradation of Resistant Starch in the Human Colon" in *The ISME Journal* 6, no. 8 (**2012**): 1535–1543.

[91] Hiippala, K. et al.; "The Potential of Gut Commensals in Reinforcing Intestinal Barrier Function and Alleviating Inflammation" in *Nutrients* 10, no. 8 (**2018**): 988.

[92] Seo, B. et al.; "Roseburia Spp. Abundance Associates with Alcohol Consumption in Humans and Its Administration Ameliorates Alcoholic Fatty Liver in Mice" in *Cell Host & Microbe* 27, no. 1 (**2020**): 25-40.e6.

[93] Ze et al., "Ruminococcus Bromii Is a Keystone Species for the Degradation of Resistant Starch in the Human Colon."

[94] LeBlanc, J.G. et al.; "Bacteria as Vitamin Suppliers to Their Host: A Gut Microbiota Perspective" in *Current Opinion in Biotechnology* 24, no. 2 (**2013**): 160–168.

known as vitamins B2, K2, B12 and B9 from the host viewpoint, involved in different biological functions such as insulin sensitivity and bone metabolism[95]. Another class of GM-derived compounds are neurotransmitters like catecholamines, amino acids, indolic compounds and γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the nervous system of mammals, implicated in gastric acid secretion and gastric emptying, intestinal motility and perception of visceral pain[96].

Another major neurotransmitter synthesized and released by the GM is nitric oxide[97] which might improve host longevity and resistance to stress in non-mammalian organisms[98]. Lactate is another remarkable metabolite produced by the GM, in particular by the milk-fermenting GM of breastfed infants. It can exert essential regulatory and metabolic effects, acting as signaling molecule and immune modulator[99]. Last but not least, gut microorganisms are able to produce polyamines, essential molecules for different host functions related to the cells like growth, proliferation and survival but, if produced at dysregulated levels, their effects could be detrimental for human health[100].

1.2 The GM in Non-Communicable Diseases

As a tremendous quantity of data have shown, disruptions in the taxonomic composition and functional capacity of the GM (*i.e.* dysbiosis) are linked to a wide range of pathological

[95] Derrien, M. & P. Veiga; "Rethinking Diet to Aid Human-Microbe Symbiosis" in *Trends in Microbiology* 25, no. 2 (**2017**): 100–112.

[96] Hyland, N.P. & J.F. Cryan; "A Gut Feeling about GABA: Focus on GABA(B) Receptors" in *Frontiers in Pharmacology* 1 (**2010**): 124.

[97] Ji, X.B. & T.C. Hollocher; "Reduction of Nitrite to Nitric Oxide by Enteric Bacteria" in *Biochemical and Biophysical Research Communications* 157, no. 1 (**1988**): 106–108.

[98] Gusarov, I. et al.; "Bacterial Nitric Oxide Extends the Lifespan of C. Elegans" in *Cell* 152, no. 4 (**2013**): 818–830.

[99] Koh et al., "From Dietary Fiber to Host Physiology."

[100] Di Martino, M.L. et al.; "Polyamines: Emerging Players in Bacteria-Host Interactions" in *International journal of medical microbiology: IJMM* 303, no. 8 (**2013**): 484–491.

phenotypes, especially NCDs. For example, Proteobacteria represent the most consistently reported obesity-associated phylum. Several members of this phylum, e.g. *Proteus mirabilis* and *Escherichia coli*, are considered potential drivers of inflammation in the gastrointestinal tract[101, 102, 103] which is a risk factor for the development of other metabolic diseases including atherosclerosis and T2D[104]. Additionally, in obese subjects with T2D was reported the presence of members from *Enterobacteriaceae* family in plasma and omental adipose tissue microbiota[105]. Findings on the correlation between the genus *Lactobacillus* and obesity, on the other hand, are still contradictory. *Lactobacillus* was reported to be an obesity-associated taxon and its abundance seems higher in the stool of patients affected by obesity. This food-derived probiotic genus shows relatively low prevalence and abundance in the commensal GM[106]. Clinical trials on the use of *Lactobacillus*, alone or in combination with *Bifidobacterium*, showed variable efficacy in weight loss in patients with obesity[107]. Furthermore, an analysis on nine randomized controlled trials revealed significant weight reduction and BMI decreases with *Lactobacillus plantarum* supplementation compared to a placebo and the effects of this reduction were enhanced using more than two strains

[101] Allegretti, J.R. et al.; "Effects of Fecal Microbiota Transplantation With Oral Capsules in Obese Patients" in *Clinical Gastroenterology and Hepatology* 18, no. 4 (**2020**): 855-863.e2.

[102] Rhodes, J.M.; "The Role of Escherichia Coli in Inflammatory Bowel Disease" in *Gut* 56, no. 5 (**2007**): 610–612.

[103] Zhang, J. et al.; "Elucidation of Proteus Mirabilis as a Key Bacterium in Crohn's Disease Inflammation" in *Gastroenterology* 160, no. 1 (**2021**): 317-330.e11.

[104] Ellulu, M.S. et al.; "Obesity and Inflammation: The Linking Mechanism and the Complications" in *Archives of Medical Science* 13, no. 4 (**2017**): 851–863.

[105] Anhê, F.F. et al.; "Type 2 Diabetes Influences Bacterial Tissue Compartmentalisation in Human Obesity" in *Nature Metabolism* 2, no. 3 (**2020**): 233–242.

[106] Qin, J. et al.; "A Metagenome-Wide Association Study of Gut Microbiota in Type 2 Diabetes" in *Nature* 490, no. 7418 (**2012**): 55–60.

[107] Borgeraas, H. et al.; "Effects of Probiotics on Body Weight, Body Mass Index, Fat Mass and Fat Percentage in Subjects with Overweight or Obesity: A Systematic Review and Meta-Analysis of Randomized Controlled Trials" in *Obesity Reviews* 19, no. 2 (**2018**): 219–232.

together[108]. Also, *Bacteroides thetaiotaomicron*, a commensal member of the Bacteroidota phylum that ferments glutamate, is inversely linked with blood glutamate levels and significantly reduced in obese people[109]. Moreover, *Akkermansia muciniphila*, a member of the Verrucomicrobiota phylum, is one of the most frequently reported lean-associated bacteria in obesity and metabolic diseases. Supplementation with *A. muciniphila* could decrease the levels of blood markers for liver dysfunction and inflammation and could reduce body weight in obese insulin-resistant patients[110]. Also, a supplementation with five strains including *A. muciniphila* was associated with improved postprandial glucose control[111]. At intestinal level, *A. muciniphila* could help modulate the gut barrier function and prevent inflammation caused by the so called "leaky" gut[112]. Other genera with a higher relative abundance consistently reported in healthy individuals compared to obese patients are *Faecalibacterium* and *Alistipes*. *Faecalibacterium*, a Firmicutes member, is one of the major butyrate producers in the human gut[113, 114], while *Alistipes* (Bacteroidota) could

[108] Li, C.-P. et al.; "The Role of Lactobacillus Plantarum in Reducing Obesity and Inflammation: A Meta-Analysis" in *International Journal of Molecular Sciences* 25, no. 14 (**2024**): 7608.

[109] Liu, R. et al.; "Gut Microbiome and Serum Metabolome Alterations in Obesity and after Weight-Loss Intervention" in *Nature Medicine* 23, no. 7 (**2017**): 859–868.

[110] Depommier, C. et al.; "Supplementation with Akkermansia Muciniphila in Overweight and Obese Human Volunteers: A Proof-of-Concept Exploratory Study" in *Nature Medicine* 25, no. 7 (**2019**): 1096–1103.

[111] Perraudeau, F. et al.; "Improvements to Postprandial Glucose Control in Subjects with Type 2 Diabetes: A Multicenter, Double Blind, Randomized Placebo-Controlled Trial of a Novel Probiotic Formulation" in *BMJ Open Diabetes Research and Care* 8, no. 1 (**2020**): e001319.

[112] Ansaldo, E. et al.; "Akkermansia Muciniphila Induces Intestinal Adaptive Immune Responses during Homeostasis" in *Science* 364, no. 6446 (**2019**): 1179–1184.

[113] De Vuyst, L. & F. Leroy; "Cross-Feeding between Bifidobacteria and Butyrate-Producing Colon Bacteria Explains Bifdobacterial Competitiveness, Butyrate Production, and Gas Production" in *International Journal of Food Microbiology* 149, no. 1, 3rd International Symposium on Propionibacteria and Bifidobacteria: Dairy and Probiotic applications, Oviedo 1-4 June 2010 (**2011**): 73–80.

[114] Walker, A.W. et al.; "Phylogeny, Culturing, and Metagenomics of the Human Gut Microbiota" in *Trends in Microbiology* 22, no. 5 (**2014**): 267–274.

produce small amounts of SCFAs as acetic, isobutyric, isovaleric, and propionic acid[115]. Finally, Xu et al. found that the genera *Bifidobacterium*, *Roseburia*, *Prevotella*, and *Ruminococcus* are consistently reported to be lean-associated microorganisms exclusively in subjects from Eastern countries[116].

An aberrant GM could also be linked to T2D, as shown in different studies. In particular, some studies focused on the so-called prediabetes, the drug-naïve early stages of T2D, in which the GM exhibits a loss of butyrate-producing taxa, an increase in bacteria with pro-inflammatory potential and a decrease in *A. municiphila* abundance[117, 118]. Reduced abundance of butyrate-producing bacteria has been consistently found, together with a reduced bacterial richness and increased opportunistic pathogens, also in patients with gestational diabetes mellitus[119, 120]. The GM of gestational diabetes mellitus patients resembles the aberrant microbiota of non-pregnant individuals with T2D[121]. Nonetheless, it is especially important to remember that different medications used to treat these conditions may have an impact on GM when searching the possible role of altered GM in the development of chronic illnesses[122]. For instance, metformin, an anti-hyperglycemic drug

[115] Parker, B.J. et al.; "The Genus Alistipes: Gut Bacteria With Emerging Implications to Inflammation, Cancer, and Mental Health" in *Frontiers in Immunology* 11 (**2020**).

[116] XU, Z. et al.; "Gut Microbiota in Patients with Obesity and Metabolic Disorders — a Systematic Review" in *Genes & Nutrition* 17, no. 1 (**2022**): 2.

[117] Allin, K.H. et al.; "Aberrant Intestinal Microbiota in Individuals with Prediabetes" in *Diabetologia* 61, no. 4 (**2018**): 810–820.

[118] Zhong, H. et al.; "Distinct Gut Metagenomics and Metaproteomics Signatures in Prediabetics and Treatment-Naïve Type 2 Diabetics" in *EBioMedicine* 47 (**2019**): 373–383.

[119] Karlsson, F.H. et al.; "Gut Metagenome in European Women with Normal, Impaired and Diabetic Glucose Control" in *Nature* 498, no. 7452 (**2013**): 99–103.

[120] Qin et al., "A Metagenome-Wide Association Study of Gut Microbiota in Type 2 Diabetes."

[121] Crusell, M.K.W. et al.; "Gestational Diabetes Is Associated with Change in the Gut Microbiota Composition in Third Trimester of Pregnancy and Postpartum" in *Microbiome* 6, no. 1 (**2018**): 89.

[122] Fan, Y. & O. Pedersen; "Gut Microbiota in Human Metabolic Health and Disease" in *Nature Reviews Microbiology* 19, no. 1 (**2021**): 55–71.

often prescribed for T2D, appears to have an effect on *Escherichia* and *Intestinibacter* genera relative abundances, with the enhancement of propionate and butyrate production, inducing intestinal gluconeogenesis[123, 124, 125]. Other conditions heavily medicated are, for instance, the cardio-metabolic diseases, such as arteriosclerosis. GM of patients treated with medications, exhibits higher abundances of *Enterobacteriaceae*, including *E. coli*, *Klebsiella* spp. and *Enterobacter aerogenes*[126] and lower abundances of *Bacteroides* spp. and *F. prausnitzii*[127].

Significant changes also occur in the development of other NCDs, like cancer. The GM plays an important role in particular in colorectal cancer, or CRC, and it is involved in its carcinogenesis and progression as well as its response to different systemic therapies. The GM-mediated carcinogenesis of CRC causes initial inflammation and modulates different signalling pathways[128, 129, 130, 131, 132]. Bacterial biomarkers have the potential to detect CRC

[123] Bryrup, T. et al.; "Metformin-Induced Changes of the Gut Microbiota in Healthy Young Men: Results of a Non-Blinded, One-Armed Intervention Study" in *Diabetologia* 62, no. 6 (**2019**): 1024–1035.

[124] Forslund, K. et al.; "Disentangling Type 2 Diabetes and Metformin Treatment Signatures in the Human Gut Microbiota" in *Nature* 528, no. 7581 (**2015**): 262–266.

[125] Wu, H. et al.; "Metformin Alters the Gut Microbiome of Individuals with Treatment-Naive Type 2 Diabetes, Contributing to the Therapeutic Effects of the Drug" in *Nature Medicine* 23, no. 7 (**2017**): 850–858.

[126] Jie, Z. et al.; "The Gut Microbiome in Atherosclerotic Cardiovascular Disease" in *Nature Communications* 8, no. 1 (**2017**): 845.

[127] Sokol, H. et al.; "Faecalibacterium Prausnitzii Is an Anti-Inflammatory Commensal Bacterium Identified by Gut Microbiota Analysis of Crohn Disease Patients" in *Proceedings of the National Academy of Sciences of the United States of America* 105, no. 43 (**2008**): 16731–16736.

[128] Gill, S.R. et al.; "Metagenomic Analysis of the Human Distal Gut Microbiome" in *Science (New York, N.Y.)* 312, no. 5778 (**2006**): 1355.

[129] Gopalakrishnan, V. et al.; "The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy" in *Cancer Cell* 33, no. 4 (**2018**): 570–580.

[130] Holmes, E. et al.; "Gut Microbiota Composition and Activity in Relation to Host Metabolic Phenotype and Disease Risk" in *Cell Metabolism* 16, no. 5 (**2012**): 559–564.

[131] Maisonneuve, C. et al.; "The Impact of the Gut Microbiome on Colorectal Cancer" in *Annual Review of Cancer Biology* 2, no. Volume 2, 2018 (**2018**): 229–249.

[132] Wong, S.H. & J. Yu; "Gut Microbiota in Colorectal Cancer: Mechanisms of Action and Clinical Applications" in *Nature Reviews. Gastroenterology & Hepatology* 16, no. 11 (**2019**): 690–704.

and predict clinical outcome[133, 134, 135, 136, 137] since the CRC microbiota has a distinct signature compared to a healthy GM, containing specific species such as *Bacteroides fragilis*, *Streptococcus gallolyticus*, *Enterococcus faecalis*, and *E. coli*, along with more recently identified taxa like *Fusobacterium nucleatum*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, and *Prevotella*. Higher abundances of these bacteria in stool and tumor samples from CRC patients are used as biomarkers for the disease[138, 139]. Certain bacterial species, such as *F. nucleatum* and *Solobacterium moorei*, are present from the early stages of CRC to the metastatic ones, while others, like *Atopobium parvulum* and *Actinomyces odontolyticus*, are abundant only in adenomas and intramucosal carcinomas[140]. Recent research[141] has explored the interplay between the Wnt/β-catenin signaling pathway, the PI3K/mTOR pathway, and GM composition at different stages of CRC development. Dysregulation of these pathways is known to contribute to CRC carcinogenesis[142, 143, 144]. In particular, simultaneous hyperactivation of the Wnt/β-catenin and PI3K/mTOR pathways in

[133] Gill et al., "Metagenomic Analysis of the Human Distal Gut Microbiome."

[134] Gopalakrishnan et al., "The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy."

[135] Holmes et al., "Gut Microbiota Composition and Activity in Relation to Host Metabolic Phenotype and Disease Risk."

[136] Rebersek, M.; "Gut Microbiome and Its Role in Colorectal Cancer" in *BMC Cancer* 21 (**2021**): 1325.

[137] Wong and Yu, "Gut Microbiota in Colorectal Cancer."

[138] Grenham, S. et al.; "Brain–Gut–Microbe Communication in Health and Disease" in *Frontiers in Physiology* 2 (**2011**).

[139] Wong and Yu, "Gut Microbiota in Colorectal Cancer."

[140] Ibid.

[141] Di Paola, F.J. et al.; "Interplay between WNT/PI3K-mTOR Axis and the Microbiota in APC-Driven Colorectal Carcinogenesis: Data from a Pilot Study and Possible Implications for CRC Prevention" in *Journal of Translational Medicine* 22, no. 1 (**2024**): 631.

[142] Francipane, M.G. & E. Lagasse; "mTOR Pathway in Colorectal Cancer: An Update" in *Oncotarget* 5, no. 1 (**2014**): 49–66.

[143] Gulhati, P. et al.; "Targeted Inhibition of Mammalian Target of Rapamycin Signaling Inhibits Tumorigenesis of Colorectal Cancer" in *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 15, no. 23 (**2009**): 7207–7216.

[144] Kinzler, K.W. & B. Vogelstein; "Lessons from Hereditary Colorectal Cancer" in *Cell* 87, no. 2 (**1996**): 159–170.

lesions associated with familial adenomatous polyposis (FAP), a condition caused by pathogenic variants in the *APC* gene that confer nearly a 100% lifetime risk of developing CRC[145]. In FAP fecal microbiota, molecular markers of the Wnt/β-catenin pathway showed a positive correlation with *Clostridium_sensu_stricto_1* and a negative correlation with *Bacteroides*. Additionally, *Alistipes*, *Lachnospiraceae*, and *Ruminococcaceae* were enriched in FAP stool samples and adenomas. The adenomas also exhibited an overabundance of *Lachnoclostridium*, which positively correlated with *cMYC* expression, a key proto-oncogene. In CRC tissues with mTOR pathway impairment, the effector protein p-S6R expression was associated with *Fusobacterium* and *Dialister*, with the latter also confirmed in the fecal microbiota[146].

Not only the cancer *per se*, but an important perturbation of the gut ecosystem is given by the therapy, especially chemotherapy. Chemotherapy is well documented to reduce GM diversity, which may lead to a loss of functional redundancy, a feature crucial for the GM's stability and resilience under stress. A lot of studies have documented declines in both GM richness (total species count) and diversity (evenness and phylogenetic spread), especially in patients treated for CRC with specific chemotherapeutic agents. For example, findings showed decreases in microbial diversity after chemotherapic treatments, but with a notable enrichment of *Bacteroides*[147, 148]. In another human study on patients undergoing chemotherapy, there was a slight increase in *Bacteroides* species, as well as the emergence

[145] Galiatsatos, P. & W.D. Foulkes; "Familial Adenomatous Polyposis" in *The American Journal of Gastroenterology* 101, no. 2 (**2006**): 385–398.

[146] Di Paola et al., "Interplay between WNT/PI3K-mTOR Axis and the Microbiota in APC-Driven Colorectal Carcinogenesis."

[147] Deng, X. et al.; "Comparison of Microbiota in Patients Treated by Surgery or Chemotherapy by 16S rRNA Sequencing Reveals Potential Biomarkers for Colorectal Cancer Therapy" in *Frontiers in Microbiology* 9 (**2018**): 1607.

[148] Fei, Z. et al.; "Gut Microbiome Associated with Chemotherapy-Induced Diarrhea from the CapeOX Regimen as Adjuvant Chemotherapy in Resected Stage III Colorectal Cancer" in *Gut Pathogens* 11 (**2019**): 18.

of potential pathogens, including *C. difficile* and *Enterococcus faecium*, not previously detected[149].

Chemotherapy-treated patients generally show reductions in beneficial microbes, such as *Bifidobacterium*, *Ruminoclostridium*, *Lachnoclostridium*, *Oscillobacter*[150], *Lactobacillus* and *Bacteroides*[151] compared to healthy controls[152], with an increase in potentially pathogenic bacteria, such as *Staphylococcus* spp. and *E. coli*[153]. Platinum-based chemotherapies have also been linked to decreased levels of SCFAs-producing taxa in patients with ovarian cancer. Interestingly, abundances of certain taxa appear associated with resistance to chemotherapy, such as increased abundances of *Coriobacteriaceae* and *Bifidobacterium*[154]. A study from 2018 compared GM profiles of untreated patients with gastrointestinal cancers to those treated with chemotherapy and radiotherapy, finding a greater presence of potentially beneficial *Lactobacillus* species in the treated group. However, this increase is likely influenced by the patients' GM status before treatment rather than a direct benefit of chemotherapy[155]. Moreover, studies on patients with acute myeloid leukemia receiving chemotherapy revealed that the GM often became dominated by opportunistic pathogens, like *Staphylococcus*, *Enterobacter*, and *Escherichia*[156]. In some cases, *Lactobacillus*

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[149] Zwielehner, J. et al.; "Changes in Human Fecal Microbiota Due to Chemotherapy Analyzed by TaqMan-PCR, 454 Sequencing and PCR-DGGE Fingerprinting" in *PloS One* 6, no. 12 (**2011**): e28654.

[150] Youssef, O. et al.; "Stool Microbiota Composition Differs in Patients with Stomach, Colon, and Rectal Neoplasms" in *Digestive Diseases and Sciences* 63, no. 11 (**2018**): 2950–2958.

[151] Stringer, A.M. et al.; "Biomarkers of Chemotherapy-Induced Diarrhoea: A Clinical Study of Intestinal Microbiome Alterations, Inflammation and Circulating Matrix Metalloproteinases" in *Supportive Care in Cancer* 21, no. 7 (**2013**): 1843–1852.

[152] Youssef et al., "Stool Microbiota Composition Differs in Patients with Stomach, Colon, and Rectal Neoplasms."

[153] Stringer et al., "Biomarkers of Chemotherapy-Induced Diarrhoea."

[154] D'Amico, F. et al.; "Gut Microbiota Dynamics during Chemotherapy in Epithelial Ovarian Cancer Patients Are Related to Therapeutic Outcome" in *Cancers* 13, no. 16 (**2021**): 3999.

[155] Youssef et al., "Stool Microbiota Composition Differs in Patients with Stomach, Colon, and Rectal Neoplasms."

[156] Galloway-Peña, J.R. et al.; "The Role of the Gastrointestinal Microbiome in Infectious Complications during Induction Chemotherapy for Acute Myeloid Leukemia" in *Cancer* 122, no. 14 (**2016**): 2186–2196.

abundance also increased, possibly aiding GM recovery, though its precise role in resilience or recovery remains unclear[157, 158]. The overall evidence emphasizes the need for further research to clarify the GM's response to chemotherapy and its potential role in treatment outcomes[159].

1.2.1 Highlights on pharmacomicrobiomics

When addressing the restoration of GM eubiosis and pharmacological treatments for NCDs, the effects that the microbiota exerts on these xenobiotics cannot be overlooked. In fact, the advancement of microbial genomics towards culture-independent methods has made possible to identify the GM's molecular fingerprint as well as understanding how the GM is linked to specific disorders or changes in the therapy response[160]. Pharmacomicrobiomics examines how variations in the microbiome can influence the overall response to drugs and xenobiotics[161, 162]. In particular, pharmacomicrobiomics examines the effects of intra- and inter-individual microbiome changes on drug action, tolerability, efficacy, and toxicity. Rather than drug-drug interactions with particular microorganisms, the primary focus is on the influence of microbiome (*i.e.*, microbial species) differences on clinical outcomes of

[157] Ibid.

[158] Youssef, O. et al.; "Stool Microbiota Composition Differs in Patients with Stomach, Colon, and Rectal Neoplasms" in *Digestive Diseases and Sciences* 63, no. 11 (**2018**): 2950–2958.

[159] Roggiani, S. et al.; "Gut Microbiota Resilience and Recovery after Anticancer Chemotherapy" in *Microbiome Research Reports* 2, no. 3 (**2023**): N/A-N/A.

[160] Scher, J.U. et al.; "Pharmacomicrobiomics in Inflammatory Arthritis: Gut Microbiome as Modulator of Therapeutic Response" in *Nature Reviews. Rheumatology* 16, no. 5 (**2020**): 282–292.

[161] Conti, G. et al.; "Pharmacomicrobiomics in Anticancer Therapies: Why the Gut Microbiota Should Be Pointed Out" in *Genes* 14, no. 1 (**2022**): 55.

[162] Rizkallah, M., R. Saad, & R. Aziz; "The Human Microbiome Project, Personalized Medicine and the Birth of Pharmacomicrobiomics" in *Current Pharmacogenomics and Personalized Medicine (Formerly Current Pharmacogenomics)* (**2010**).

pharmacokinetics and pharmacodynamics of pharmaceuticals and xenobiotics[163]. The GM can regulate and affect the metabolic processes of drugs via biotransformation through mechanisms including hydrolysis, demethylation, deamination, and reactive reactions. Nonetheless, the GM can also control the efficacy of several drugs by modulating the host's metabolism and establishing a competitive environment for the drug receptor[164, 165]. The **Figure 2** shows these interactions.

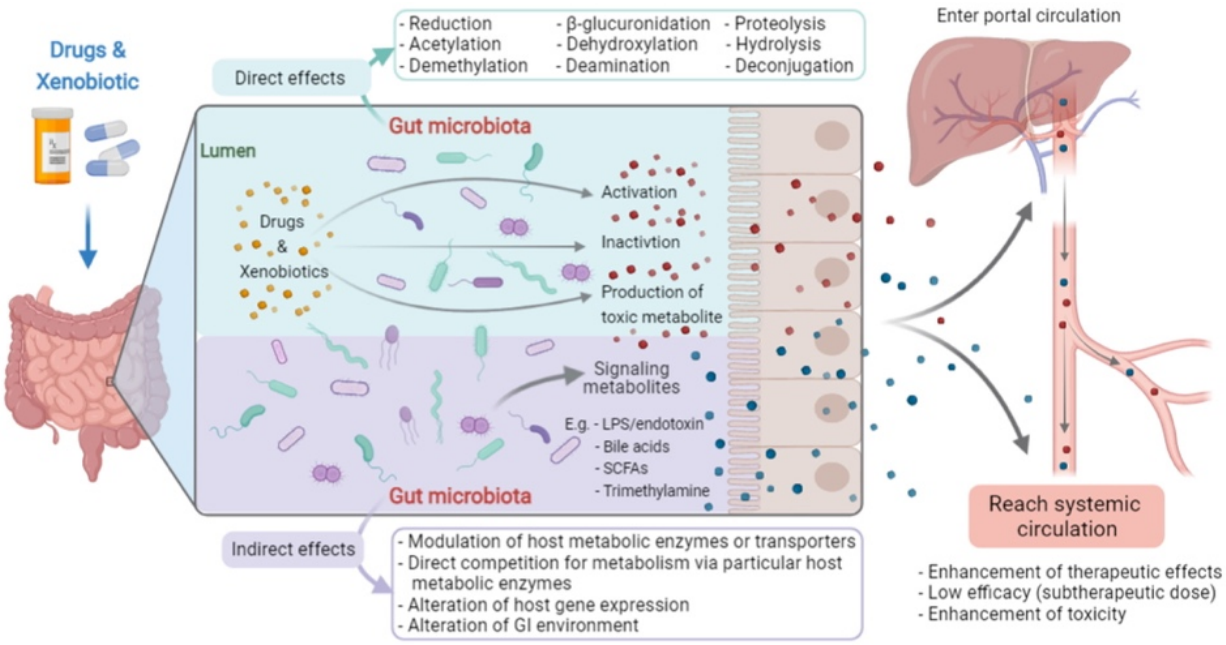

Figure 2. Influence of the GM on drug and xenobiotic metabolism[164].

The impact of the GM on drug metabolism was first observed in 1930 with the biotransformation of prontosil[166]. The interplay between drugs and the GM can yield both

[163] Aziz, R.K.; "Interview with Prof. Ramy K. Aziz, Cairo University. The Dawn of Pharmacomicrobiomics" in *Omics: A Journal of Integrative Biology* 22, no. 4 (**2018**): 295–297.

[164] Dikeocha, I.J. et al.; "Pharmacomicrobiomics: Influence of Gut Microbiota on Drug and Xenobiotic Metabolism" in *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* 36, no. 6 (**2022**): e22350.

[165] Wilson, I.D. & J.K. Nicholson; "Gut Microbiome Interactions with Drug Metabolism, Efficacy, and Toxicity" in *Translational Research* 179 (**2017**): 204–222.

[166] Atkinson, A.J. et al.; *Principles of Clinical Pharmacology* (Elsevier Inc., **2007**).

beneficial and detrimental effects. Beneficial outcomes include the conversion of prontosil to the antibiotic PABA or the activation of sulfasalazine into its active component, 5-aminosalicylic acid[167]. On the other hand, gut microbial metabolism can enhance drug toxicity; for instance, the bacterial β-glucuronidase enzyme can convert irinotecan's inactive metabolite, SN-38G, back into the toxic form SN-38, causing significant intestinal damage and severe diarrhea in approximately 80% of treated patients[168]. Similarly, the bacterial β-glucuronidase enzyme contributes to the adverse effects associated with non-steroidal anti-inflammatory drugs (NSAIDs), including gastroduodenal mucosal damage, affecting nearly half of regular NSAID users[169]. The widespread production of β-glucuronidase by gut bacteria complicates the development of drug formulations that selectively reduce toxicity[170]. Research has demonstrated the potential of β-glucuronidase inhibitors that specifically target bacterial enzymes without harming host cells, as evidenced by studies in mice showing reduced irinotecan toxicity when co-administered with *E. coli*-derived β-glucuronidase inhibitors (*E. coli* GUS)[171, 172]. The effect of pharmacomicrobiomics on compounds other than drugs will be discussed deeply in the next chapter, regarding the GM action on polyphenols, compounds often used in NCDs treatment.

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[167] Collins, S.L. & A.D. Patterson; "The Gut Microbiome: An Orchestrator of Xenobiotic Metabolism" in *Acta Pharmaceutica Sinica B* 10, no. 1, SI: Drug metabolism and disposition in diseases (**2020**): 19–32.

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[169] Higuchi, K. et al.; "Present Status and Strategy of NSAIDs-Induced Small Bowel Injury" in *Journal of Gastroenterology* 44, no. 9 (**2009**): 879–888.

[170] Dabek, M. et al.; "Distribution of Beta-Glucosidase and Beta-Glucuronidase Activity and of Beta-Glucuronidase Gene Gus in Human Colonic Bacteria" in *FEMS microbiology ecology* 66, no. 3 (**2008**): 487–495.

[171] Wallace, B.D. et al.; "Alleviating Cancer Drug Toxicity by Inhibiting a Bacterial Enzyme" in *Science (New York, N.Y.)* 330, no. 6005 (**2010**): 831–835.

[172] Woo, A.Y.M. et al.; "Targeting the Human Gut Microbiome with Small-Molecule Inhibitors" in *Nature Reviews Chemistry* 7, no. 5 (**2023**): 319–339.

1.3 Interventions for the restoration of the gut microbiota dysbiosis

As previously discussed, diets, NCDs and other factors can seriously compromise the stability of the GM, pushing it towards an unstable dysbiotic state. Once the perturbation has ceased, the GM can recover to its original state or stabilize in a new alternative, healthy or dysbiotic (or disease-associated) state[173, 174]. Indeed, resilience is defined as the property of a microbial ecosystem that describes how quickly and to what extent it will recover its initial taxonomical and/or functional composition following perturbations[175]. The GM is defined as resistant when it remains substantially unchanged in the face of perturbations. The genus *Bacteroides*, in particular, has emerged as a crucial player in GM recovery. It appears to have a unique ability to endure different therapies like chemotherapy and antibiotic therapies. Within *Bacteroides* genus, 21 bacterial species (*i.e., B. intestinalis*, *B. thetaiotaomicron*, *B. uniformis*, *B. stercoris*, *B. coprocola*, *B. egghertii* and *B. caccae*), that are strongly linked to post-antibiotic recovery have been identified[176]. The persistence of these species is thought to be due to their ability to penetrate and reside within the colonic mucus layer, particularly in the crypt channels, which are less exposed to environmental stressors[177, 178].

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[173] Fassarella, M. et al.; "Gut Microbiome Stability and Resilience: Elucidating the Response to Perturbations in Order to Modulate Gut Health" in *Gut* 70, no. 3 (**2021**): 595–605.

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[177] Donaldson, G.P., S.M. Lee, & S.K. Mazmanian; "Gut Biogeography of the Bacterial Microbiota" in *Nature Reviews. Microbiology* 14, no. 1 (**2016**): 20–32.

[178] Lee, S.M. et al.; "Bacterial Colonization Factors Control Specificity and Stability of the Gut Microbiota" in *Nature* 501, no. 7467 (**2013**): 426–429.

Some interventions aimed at restoring a eubiotic profile within a resilient GM include the use of prebiotics and probiotics. Prebiotics are non-living food material defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”[179]. The majority of the prebiotics currently used are carbohydrates-based such as fructo-oligosaccharides, galacto-oligosaccharides, lactulose, inulin, human milk oligosaccharides[180, 181, 182, 183] as well as galactofructose, mannan-oligosaccharides and xylooligosaccharides[184]. However, other substances, such as polyphenols[185] and polyunsaturated fatty acids[186], are being studied for their beneficial effects on host health. These substances almost entirely pass through the small intestine's digestive process before entering the colon, where they can be digested into SCFAs by a variety of bacterial taxa[187]. The development of syntrophic cross-feeding interactions, in particular, is known to

[179] Gibson, G.R. et al.; “Expert Consensus Document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) Consensus Statement on the Definition and Scope of Prebiotics” in *Nature Reviews Gastroenterology & Hepatology* 14, no. 8 (**2017**): 491–502.

[180] García-Peris, P. et al.; “Effect of a Mixture of Inulin and Fructo-Oligosaccharide on Lactobacillus and Bifidobacterium Intestinal Microbiota of Patients Receiving Radiotherapy: A Randomised, Double-Blind, Placebo-Controlled Trial” in *Nutricion Hospitalaria* 27, no. 6 (**2012**): 1908–1915.

[181] Messaoudene, M. et al.; “A Natural Polyphenol Exerts Antitumor Activity and Circumvents Anti–PD-1 Resistance through Effects on the Gut Microbiota” in *Cancer Discovery* 12, no. 4 (**2022**): 1070–1087.

[182] Sanders, M.E. et al.; “Probiotics and Prebiotics in Intestinal Health and Disease: From Biology to the Clinic” in *Nature Reviews Gastroenterology & Hepatology* 16, no. 10 (**2019**): 605–616.

[183] Wang, Y. & H. Li; “Gut Microbiota Modulation: A Tool for the Management of Colorectal Cancer” in *Journal of Translational Medicine* 20, no. 1 (**2022**): 178.

[184] Davani-Davari, D. et al.; “Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications” in *Foods* 8, no. 3 (**2019**): 92.

[185] Fabbrini, M. et al.; “Polyphenol and Tannin Nutraceuticals and Their Metabolites: How the Human Gut Microbiota Influences Their Properties” in *Biomolecules* 12, no. 7 (**2022**): 875.

[186] Rinninella, E. & L. Costantini; “Polyunsaturated Fatty Acids as Prebiotics: Innovation or Confirmation?” in *Foods* 11, no. 2 (**2022**): 146.

[187] Holscher, H.D.; “Dietary Fiber and Prebiotics and the Gastrointestinal Microbiota” in *Gut Microbes* 8, no. 2 (**2017**): 172–184.

be a component of their metabolism[188, 189, 190]. Since these interactions are critical to the ecological health of GM, they may encourage the persistence and/or repopulation of advantageous commensals, which could accelerate the restoration of microbial diversity and abundance[191]. In addition, nutraceuticals represent another class of compounds that are often used for NCDs treatment. Nutraceuticals are generally defined as "a food or part of a food that provides benefits to health in addition to its nutritional content"[192]. In this sense, prebiotics and plant-derived molecules, like phenolic compounds are included in this definition. The definition of phenolic compounds generally refers to ubiquitously distributed plant-derived phytochemicals, which are part of the daily diet due to their presence in vegetables and fruits[193]. The health benefits of various flavonoids, including quercetin, kaempferol, flavanones, flavan-3-ols, anthocyanins, and tannins are well known. Quercetin and kaempferol exhibit anticancer, antioxidant, and anti-inflammatory effects *in vitro*, while clinical trials show that flavanol intake may reduce cardiovascular risk. Key gut bacteria, such as those from *Lachnospiraceae* family (specifically, the genera *Lachnoclostridium* and *Eisenbergiella*), *Enterobacteriaceae* family (*Escherichia*) and *Bifidobacterium dentium*, are capable of metabolizing flavonoids, enhancing their bioavailability and therapeutic impact[194,

[188] Guarino, M.P.L. et al.; "Mechanisms of Action of Prebiotics and Their Effects on Gastro-Intestinal Disorders in Adults" in *Nutrients* 12, no. 4 (**2020**): 1037.

[189] Jean-Pierre, F., M.A. Henson, & G.A. O'Toole; "Metabolic Modeling to Interrogate Microbial Disease: A Tale for Experimentalists" in *Frontiers in Molecular Biosciences* 8 (**2021**).

[190] Klünemann, M. et al.; "Bioaccumulation of Therapeutic Drugs by Human Gut Bacteria" in *Nature* 597, no. 7877 (**2021**): 533–538.

[191] Roggiani et al., "Gut Microbiota Resilience and Recovery after Anticancer Chemotherapy."

[192] Santini, A., G.C. Tenore, & E. Novellino; "Nutraceuticals: A Paradigm of Proactive Medicine" in *European Journal of Pharmaceutical Sciences: Official Journal of the European Federation for Pharmaceutical Sciences* 96 (**2017**): 53–61.

[193] Soto-Hernandez, M. et al.; *Phenolic Compounds - Biological Activity*, **2017**.

[194] Bang, S.-H. et al.; "Metabolism of Rutin and Poncirin by Human Intestinal Microbiota and Cloning of Their Metabolizing α-L-Rhamnosidase from Bifidobacterium Dentium" in *Journal of Microbiology and Biotechnology* 25, no. 1 (**2015**): 18–25.

[195, 196]. Flavanones are known for their anti-inflammatory and anticancer potential[197], forming the basis for developing new therapeutic agents. Specific bacterial species are associated with metabolic steps that produce active flavanone metabolites, aiding in their absorption and utilization by the body (*i.e. Parabacteroides distasonis*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum* for O-deglycosylation, *Eubacterium cellulosolvens* for C-deglycosylation)[198]. Similarly, flavan-3-ols have shown promising anti-cancer effects, particularly in combination with other compounds like procyanidin B2 (an anthocyanin flavonoid), in breast and prostate cancer cell lines[199] and the intake of foods rich in flavan-3-ols has been linked to reduced risk of T2D[200] and cardiovascular disease[201] in case-control studies. Flavan-3-ols also promote the growth of *Bacteroides*, *Bifidobacterium*, *Faecalibacterium* and *Parabacteroides* in *in vitro* studies, suggesting a role in the gut metabolisms of these biomolecules[202]. Anthocyanins demonstrate neuroprotective and anti-

[195] Beekwilder, J. et al.; "Characterization of Rhamnosidases from Lactobacillus Plantarum and Lactobacillus Acidophilus" in *Applied and Environmental Microbiology* 75, no. 11 (**2009**): 3447–3454.

[196] Riva, A. et al.; "Conversion of Rutin, a Prevalent Dietary Flavonol, by the Human Gut Microbiota" in *Frontiers in Microbiology* 11 (**2020**): 585428.

[197] Denaro, M., A. Smeriglio, & D. Trombetta; "Antioxidant and Anti-Inflammatory Activity of Citrus Flavanones Mix and Its Stability after In Vitro Simulated Digestion" in *Antioxidants* 10, no. 2 (**2021**): 140.

[198] Braune, A. & M. Blaut; "Bacterial Species Involved in the Conversion of Dietary Flavonoids in the Human Gut" in *Gut Microbes* 7, no. 3 (**2016**): 216–234.

[199] Núñez-Iglesias, M.J. et al.; "Co-Adjuvant Therapy Efficacy of Catechin and Procyanidin B2 with Docetaxel on Hormone-Related Cancers In Vitro" in *International Journal of Molecular Sciences* 22, no. 13 (**2021**): 7178.

[200] Nguyen, C.T. et al.; "Habitual Tea Drinking Associated with a Lower Risk of Type 2 Diabetes in Vietnamese Adults" in *Asia Pacific Journal of Clinical Nutrition* 27, no. 3 (**2018**): 701–706.

[201] Wang, X. et al.; "Flavonoid Intake and Risk of CVD: A Systematic Review and Meta-Analysis of Prospective Cohort Studies" in *The British Journal of Nutrition* 111, no. 1 (**2014**): 1–11.

[202] Liu, Z. et al.; "Microbial Metabolism of Theaflavin-3,3′-Digallate and Its Gut Microbiota Composition Modulatory Effects" in *Journal of Agricultural and Food Chemistry* 69, no. 1 (**2021**): 232–245.

inflammatory effects[203, 204] and epidemiological and human intervention studies indicate that they may reduce cardiovascular disease risk due to their anti-thrombotic properties[205, 206, 207, 208]. Bacterial fermentation of anthocyanins produces compounds like gallic and syringic acids, which are linked to various health-promoting properties[209]. The main bacterial genera presumably involved have been identified by *in vitro* microbial cultivations and include *Lactobacillus* and *Bifidobacterium*[210], most likely because these genera typically possess β-glucosidases and ring-fission catabolic activities[211]. To conclude, tannins possess significant anticancer properties, leading to the prevention of cardiovascular disease, cancer and osteoporosis. Especially tannic acid has been found to be useful in cancer prophylaxis and

[203] Demeilliers, C. et al.; "Ethanol Drinking, Brain Mitochondrial DNA, Polyunsaturated Fatty Acids and Effects of Dietary Anthocyanins" in *Clinical Nutrition Experimental* 12 (**2017**): 11–19.

[204] Magni, G. et al.; "Purple Corn Extract as Anti-Allodynic Treatment for Trigeminal Pain: Role of Microglia" in *Frontiers in Cellular Neuroscience* 12 (**2018**).

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[206] García-Conesa, M.-T. et al.; "Meta-Analysis of the Effects of Foods and Derived Products Containing Ellagitannins and Anthocyanins on Cardiometabolic Biomarkers: Analysis of Factors Influencing Variability of the Individual Responses" in *International Journal of Molecular Sciences* 19, no. 3 (**2018**): 694.

[207] Thompson, K. et al.; "Anthocyanin Supplementation in Alleviating Thrombogenesis in Overweight and Obese Population: A Randomized, Double-Blind, Placebo-Controlled Study" in *Journal of Functional Foods* 32 (**2017**): 131–138.

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[211] Tian, L. et al.; "Metabolism of Anthocyanins and Consequent Effects on the Gut Microbiota" in *Critical Reviews in Food Science and Nutrition* 59, no. 6 (**2019**): 982–991.

used as an adjuvant in cancer therapy in human studies[212, 213]. Gut colonizers such as members of *Akkermansia* genus, *Lachnospiraceae* and *Ruminococcaceae* families[214] play an essential role in metabolizing tannins into bioavailable metabolites, like catechins and phenolic acids, which can exert systemic health benefits[215, 216].

In contrast, probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" by the Food and Agriculture Organization of the United Nations/World Health Organization[217,218]. Probiotic strains should have certain qualities like the ability to survive the severe circumstances found in the gastrointestinal tract. Certainly, native (to humans) bacterial strains have these characteristics, and undoubtedly they confer stability and long-lasting beneficial effects on human digestive tract[219]. Probiotic organisms that are frequently utilized include yeasts like *Saccharomyces boulardii* and lactic acid bacteria like *Lactobacillus* (e.g., *L. paracasei*[220], *L.*

[212] A. Youness, R. et al.; "Recent Advances in Tannic Acid (Gallotannin) Anticancer Activities and Drug Delivery Systems for Efficacy Improvement; A Comprehensive Review" in *Molecules* 26, no. 5 (**2021**): 1486.

[213] Baer-Dubowska, W. et al.; "Tannic Acid: Specific Form of Tannins in Cancer Chemoprevention and Therapy-Old and New Applications" in *Current Pharmacology Reports* 6, no. 2 (**2020**): 28–37.

[214] Molino, S. et al.; "Enrichment of Food With Tannin Extracts Promotes Healthy Changes in the Human Gut Microbiota" in *Frontiers in Microbiology* 12 (**2021**): 625782.

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[216] Sallam, I.E. et al.; "Effect of Gut Microbiota Biotransformation on Dietary Tannins and Human Health Implications" in *Microorganisms* 9, no. 5 (**2021**): 965.

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rhamnosus[221, 222] *L. reuteri*[223])[224], *Bifidobacterium* (e.g., *B. breve*[225], *B. longum*[226] and *B. infantis*[227]), *Streptococcus (*e.g., *S. thermophilus*[228]), and *Enterococcus* strain (e.g., *E. faecium*[229,230])[231]. Also, their selection must be rationally guided by the knowledge of which are the keystone species associated with GM recovery, which most likely do not include lactobacilli and bifidobacteria (generally subdominant taxa if not absent in adult GMs). For instance, a healthy gut repopulation may be favored by some *Bacteroides* species, as discussed before, such as *B. fragilis* and *B. thetaiotaomicron*, which have demonstrated

[221] Basu, S. et al.; "Efficacy of High-Dose Lactobacillus Rhamnosus GG in Controlling Acute Watery Diarrhea in Indian Children: A Randomized Controlled Trial" in *Journal of Clinical Gastroenterology* 43, no. 3 (**2009**): 208.

[222] Zocco, M.A. et al.; "Efficacy of Lactobacillus GG in Maintaining Remission of Ulcerative Colitis" in *Alimentary Pharmacology & Therapeutics* 23, no. 11 (**2006**): 1567–1574.

[223] Gutierrez-Castrellon, P. et al.; "Diarrhea in Preschool Children and Lactobacillus Reuteri: A Randomized Controlled Trial" in *Pediatrics* 133, no. 4 (**2014**): e904–e909.

[224] Jeong, J.-J. et al.; "The Lactobacillus as a Probiotic: Focusing on Liver Diseases" in *Microorganisms* 10, no. 2 (**2022**): 288.

[225] Muñoz-Quezada, S. et al.; "Isolation, Identification and Characterisation of Three Novel Probiotic Strains (Lactobacillus Paracasei CNCM I-4034, Bifidobacterium Breve CNCM I-4035 and Lactobacillus Rhamnosus CNCM I-4036) from the Faeces of Exclusively Breast-Fed Infants" in *British Journal of Nutrition* 109, no. S2 (**2013**): S51–S62.

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[230] Vimont, A. et al.; "Bacteriocin-Producing Enterococcus Faecium LCW 44: A High Potential Probiotic Candidate from Raw Camel Milk" in *Frontiers in Microbiology* 8 (**2017**): 865.

[231] Dixit, K. et al.; "Restoration of Dysbiotic Human Gut Microbiome for Homeostasis" in *Life Sciences* 278 (**2021**): 119622.

promising therapeutic benefits on immunological dysregulation and intestinal epithelial barrier impairment[232, 233, 234, 235, 236].

Furthermore, in 2017 the concept of "next-generation probiotics" (NGPs) was introduced. This concept refers to live bacterial strains identified through comparative microbiota analyses that, when administered in appropriate amounts, provide specific health benefits[237]. In contrast to traditional probiotics, which generally exhibit limited therapeutic effects, NGPs are increasingly regarded as innovative tools for disease prevention and treatment. Certain microorganisms, including *A. muciniphila*, *Eubacterium hallii*, and *F. prausnitzii*, have been identified as promising NGP candidates due to their potential in preventing and treating dysbiosis-associated diseases[238]. However, their application remains constrained by high sensitivity to oxygen exposure and the adverse gastric conditions encountered during ingestion[239]. Other NGPs including *B. thetaiotaomicron* and *Christensenella minuta* have been highlighted for their potential in preventing and managing

[232] Chng et al., "Metagenome-Wide Association Analysis Identifies Microbial Determinants of Post-Antibiotic Ecological Recovery in the Gut."

[233] Deng, H. et al.; "A Novel Strain of Bacteroides Fragilis Enhances Phagocytosis and Polarises M1 Macrophages" in *Scientific Reports* 6, no. 1 (**2016**): 29401.

[234] Liu, Q. et al.; "Surface Components and Metabolites of Probiotics for Regulation of Intestinal Epithelial Barrier" in *Microbial Cell Factories* 19, no. 1 (**2020**): 23.

[235] Sofi, M.H. et al.; "A Single Strain of Bacteroides Fragilis Protects Gut Integrity and Reduces GVHD" in *JCI Insight* 6, no. 3 (**2021**).

[236] Zhou, Q. et al.; "Bacteroides Fragilis Strain ZY-312 Promotes Intestinal Barrier Integrity via Upregulating the STAT3 Pathway in a Radiation-Induced Intestinal Injury Mouse Model" in *Frontiers in Nutrition* 9 (**2022**).

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[238] Almeida, D. et al.; "Evolving Trends in Next-Generation Probiotics: A 5W1H Perspective" in *Critical Reviews in Food Science and Nutrition* 60, no. 11 (**2020**): 1783–1796.

[239] Torp, A.M. et al.; "Optimizing Oral Delivery of next Generation Probiotics" in *Trends in Food Science & Technology* 119 (**2022**): 101–109.

conditions such as colitis, obesity, liver disorders, and diabetes[240]. By contrast, conventional probiotics lack the specificity required to effectively target particular diseases[241].

Moreover, live biotherapeutic products (LBPs) have recently attracted considerable attention within the scientific community. These products are defined as live microorganisms specifically designed to treat, cure, or prevent diseases or conditions in humans[242]. LBPs are distinguished from probiotic supplements by their labeling claims, as probiotics are typically regulated as dietary supplements and therefore cannot include claims related to disease treatment or prevention[243, 244]. However, certain probiotics may meet the criteria for classification as LBPs if they demonstrate therapeutic efficacy and are developed accordingly[245]. LBPs may also include genetically modified organisms (recombinant LBPs), which are engineered through the addition, deletion, or modification of genetic material within the microbial chassis[246, 247]. Examples of engineered bacterial therapeutics include *Lactobacillus casei*[248] and *Lactococcus lactis*. Notably, *L. lactis* is recognized for its ability

[240] Zhai, Q. et al.; "A next Generation Probiotic, Akkermansia Muciniphila" in *Critical Reviews in Food Science and Nutrition* 59, no. 19 (**2019**): 3227–3236.

[241] Jan, T. et al.; "Next Generation Probiotics for Human Health: An Emerging Perspective" in *Heliyon* 10, no. 16 (**2024**): e35980.

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[243] Ibid.

[244] Ross, J.J. et al.; "Considerations in the Development of Live Biotherapeutic Products for Clinical Use" in *Current Issues in Molecular Biology* 10, no. 1–2 (**2008**): 13–16.

[245] Food and Drug Administration (FDA), "Early Clinical Trials With Live Biotherapeutic Products."

[246] Ibid.

[247] Charbonneau, M.R. et al.; "Developing a New Class of Engineered Live Bacterial Therapeutics to Treat Human Diseases" in *Nature Communications* 11, no. 1 (**2020**): 1738.

[248] Tan, Y. et al.; "Engineered Live Biotherapeutics: Progress and Challenges" in *Biotechnology Journal* 15, no. 10 (**2020**): 2000155.

to secrete an anti-enterococcal peptide that inhibits the growth of enterococci, demonstrating antimicrobial activity against both *E. faecalis* and multidrug-resistant strains of *E. faecium*[249]. Lastly, one of the most effective GM restoring intervention is the Fecal Microbiota Transplantation (FMT), defined as the transfer of minimally altered feces from healthy donors into a recipient's gut with the intention of curing a disease linked to changes in the recipient's gut microbiota[250]. FMT has been studied for a variety of conditions, both communicable and non-communicable diseases, with varying results. FMT was originally looked at as a possible treatment for recurrent *Clostridioides difficile* infection (CDI), where it has consistently demonstrated a success rate of around 90%[251,252, 253] thanks to the early successful reports on pseudo-membranous colitis[254]. The increased demand for a recurrent CDI cure in clinical practice has led to a progressive evolution of FMT throughout time. The FMT modernization starts with the use of frozen stools over fresh feces[255]. With this change, managing a large number of patients is possible without running the risk of a stool shortage. Additionally, sample freezing has strengthened safety protocols by allowing the

[249] Navalkele, B.D. & T. Chopra; "Clinical Application of Live Biotherapeutic Products in Infectious Diseases" in *Frontiers in Microbiomes* 3 (**2024**).

[250] Porcari, S. et al.; "Key Determinants of Success in Fecal Microbiota Transplantation: From Microbiome to Clinic" in *Cell Host & Microbe* 31, no. 5 (**2023**): 712–733.

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[253] Quraishi, M.N. et al.; "Systematic Review with Meta-Analysis: The Efficacy of Faecal Microbiota Transplantation for the Treatment of Recurrent and Refractory Clostridium Difficile Infection" in *Alimentary Pharmacology & Therapeutics* 46, no. 5 (**2017**): 479–493.

[254] Eiseman, B. et al.; "Fecal Enema as an Adjunct in the Treatment of Pseudomembranous Enterocolitis" in *Surgery* 44, no. 5 (**1958**): 854–859.

[255] Lee, C.H. et al.; "Frozen vs Fresh Fecal Microbiota Transplantation and Clinical Resolution of Diarrhea in Patients With Recurrent Clostridium Difficile Infection: A Randomized Clinical Trial" in *JAMA* 315, no. 2 (**2016**): 142–149.

quarantine[256, 257] of the conserved feces, and increased donor screening through molecular testing[258], which can avoid the transmission of multi-drug resistant bacteria, identified from the US Food and Drug Administration (FDA) as a potential risk of FMT[259]. The success rate of FTM outcomes depends on different factors, both donor-related and recipient-related, resumed in **Figure 3**. In fact, one of the most challenging step of FMT is the donor screening, defined by the clinical practice FMT guidelines[260] a necessary practice to avoid the transmission of pathogens and other infectious agents. This practice has become urgent after the transmission of a drug-resistant *E. coli* strain to two immunocompromised recipients, with a fatal outcome in one case after the development of bacteremia[261].

[256] Kuijper, E.J. et al.; "A Necessary Discussion after Transmission of Multidrug-Resistant Organisms through Faecal Microbiota Transplantations" in *The Lancet Infectious Diseases* 19, no. 11 (**2019**): 1161–1162.

[257] Vendrik, K.E.W. et al.; "Periodic Screening of Donor Faeces with a Quarantine Period to Prevent Transmission of Multidrug-Resistant Organisms during Faecal Microbiota Transplantation: A Retrospective Cohort Study" in *The Lancet Infectious Diseases* 21, no. 5 (**2021**): 711–721.

[258] Ianiro, G. et al.; "Donor Program for Fecal Microbiota Transplantation: A 3-Year Experience of a Large-Volume Italian Stool Bank" in *Digestive and Liver Disease* 53, no. 11 (**2021**): 1428–1432.

[259] Center for Biologics Evaluation and Research; "Important Safety Alert Regarding Use of Fecal Microbiota for Transplantation and Risk of Serious Adverse Reactions Due to Transmission of Multi-Drug Resistant Organisms" in *FDA* (**2020**).

[260] Cammarota, G. et al.; "European Consensus Conference on Faecal Microbiota Transplantation in Clinical Practice" in *Gut* 66, no. 4 (**2017**): 569–580.

[261] DeFilipp et al.; "Drug-Resistant E. Coli Bacteremia Transmitted by Fecal Microbiota Transplant" in *New England Journal of Medicine* 381, no. 21 (**2019**): 2043–2050.

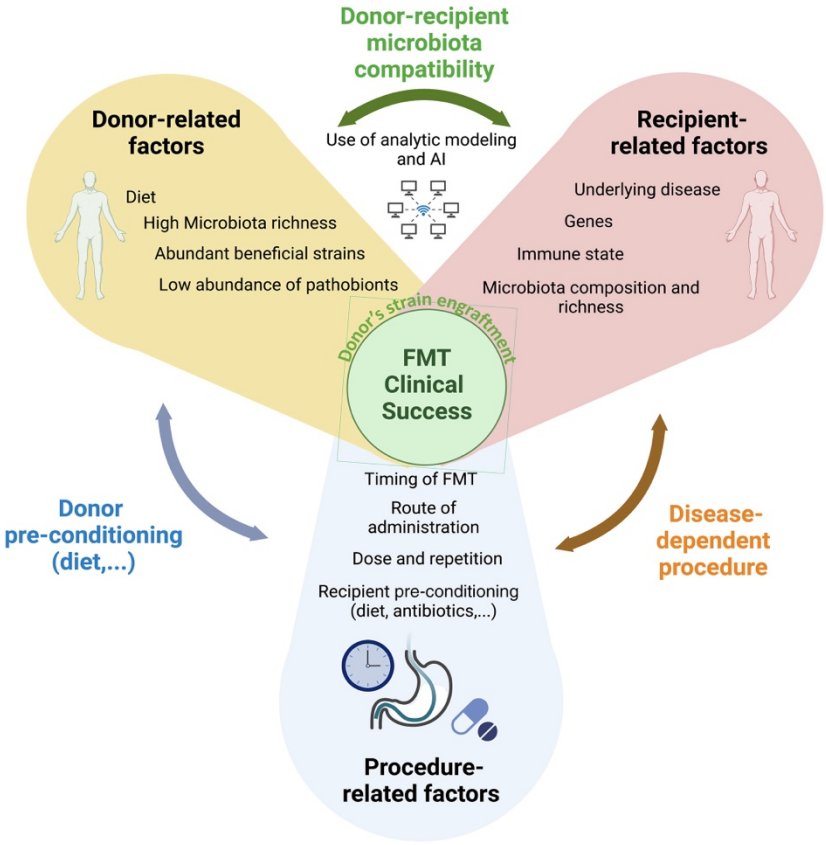

Figure 3. Factors that influence FMT success[262].

This fact encouraged FDA to mandate for multi-drug resistant organisms screening in FMT study protocols, a step already recommended by international guidelines and followed by major stool banks[263]. Additionally, in response to the COVID-19 pandemic[264, 265] FDA also introduced guidelines to prevent the transmission of SARS-CoV-2 through FMT, which have been effective[266]. Notably, a recent work indicated that serious adverse events related to

[262] Porcari et al., "Key Determinants of Success in Fecal Microbiota Transplantation."

[263] Kuijper et al., "A Necessary Discussion after Transmission of Multidrug-Resistant Organisms through Faecal Microbiota Transplantations."

[264] Food and Drug Administration (FDA); "Safety Alert Regarding Use of Fecal Microbiota for Transplantation and Additional Safety Protections Pertaining to SARS-CoV-2 and COVID-19" in *FDA* (**2020**).

[265] Ianiro, G. et al.; "Reorganisation of Faecal Microbiota Transplant Services during the COVID-19 Pandemic" in *Gut* 69, no. 9 (**2020**): 1555–1563.

[266] Ianiro, G. et al.; "Maintaining Standard Volumes, Efficacy and Safety, of Fecal Microbiota Transplantation for C. Difficile Infection during the COVID-19 Pandemic: A Prospective Cohort Study" in *Digestive and Liver Disease* 52, no. 12 (**2020**): 1390–1395.

FMT occurred predominantly in patients with compromised mucosal barriers[267]. As the integrity of the gut barrier is crucial in many gastrointestinal and extraintestinal disorders[268], it is essential to assess gut injuries when selecting FMT candidates[269].

1.4 How to study the GM

For the analysis of the GM, exploring the correlations between the microbial ecosystem and diseases, as well as developing microbial consortia and NGPs, remains a significant challenge. Achieving this requires the use of well-defined and suitable cultural conditions, since anaerobes constitute the predominant members of the healthy human GM, though only a few are associated with human infections, such as *C. difficile*, which will be examined in detail in the following chapter. Initial efforts to culture and study members of the GM date back to the 1970s[270]. These early studies investigated the impact of diet on the gut microbial ecosystem composition. During this period, members of the *Enterobacteriaceae* and *Veillonellaceae* families were found to be prevalent among approximately 400 species cultured from the human GM[271]. It was generally believed that the GM was dominated by non-spore-forming, anaerobic, rod-shaped bacteria. Notably, many of the dominant species

[267] Marcella, C. et al.; "Systematic Review: The Global Incidence of Faecal Microbiota Transplantation-Related Adverse Events from 2000 to 2020" in *Alimentary Pharmacology & Therapeutics* 53, no. 1 (**2021**): 33–42.

[268] Di Tommaso, N., A. Gasbarrini, & F.R. Ponziani; "Intestinal Barrier in Human Health and Disease" in *International Journal of Environmental Research and Public Health* 18, no. 23 (**2021**): 12836.

[269] Porcari et al., "Key Determinants of Success in Fecal Microbiota Transplantation."

[270] Moore, W.E. & L.V. Holdeman; "Human Fecal Flora: The Normal Flora of 20 Japanese-Hawaiians" in *Applied Microbiology* 27, no. 5 (**1974**): 961–979.

[271] Rajilić-Stojanović, M., H. Smidt, & W.M. de Vos; "Diversity of the Human Gastrointestinal Tract Microbiota Revisited" in *Environmental Microbiology* 9, no. 9 (**2007**): 2125–2136.

identified in 1995[272] corresponded to species later detected through metagenomic techniques[273].

Cultivating obligate anaerobes necessitates specific microbiological equipment to maintain an oxygen-free environment and specialized culture media enriched with various supplements, such as carbon sources, macro-elements, trace metals, and growth factors. Historically, culture media were supplemented with animal tissues (e.g., brain, meat, or heart tissue) to create the earliest forms of nonselective media in the 1890s. Selective culture conditions have been developed over time, utilizing substances like antibiotics, bile salts, and dyes (such as crystal violet), along with physical processes like heat shock. For instance, to culture *Clostridium* and *Fusobacterium* species, egg-based media were introduced. In 1968, Hungate revolutionized the cultivation of highly oxygen-sensitive microorganisms, such as sulfate-reducing bacteria and methanogenic archaea, through the use of roll tube techniques that substituted atmospheric oxygen with inert gases like nitrogen (N_2), carbon dioxide (CO_2), or hydrogen (H_2). Despite its innovation, this method is resource-intensive, requires advanced expertise, and is primarily limited to specialized laboratories[274]. Modern approaches to cultivating bacteria under anaerobic conditions have evolved, including the development of anaerobic jars, GasPak systems, and anaerobic chambers[275]. Despite typically requiring strict anaerobic conditions, some bacterial species, such as *Ruminococcus gnavus*, *Prevotella nigrescens*, *Fusobacterium necrophorum*, *Finegoldia magna*, *Solobacterium moorei*, and *Atopobium vaginae*, have been successfully cultured in

[272] Moore, W.E. & L.H. Moore; "Intestinal Floras of Populations That Have a High Risk of Colon Cancer" in *Applied and Environmental Microbiology* 61, no. 9 (**1995**): 3202–3207.

[273] Walker et al., "Phylogeny, Culturing, and Metagenomics of the Human Gut Microbiota."

[274] Nottingham, P.M. & R.E. Hungate; "Isolation of Methanogenic Bacteria from Feces of Man" in *Journal of Bacteriology* 96, no. 6 (**1968**): 2178.

[275] Lagier, J.-C. et al.; "Current and Past Strategies for Bacterial Culture in Clinical Microbiology" in *Clinical Microbiology Reviews* 28, no. 1 (**2015**): 208–236.

aerobic environments[276]. In another elegant study, the addition of uric acid to culture media enabled the aerobic cultivation of 276 bacterial species, 82 of which were strict anaerobes[277]. Another study conducted in 2016 further demonstrated the isolation of highly oxygen-sensitive methanogenic archaea, utilizing *B. thetaiotaomicron* as a hydrogen donor in a specially designed double-chamber flask[278,279].

In recent years, culturomics has emerged as a valuable approach in studying the human microbiota[280, 281]. Culturomics involves the application of high-throughput culture conditions to study the human microbiota, using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) or 16S rRNA amplification and sequencing for the identification of growing colonies, including numerous previously uncharacterized species[282]. One of the first study using this technique was the one of Lagier et al., which assessed 212 distinct culture conditions, identifying 18 that successfully promoted the growth of the most phylogenetically diverse organisms[283]. Culturomics frequently integrates MALDI-TOF for the identification of

[276] La Scola, B. et al.; "Aerobic Culture of Anaerobic Bacteria Using Antioxidants: A Preliminary Report" in *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology* 33, no. 10 (**2014**): 1781–1783.

[277] Dione, N. et al.; "A Quasi-Universal Medium to Break the Aerobic/Anaerobic Bacterial Culture Dichotomy in Clinical Microbiology" in *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 22, no. 1 (**2016**): 53–58.

[278] Khelaifia, S. et al.; "Aerobic Culture of Methanogenic Archaea without an External Source of Hydrogen" in *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology* 35, no. 6 (**2016**): 985–991.

[279] Lagier, J.-C. et al.; "Culturing the Human Microbiota and Culturomics" in *Nature Reviews. Microbiology* 16 (**2018**): 540–550.

[280] Lagier, J.-C. et al.; "Microbial Culturomics: Paradigm Shift in the Human Gut Microbiome Study" in *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 18, no. 12 (**2012**): 1185–1193.

[281] Lagier, J.-C. et al.; "Culture of Previously Uncultured Members of the Human Gut Microbiota by Culturomics" in *Nature Microbiology* 1, no. 12 (**2016**): 1–8.

[282] Lagier, J.-C. et al.; "The Rebirth of Culture in Microbiology through the Example of Culturomics to Study Human Gut Microbiota" in *Clinical Microbiology Reviews* 28, no. 1 (**2015**): 237–264.

[283] Lagier et al., "Culture of Previously Uncultured Members of the Human Gut Microbiota by Culturomics."

bacterial colonies, supplemented by 16S rRNA gene sequencing when databases lack reference spectra[284], to increase the bacterial repertoire. This approach has notably advanced the characterization of the human GM, facilitating the isolation of numerous previously unreported bacterial species and strains that had not been isolated from humans before[285, 286, 287]. Using living microorganisms, culturomics enables in-depth study and accurate classification of newly discovered species[288]. Consequently, culturomics stands out as a valuable complement to metagenomics, given its capacity to continually expand genomic databases[289, 290]. In 2020, Diakite et al. optimized culturomics by reducing the required culture conditions to 16 highly efficient ones, capable of recovering 98% of the previously isolated species from human gut samples[291]. Notably, other research groups have contributed to enhancing culturomics by refining both culture conditions and isolation techniques[292, 293]. For instance, Watterson et al. developed an innovative droplet-based cultivation method, which enables the isolation of extensive bacterial populations within millions of picoliter-sized droplets using a microfluidic platform[294]. Similarly, Poyet et al.

[284] Ibid.

[285] Bilen, M. et al.; "The Contribution of Culturomics to the Repertoire of Isolated Human Bacterial and Archaeal Species" in *Microbiome* 6, no. 1 (**2018**): 94.

[286] Lagier et al., "Culture of Previously Uncultured Members of the Human Gut Microbiota by Culturomics"; Lagier et al., "Culturing the Human Microbiota and Culturomics."

[287] Lagier et al., "Culturing the Human Microbiota and Culturomics."

[288] Lagier et al., "Culture of Previously Uncultured Members of the Human Gut Microbiota by Culturomics."

[289] Ibid.

[290] Lagier et al., "Culturing the Human Microbiota and Culturomics."

[291] Diakite, A. et al.; "Optimization and Standardization of the Culturomics Technique for Human Microbiome Exploration" in *Scientific Reports* 10, no. 1 (**2020**): 9674.

[292] Chang, Y. et al.; "Optimization of Culturomics Strategy in Human Fecal Samples" in *Frontiers in Microbiology* 10 (**2019**).

[293] Lau, J.T. et al.; "Capturing the Diversity of the Human Gut Microbiota through Culture-Enriched Molecular Profiling" in *Genome Medicine* 8, no. 1 (**2016**): 72.

[294] Watterson, W.J. et al.; "Droplet-Based High-Throughput Cultivation for Accurate Screening of Antibiotic Resistant Gut Microbes" ed. Cooper et al. in *eLife* 9 (**2020**): e56998.

achieved the isolation of a substantial variety of organisms from human fecal samples by employing 19 different culture conditions[295, 296].

Regarding NGS-based microbial genotyping, two principal methodologies dominate: gene amplicon sequencing (such as 16S rRNA analysis) and shotgun metagenomics (which are described in **Figure 4**). Over the last 25 years, gene amplicon sequencing has been the primary tool for studying the phylogeny and taxonomy of complex microbiomes that were previously difficult to analyze[297]. For bacteria, archaea, fungi, and mycobacteria, specific marker genes are targeted, with the 16S rRNA gene used as the gold standard for bacterial typing. This gene, encoding the small 30S subunit of the 70S ribosomal complex in bacteria and archaea, is highly conserved and distinct from its eukaryotic homologue, the 18S rRNA gene (encoding the small eukaryotic ribosomal subunit (40S)), making it a molecular clock for studying evolutionary changes and transitions[298, 299]. Its relatively short sequence (~1500 bp) and highly conserved primer binding sites, interspersed with nine variable regions (V1-

[295] Matar, G. & M. Bilen; "Culturomics, a Potential Approach Paving the Way toward Bacteriotherapy" in *Current Opinion in Microbiology* 69 (**2022**): 102194.

[296] Poyet, M. et al.; "A Library of Human Gut Bacterial Isolates Paired with Longitudinal Multiomics Data Enables Mechanistic Microbiome Research" in *Nature Medicine* 25, no. 9 (**2019**): 1442–1452.

[297] Weisburg, W.G. et al.; "16S Ribosomal DNA Amplification for Phylogenetic Study" in *Journal of Bacteriology* 173, no. 2 (**1991**): 697–703.

[298] Baker, G.C., J.J. Smith, & D.A. Cowan; "Review and Re-Analysis of Domain-Specific 16S Primers" in *Journal of Microbiological Methods* 55, no. 3 (**2003**): 541–555.

[299] Pel, J. et al.; "Rapid and Highly-Specific Generation of Targeted DNA Sequencing Libraries Enabled by Linking Capture Probes with Universal Primers" in *PloS One* 13, no. 12 (**2018**): e0208283.

V9), facilitate large-scale sequencing. Typically, the V3-V4, V4, or V5-V6 hypervariable regions are sequenced for profiling microbial communities[300, 301].

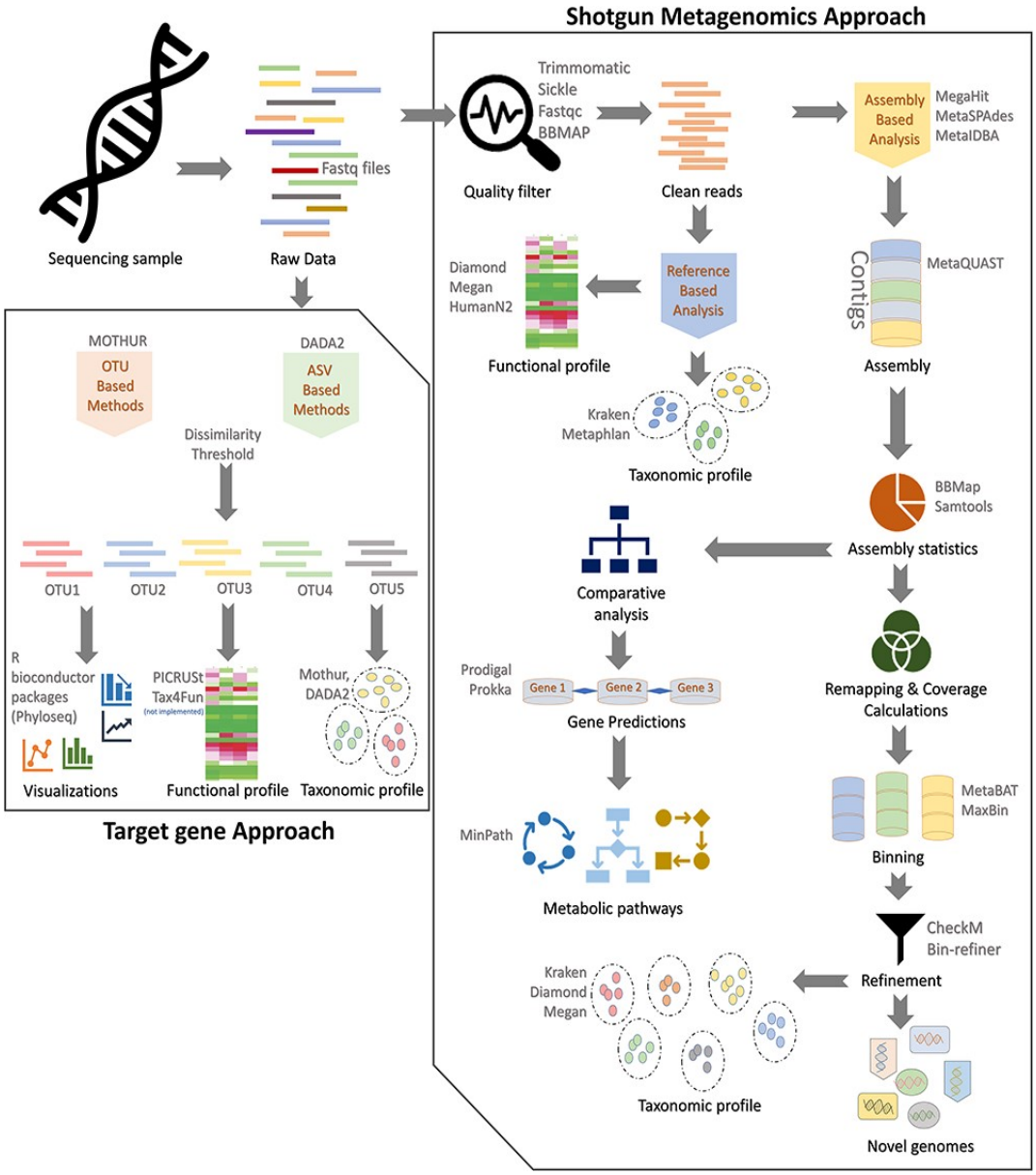

Figure 4. Best-practice protocol for the acquisition and analysis of targeted amplicon and shotgun metagenomics data from sequencing to functional annotation[302].

[300] Janda, J.M. & S.L. Abbott; "16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls" in *Journal of Clinical Microbiology* 45, no. 9 (**2007**): 2761–2764.

[301] Woo, P.C.Y. et al.; "Then and Now: Use of 16S rDNA Gene Sequencing for Bacterial Identification and Discovery of Novel Bacteria in Clinical Microbiology Laboratories" in *Clinical Microbiology and Infection* 14, no. 10 (**2008**): 908–934.

[302] Bharti, R. & D.G. Grimm; "Current Challenges and Best-Practice Protocols for Microbiome Analysis" in *Briefings in Bioinformatics* 22, no. 1 (**2021**): 178–193.

Over the last 15 years, numerous bioinformatics tools have been developed to facilitate the analysis of 16S rRNA sequencing data, with most frameworks consisting of three main steps: data preprocessing and quality control, taxonomic assignment, and community characterization. Quality control serves as the initial stage in this pipeline, encompassing processes such as quality assessment, adapter removal, filtering, and trimming. These steps help eliminate artifacts, low-quality reads, and contaminant sequences, which may stem from sample impurities or inadequate sample preparation techniques[303]. Currently, two primary strategies are employed for taxonomic assignment: operational taxonomic unit (OTU)-based analysis and amplicon sequence variant (ASV)-based analysis. OTU-based analysis groups sequences based on similarity, assigning sequences to the same OTU if they meet a preset similarity threshold, typically 97%[304]. With this approach, sequences are first clustered into OTUs, followed by taxonomic assignment. By contrast, ASV-based analysis applies a denoising technique that infers the biological sequences present in the sample prior to the introduction of errors made from amplification and sequencing. This method enables the resolution of sequences differing by even a single nucleotide[305], thus providing finer taxonomic resolution than OTU-based analysis. Widely used tools that support comprehensive 16S rRNA analysis from raw sequencing data include Quantitative

[303] Zhou, Q., X. Su, & K. Ning; "Assessment of Quality Control Approaches for Metagenomic Data Analysis" in *Scientific Reports* 4, no. 1 (**2014**): 6957.

[304] Stackebrandt, E. & B.M. Goebel; "Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology" in *International Journal of Systematic and Evolutionary Microbiology* 44, no. 4 (**1994**): 846–849.

[305] Callahan, B.J., P.J. McMurdie, & S.P. Holmes; "Exact Sequence Variants Should Replace Operational Taxonomic Units in Marker-Gene Data Analysis" in *The ISME Journal* 11, no. 12 (**2017**): 2639–2643.

Insights Into Microbial Ecology (QIIME)[306, 307] and DADA2[308]. Briefly, QIIME 1, and its successor, QIIME 2, are open-source platforms designed for microbial community analysis and visualization. The standard 16S workflow in QIIME 1 includes demultiplexing and quality filtering to assign reads to samples and exclude low-quality sequences, chimera detection using ChimeraSlayer or USEARCH 6.1 to remove chimeric sequences, OTU clustering and taxonomic assignment based on sequence similarity and community analysis for assessing community composition, phylogenic tree, and α- and β-diversity through OTU tables[309]. Both QIIME versions also provide robust visualization tools, enabling users to create principal coordinate analysis (PCoA) plots, α-diversity rarefaction curves, and taxonomic composition bar plots. DADA2 is an analysis package based on amplicon sequence variants (ASVs), employing a model-based algorithm that corrects amplicon errors without relying on OTU clustering[310]. Its workflow includes quality control to filter and trim low-quality reads, sample inference to construct ASV tables, removal of chimeric ASVs, and taxonomic assignment to create taxonomy tables. DADA2's approach allows for the detection of fine-scale sequence variations, yielding greater accuracy than OTU-based methods[311].

While 16S rRNA sequencing focuses on a specific marker gene rather than entire genomes, metagenomics employs a culture-independent, genome-wide shotgun sequencing

[306] Bolyen, E. et al.; "Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2" in *Nature Biotechnology* 37, no. 8 (**2019**): 852–857; Caporaso, J.G. et al.; "QIIME Allows Analysis of High-Throughput Community Sequencing Data" in *Nature Methods* 7, no. 5 (**2010**): 335–336.

[307] Caporaso et al., "QIIME Allows Analysis of High-Throughput Community Sequencing Data."

[308] Callahan, B.J. et al.; "DADA2: High-Resolution Sample Inference from Illumina Amplicon Data" in *Nature Methods* 13, no. 7 (**2016**): 581–583.

[309] Caporaso et al., "QIIME Allows Analysis of High-Throughput Community Sequencing Data."

[310] Callahan et al., "DADA2."

[311] Gao, B. et al.; "An Introduction to Next Generation Sequencing Bioinformatic Analysis in Gut Microbiome Studies" in *Biomolecules* 11, no. 4 (**2021**): 530.

approach to directly analyze samples[312, 313], sequencing the sample's entire metagenome without a specific primer, which lessens biases from primer choices[314]. This method identifies all microorganisms within complex samples, including both culturable and unculturable species. Unlike the single-gene approach of 16S rRNA sequencing, metagenomics captures the full genetic composition of microbial communities, thereby providing greater taxonomic detail and functional insights[315, 316], although it is more expensive and time-consuming than marker gene amplification. It also facilitates the detection of viruses, which are often missed by single-gene approaches due to their genetic diversity[317]. Gene composition analysis enables the prediction of potential functional pathways. Shotgun metagenomic sequencing has been applied to investigate functional alterations in the GM associated with various diseases, including inflammatory bowel disease[318], irritable bowel syndrome[319], Crohn's disease[320, 321], non-alcoholic fatty liver

[312] Escobar-Zepeda, A., A. Vera-Ponce de León, & A. Sanchez-Flores; "The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics" in *Frontiers in Genetics* 6 (**2015**): 348.

[313] Gilbert, J.A. & C.L. Dupont; "Microbial Metagenomics: Beyond the Genome" in *Annual Review of Marine Science* 3 (**2011**): 347–371.

[314] Gao et al., "An Introduction to Next Generation Sequencing Bioinformatic Analysis in Gut Microbiome Studies."

[315] Quince, C. et al.; "Shotgun Metagenomics, from Sampling to Analysis" in *Nature Biotechnology* 35, no. 9 (**2017**): 833–844.

[316] Riesenfeld, C.S., P.D. Schloss, & J. Handelsman; "Metagenomics: Genomic Analysis of Microbial Communities" in *Annual Review of Genetics* 38 (**2004**): 525–552.

[317] Kristensen, D.M. et al.; "New Dimensions of the Virus World Discovered through Metagenomics" in *Trends in Microbiology* 18, no. 1 (**2010**): 11–19.

[318] Franzosa, E.A. et al.; "Gut Microbiome Structure and Metabolic Activity in Inflammatory Bowel Disease" in *Nature Microbiology* 4, no. 2 (**2019**): 293–305.

[319] Vich Vila, A. et al.; "Gut Microbiota Composition and Functional Changes in Inflammatory Bowel Disease and Irritable Bowel Syndrome" in *Science Translational Medicine* 10, no. 472 (**2018**): eaap8914.

[320] Lewis, J.D. et al.; "Inflammation, Antibiotics, and Diet as Environmental Stressors of the Gut Microbiome in Pediatric Crohn's Disease" in *Cell Host & Microbe* 18, no. 4 (**2015**): 489–500.

[321] Ni, J. et al.; "A Role for Bacterial Urease in Gut Dysbiosis and Crohn's Disease" in *Science Translational Medicine* 9, no. 416 (**2017**): eaah6888.

disease[322, 323], Parkinson's disease[324] and melanoma[325], just to cite few. Modern NGS platforms, such as Illumina/Solexa and previously 454/Roche, have largely replaced classical Sanger sequencing for metagenomic shotgun analysis, expanding the capability to study microbiomes across diverse environments[326, 327]. The shotgun metagenomic sequencing workflow includes sample collection and storage, nucleic acid extraction, metagenomic library preparation, quality control, and data analysis. Quality control serves as the initial stage of the pipeline and employs tools like Trimmomatic[328], Ktrim[329], Cutadapt[330] and MultiQC[331] to ensure high-quality reads. These processed reads can then be analyzed through either mapping to reference genomes or by using assembly tools. Consequently, shotgun metagenomic sequencing is typically divided into two approaches:

[322] Loomba, R. et al.; "Gut Microbiome-Based Metagenomic Signature for Non-Invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease" in *Cell Metabolism* 25, no. 5 (**2017**): 1054-1062.e5.

[323] Schwimmer, J.B. et al.; "Microbiome Signatures Associated With Steatohepatitis and Moderate to Severe Fibrosis in Children With Nonalcoholic Fatty Liver Disease" in *Gastroenterology* 157, no. 4 (**2019**): 1109–1122.

[324] Bedarf, J.R. et al.; "Functional Implications of Microbial and Viral Gut Metagenome Changes in Early Stage L-DOPA-Naïve Parkinson's Disease Patients" in *Genome Medicine* 9, no. 1 (**2017**): 39.

[325] Frankel, A.E. et al.; "Metagenomic Shotgun Sequencing and Unbiased Metabolomic Profiling Identify Specific Human Gut Microbiota and Metabolites Associated with Immune Checkpoint Therapy Efficacy in Melanoma Patients" in *Neoplasia* 19, no. 10 (**2017**): 848–855.

[326] Bharti and Grimm, "Current Challenges and Best-Practice Protocols for Microbiome Analysis."

[327] Luo, C. et al.; "Direct Comparisons of Illumina vs. Roche 454 Sequencing Technologies on the Same Microbial Community DNA Sample" in *PLOS ONE* 7, no. 2 (**2012**): e30087.

[328] Bolger, A.M., M. Lohse, & B. Usadel; "Trimmomatic: A Flexible Trimmer for Illumina Sequence Data" in *Bioinformatics* 30, no. 15 (**2014**): 2114–2120.

[329] Sun, K.; "Ktrim: An Extra-Fast and Accurate Adapter- and Quality-Trimmer for Sequencing Data" in *Bioinformatics* 36, no. 11 (**2020**): 3561–3562.

[330] Martin, M.; "Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads" in *EMBnet.journal* 17, no. 1 (**2011**): 10–12.

[331] Ewels, P. et al.; "MultiQC: Summarize Analysis Results for Multiple Tools and Samples in a Single Report" in *Bioinformatics* 32, no. 19 (**2016**): 3047–3048.

read-mapping and assembly-based. Combining both approaches is often recommended to achieve the most accurate results[332, 333].

Shortly, the read-mapping approach in shotgun metagenomics classifies sequencing reads by mapping them to known microbial genomes or protein family databases using tools like Bowtie2[334], DIAMOND[335] and BBMap[336]. Taxonomic and functional annotations are achieved through marker gene and protein databases, such as KEGG[337] and COG[338]. In contrast, the assembly-based approach reconstructs entire genomes, including those previously uncharacterized, by assembling short reads into contigs. Subsequently, sequence-intrinsic features, including coverage and tetranucleotide frequency, are utilized to group contigs originating from the same microbial genome, resulting in the construction of metagenome-assembled genomes (MAGs)[339]. Taxonomic classification is then assigned to each genome. To estimate abundance, sequencing reads are aligned to the assembled genomes. The final outcome of all approaches is an abundance table detailing taxa across samples[340].

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[332] Jünemann, S. et al.; "Bioinformatics for NGS-Based Metagenomics and the Application to Biogas Research" in *Journal of Biotechnology* 261, Bioinformatics Solutions for Big Data Analysis in Life Sciences presented by the German Network for Bioinformatics Infrastructure (**2017**): 10–23.

[333] Quince et al., "Shotgun Metagenomics, from Sampling to Analysis."

[334] Langmead, B. & S.L. Salzberg; "Fast Gapped-Read Alignment with Bowtie 2" in *Nature Methods* 9, no. 4 (**2012**): 2.

[335] Buchfink, B., C. Xie, & D.H. Huson; "Fast and Sensitive Protein Alignment Using DIAMOND" in *Nature Methods* 12, no. 1 (**2015**): 59–60.

[336] Bushnell, B.; "BBMap: A Fast, Accurate, Splice-Aware Aligner" (**2014**).

[337] Kanehisa, M. & S. Goto; "KEGG: Kyoto Encyclopedia of Genes and Genomes" in *Nucleic Acids Research* 28, no. 1 (**2000**): 27–30.

[338] Galperin, M.Y. et al.; "COG Database Update: Focus on Microbial Diversity, Model Organisms, and Widespread Pathogens" in *Nucleic Acids Research* 49, no. D1 (**2021**): D274–D281.

[339] Tyson, G.W. et al.; "Community Structure and Metabolism through Reconstruction of Microbial Genomes from the Environment" in *Nature* 428, no. 6978 (**2004**): 37–43.

[340] Pinto, Y. & A.S. Bhatt; "Sequencing-Based Analysis of Microbiomes" in *Nature Reviews Genetics* 25, no. 12 (**2024**): 829–845.

An essential parameter to measure the quality of genome assemblies is N50, which refers to the smallest contig size in a set of contigs that represents at least 50% of the assembly[341]. Quality assessment tools, such as MetaQUAST[342], are used to evaluate assembly quality. The assembled genomes can be further annotated with gene family databases, while metagenomic reads can also be mapped back to the assemblies to estimate their abundance. Comprehensive automated pipelines, such as MEGAN[343] and MetaPhlAn[344], integrate these steps for streamlined analysis. Strain identification from shotgun metagenomic data can be achieved using marker gene-based or genome-wide methods. Marker gene approaches, such as StrainPhlAn[345], identify dominant strains by aligning sequencing reads to marker gene sequences and constructing sample-specific consensus sequences from multiple sequence alignments. These consensus sequences enable strain tracking and comparisons across samples, providing insights into factors like the success of clinical FMT[346]. In contrast, genome-wide tools, such as inStrain[347] and

[341] Miller, J.R., S. Koren, & G. Sutton; "Assembly Algorithms for Next-Generation Sequencing Data" in *Genomics* 95, no. 6 (**2010**): 315–327.

[342] Mikheenko, A., V. Saveliev, & A. Gurevich; "MetaQUAST: Evaluation of Metagenome Assemblies" in *Bioinformatics* 32, no. 7 (**2016**): 1088–1090.

[343] Huson, D.H. et al.; "MEGAN Analysis of Metagenomic Data" in *Genome Research* 17, no. 3 (**2007**): 377–386.

[344] Beghini, F. et al.; "Integrating Taxonomic, Functional, and Strain-Level Profiling of Diverse Microbial Communities with bioBakery 3" in *eLife* 10 (**2021**): 3.

[345] Truong, D.T. et al.; "Microbial Strain-Level Population Structure and Genetic Diversity from Metagenomes" in *Genome Research* 27, no. 4 (**2017**): 626–638.

[346] Ianiro, G. et al.; "Variability of Strain Engraftment and Predictability of Microbiome Composition after Fecal Microbiota Transplantation across Different Diseases" in *Nature Medicine* 28, no. 9 (**2022**): 1913–1923.

[347] Olm, M.R. et al.; "inStrain Profiles Population Microdiversity from Metagenomic Data and Sensitively Detects Shared Microbial Strains" in *Nature Biotechnology* 39, no. 6 (**2021**): 727–736.

MIDAS[348]/MIDAS2[349], analyze variants across the entire genome, offering a broader perspective beyond the conserved marker genes[350].

[348] Nayfach, S. et al.; "An Integrated Metagenomics Pipeline for Strain Profiling Reveals Novel Patterns of Bacterial Transmission and Biogeography" in *Genome Research* 26, no. 11 (**2016**): 1612–1625.

[349] Zhao, C. et al.; "MIDAS2: Metagenomic Intra-Species Diversity Analysis System" in *Bioinformatics (Oxford, England)* 39, no. 1 (**2023**): btac713.

[350] Pinto and Bhatt, "Sequencing-Based Analysis of Microbiomes."

Chapter 2: AIM OF THE STUDY

Considering what was discussed in the previous chapter, it has been demonstrated how various NCDs can contribute to dysbiosis, while, at the same time, dysbiosis has been associated to the onset and progression of a plethora of NCDs. Gut eubiosis, however, can be maintained through innovative approaches aimed at NCDs prevention and by NCDs treatment, either via pharmacological interventions or through the development of *ad hoc* probiotic consortia. Two studies are then presented focused on innovative drug-microbiome-based strategies, which have the potential to pave the way for new microbiota-targeted therapeutic drugs and consortia.

Especially:

The aim of the first study was the evaluation of *in vitro* efficacy of four novel rifaximin-derived drugs against antibiotic-resistant (AR) *C. difficile*, using Batch Gut Model cultures, a novel *in vitro-ex vivo* model useful for the study of the modulation of the gut microbiome[351]. A *C. difficile* AR strain was isolated from a stool sample of a patient who, after unsuccessful antibiotic therapy for CDI, underwent FMT. In particular, fecal samples were collected from patients at IRCCS Azienda Ospedaliero-Universitaria di Bologna-Policlinico di Sant'Orsola in an attempt to isolate AR *C. difficile*, while the four novel compounds were supplied by the company Alfasigma S.p.A. (Bologna, Italy).

[351] Barone, M. et al.; "Searching for New Microbiome-Targeted Therapeutics through a Drug Repurposing Approach" in *Journal of Medicinal Chemistry* 64, no. 23 (**2021**): 17277–17286.

As discussed in Chapter 1.3, probiotics have traditionally been employed as an alternative to conventional drug-based treatments in this context. More recently, innovative strategies have been developed to design targeted and personalized microbial consortia.

Indeed, the second study was conducted in the frame of the MicroWean Project (Altered vertical microbiota transmission due to early weaning and its effects on host health), in collaboration with the Commensals and Probiotics-Host Interactions Laboratory at Micalis Institute, INRAE (Jouy-en-Josas, France). This study constitutes the second part of the MicroWean Project. While in the first part it has been clarified how the vertical microbiota transmission is altered due to early weaning, including the microbial functions perturbed by this phenomenon and the consequences on the host, this study focused on the restoration of the altered vertical microbiota transmission by a nutritional approach, following mice through several generations. Employing an *in vivo* animal model is essential for studying the GM, as it provides a more comprehensive understanding of gut dynamics and enables a deeper investigation into the physiological consequences of dysbiosis, including mechanistic insights.

Chapter 3: *IN VITRO* EFFICACY OF FOUR COMPOUNDS AGAINST ANTIBIOTIC RESISTANT *C. DIFFICILE* ON HUMAN GUT MICROBIOTA USING BATCH GUT MODEL CULTURES

3.1 Brief introduction

One of the most frequent causes of infections linked to healthcare is the obligate anaerobic pathogen *C. difficile*, formerly known as *Clostridium difficile*[352, 353]. *C. difficile* is a rod-shaped bacterium, spore-forming and Gram-positive. Because of their low water content and other characteristics including high quantities of dipicolinic acid and saturation of DNA with soluble proteins, *C. difficile* spores are particularly resistant to heat, oxygen, and conventional disinfectants like ethanol[354, 355, 356, 357]. The CDI is mainly caused by its toxins, namely toxin A (TcdA) and toxin B (TcdB), both cytotoxic and enterotoxic. Some strains are also capable of producing a third toxin, the *C. difficile* transferase (CDT or binary toxin). These toxins carry out their action damaging the epithelial cell cytoskeleton, following, for instance, the breakdown of tight junctions, fluid secretion and adhesion of neutrophiles. This results in a

[352] Czepiel, J. et al.; "Clostridium Difficile Infection: Review" in *European Journal of Clinical Microbiology & Infectious Diseases* 38, no. 7 (**2019**): 1211–1221.

[353] Lawson, P.A. et al.; "Reclassification of Clostridium Difficile as Clostridioides Difficile (Hall and O'Toole 1935) Prévot 1938" in *Anaerobe* 40 (**2016**): 95–99.

[354] Dawson, L.F. et al.; "Hypervirulent Clostridium Difficile PCR-Ribotypes Exhibit Resistance to Widely Used Disinfectants" in *PloS One* 6, no. 10 (**2011**): e25754.

[355] Paredes-Sabja, D., A. Shen, & J.A. Sorg; "Clostridium Difficile Spore Biology: Sporulation, Germination, and Spore Structural Proteins" in *Trends in Microbiology* 22, no. 7 (**2014**): 406–416.

[356] Rodriguez-Palacios, A. & J.T. Lejeune; "Moist-Heat Resistance, Spore Aging, and Superdormancy in Clostridium Difficile" in *Applied and Environmental Microbiology* 77, no. 9 (**2011**): 3085–3091.

[357] Setlow, P.; "I Will Survive: DNA Protection in Bacterial Spores" in *Trends in Microbiology* 15, no. 4 (**2007**): 172–180.

disruption of the intestinal barrier, loss of function and local inflammation[358, 359, 360]. The consequences of CDI could also affect the nervous system. In fact, by targeting gut-innervating afferent neurons and pericytes through receptors, such as the Frizzled receptors (FZD1, FZD2, and FZD7) in neurons and chondroitin sulfate proteoglycan 4 (CSPG4) in pericytes, TcdB may cause neurogenic inflammation, according to a recent study on CDI in mice[361]. With these prerequisites, CDI can manifest from moderate asymptomatic carrier status with diarrhea or fulminant colitis with shock, hypotension or even megacolon. Critical symptoms (e.g., colon perforation, intestinal paralysis, septicemia) in the most serious cases of CDI can be fatal[362]. The death rate from CDI is thought to be 5%, whereas the death rate from CDI complications varies from 15% to 25%, reaching 34% in intensive care units[363, 364, 365, 366].

[358] Baktash, A. et al.; "Mechanistic Insights in the Success of Fecal Microbiota Transplants for the Treatment of Clostridium Difficile Infections" in *Frontiers in Microbiology* 9 (**2018**): 1242.

[359] Czepiel et al., "Clostridium Difficile Infection."

[360] Smits, W.K. et al.; "Clostridium Difficile Infection" in *Nature Reviews Disease Primers* 2, no. 1 (**2016**): 1–20.

[361] Manion, J. et al.; "C. Difficile Intoxicates Neurons and Pericytes to Drive Neurogenic Inflammation" in *Nature* 622, no. 7983 (**2023**): 611–618.

[362] McDonald, L.C. et al.; "Clinical Practice Guidelines for Clostridium Difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA)" in *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 66, no. 7 (**2018**): e1–e48.

[363] Czepiel, J. et al.; "Epidemiology of Clostridium Difficile Infection: Results of a Hospital-Based Study in Krakow, Poland" in *Epidemiology and Infection* 143, no. 15 (**2015**): 3235–3243.

[364] Mengoli, M. et al.; "Make It Less Difficile: Understanding Genetic Evolution and Global Spread of Clostridioides Difficile" in *Genes* 13, no. 12 (**2022**): 2200.

[365] Sidler, J.A. et al.; "Enterococci, Clostridium Difficile and ESBL-Producing Bacteria: Epidemiology, Clinical Impact and Prevention in ICU Patients" in *Swiss Medical Weekly* 144, no. 3940 (**2014**): w14009–w14009.

[366] Vincent, J.-L. et al.; "International Study of the Prevalence and Outcomes of Infection in Intensive Care Units" in *JAMA* 302, no. 21 (**2009**): 2323–2329.

It is well recognized that the GM is crucial to the pathogenesis of CDI. For example, the growth of *C. difficile* may be reduced by the presence of bacterial consortia made up of members of *Lactobacillus* and *Alistipes* genera, as well as *Lachnospiraceae* and *Porphyromonadaceae* families[367]. However, when primary fermenters like *Lactobacillus* and *Lachnospiridae* decrease, a niche is created where other primary fermenters like *B. thetaiotaomicron* are able to grow. This is particularly significant when dysbiosis is made worse by these losses. These bacteria can generate a range of metabolic byproducts, which *C. difficile* has been demonstrated to exploit as a source of carbon[368, 369, 370]. Additionally, some bacteria in the gut can produce secondary bile acids (SBAs), which can affect the germination of *C. difficile* spores[371]. Specifically, it seems that bacterial 7-α-dehydroxylation of primary bile acids (PBAs) protects against CDI[372], and it might be a biomarker for an environment resistant to *C. difficile*. On the other hand, it has been demonstrated that cefoperazone and other broad-spectrum antibiotics interfere with the GM's ability to alter bile acids, promoting *C. difficile* colonization and proliferation[373]. According to Spigaglia et al.[374], compared to other pharmacological therapies, the administration of specific

[367] Jukes, C.A. et al.; "Bile Salt Metabolism Is Not the Only Factor Contributing to Clostridioides (Clostridium) Difficile Disease Severity in the Murine Model of Disease" in *Gut Microbes* (**2020**).

[368] Curtis, M.M. et al.; "The Gut Commensal Bacteroides Thetaiotaomicron Exacerbates Enteric Infection through Modification of the Metabolic Landscape" in *Cell Host & Microbe* 16, no. 6 (**2014**): 759–769.

[369] Ferreyra, J.A. et al.; "Gut Microbiota-Produced Succinate Promotes *C. Difficile* Infection after Antibiotic Treatment or Motility Disturbance" in *Cell Host & Microbe* 16, no. 6 (**2014**): 770–777.

[370] Jukes et al., "Bile Salt Metabolism Is Not the Only Factor Contributing to Clostridioides (Clostridium) Difficile Disease Severity in the Murine Model of Disease."

[371] Ibid.

[372] Aguirre, A.M. et al.; "Bile Acid-Independent Protection against Clostridioides Difficile Infection" in *PLOS Pathogens* 17, no. 10 (**2021**): e1010015.

[373] Theriot, C.M., A.A. Bowman, & V.B. Young; "Antibiotic-Induced Alterations of the Gut Microbiota Alter Secondary Bile Acid Production and Allow for Clostridium Difficile Spore Germination and Outgrowth in the Large Intestine" in *mSphere* 1, no. 1 (**2016**): 10.1128/msphere.00045-15.

[374] Spigaglia, P., P. Mastrantonio, & F. Barbanti; "Antibiotic Resistances of Clostridium Difficile" in *Advances in Experimental Medicine and Biology* 1050 (**2018**): 137–159.

antimicrobials (such as cephalosporins and carbapenems) is actually more frequently linked to the induction of CDI.

As mentioned above, PBAs are necessary for the germination of *C. difficile* spores. PBAs aid in the breakdown of fats and are generated by the liver and released into the small intestine[375]. In contrast, SBAs inhibit *C. difficile* spore germination[376]. Metabolization of PBAs to SBAs is provided by populations from the Firmicutes phylum like *Ruminococcaceae* and *Lachnospiraceae*[377], which are shown to be decreased in patients with CDI. Reduced abundances of these bacterial groups lead to a drop in SBAs and, thus, to a reduction of the ability to suppress germination in the ileum. So, CDI can develop more easily as a result of this decline in SBAs[378, 379, 380, 381]. The decreases in these families also lead to a decrease in the concentration of SCFAs, providing advantages to taxa of the Proteobacteria phylum (*Enterobacteriaceae* family), in particular, *Escherichia*/*Shigella*, *Proteus* spp., *Klebsiella* spp.

[375] Crobach, M.J.T. et al.; "Understanding Clostridium Difficile Colonization" in *Clinical Microbiology Reviews* 31, no. 2 (**2018**): 10.1128/cmr.00021-17.

[376] Francis, M.B. et al.; "Bile Acid Recognition by the Clostridium Difficile Germinant Receptor, CspC, Is Important for Establishing Infection" in *PLOS Pathogens* 9, no. 5 (**2013**): e1003356.

[377] Crobach et al., "Understanding Clostridium Difficile Colonization"; Theriot, C.M. et al.; "Antibiotic-Induced Shifts in the Mouse Gut Microbiome and Metabolome Increase Susceptibility to Clostridium Difficile Infection" in *Nature Communications* 5, no. 1 (**2014**): 3114.

[378] Buffie, C.G. et al.; "Precision Microbiome Reconstitution Restores Bile Acid Mediated Resistance to Clostridium Difficile" in *Nature* 517, no. 7533 (**2015**): 205–208.

[379] Crobach et al., "Understanding Clostridium Difficile Colonization."

[380] Francis et al., "Bile Acid Recognition by the Clostridium Difficile Germinant Receptor, CspC, Is Important for Establishing Infection."

[381] Martinez, E. et al.; "Gut Microbiota Composition Associated with Clostridioides Difficile Colonization and Infection" in *Pathogens* 11, no. 7 (**2022**): 781.

and *Providencia* spp.[382, 383, 384, 385, 386, 387, 388, 389, 390, 391]. On the other hand, *Clostridium scindens* can stop the germination of *C. difficile* and restore the metabolism of SBAs[392, 393, 394, 395].

The pathophysiology and dissemination of *C. difficile* are significantly influenced by antibiotic resistance, which is one of the main problems associated with CDI (and recurrent CDI)[396]. Even though it is still uncommon, the resistance to antimicrobials advised by guidelines for

[382] Crobach et al., "Understanding Clostridium Difficile Colonization."

[383] Gu, S. et al.; "Identification of Key Taxa That Favor Intestinal Colonization of *Clostridium Difficile* in an Adult Chinese Population" in *Microbes and Infection* 18, no. 1 (**2016**): 30–38.

[384] Han, S.-H. et al.; "Composition of Gut Microbiota in Patients with Toxigenic Clostridioides (Clostridium) Difficile: Comparison between Subgroups According to Clinical Criteria and Toxin Gene Load" in *PLOS ONE* 14, no. 2 (**2019**): e0212626.

[385] Hernandez, D.R.; "Gut Check: *In Vitro* Diagnostics for Gut Microbiome Analysis" in *Clinical Microbiology Newsletter* 41, no. 7 (**2019**): 57–62.

[386] Jeon, Y.D. et al.; "Characteristics of Faecal Microbiota in Korean Patients with Clostridioides Difficile-Associated Diarrhea" in *Infection & Chemotherapy* 51, no. 4 (**2019**): 365–375.

[387] Kim, J. et al.; "Quantitative Characterization of Clostridioides Difficile Population in the Gut Microbiome of Patients with C. Difficile Infection and Their Association with Clinical Factors" in *Scientific Reports* 10, no. 1 (**2020**): 17608.

[388] Milani, C. et al.; "Gut Microbiota Composition and Clostridium Difficile Infection in Hospitalized Elderly Individuals: A Metagenomic Study" in *Scientific Reports* 6, no. 1 (**2016**): 25945.

[389] Stewart, D.B. et al.; "Integrated Meta-Omics Reveals a Fungus-Associated Bacteriome and Distinct Functional Pathways in Clostridioides Difficile Infection" in *mSphere* 4, no. 4 (**2019**): 10.1128/msphere.00454-19.

[390] Vakili, B. et al.; "Characterization of Gut Microbiota in Hospitalized Patients with Clostridioides Difficile Infection" in *Current Microbiology* 77, no. 8 (**2020**): 1673–1680.

[391] Zhang, L. et al.; "Insight into Alteration of Gut Microbiota in *Clostridium Difficile* Infection and Asymptomatic *C. Difficile* Colonization" in *Anaerobe* 34 (**2015**): 1–7.

[392] Buffie et al., "Precision Microbiome Reconstitution Restores Bile Acid Mediated Resistance to Clostridium Difficile."

[393] Crobach et al., "Understanding Clostridium Difficile Colonization."

[394] Francis et al., "Bile Acid Recognition by the Clostridium Difficile Germinant Receptor, CspC, Is Important for Establishing Infection."

[395] Martinez et al., "Gut Microbiota Composition Associated with Clostridioides Difficile Colonization and Infection."

[396] Imwattana, K. et al.; "A Species-Wide Genetic Atlas of Antimicrobial Resistance in Clostridioides Difficile" in *Microbial Genomics* 7, no. 11 (**2021**): 000696.

treating CDI has been addressed in revisions throughout time[397, 398], also to deal with this problem. Specifically, the first guidance (1995-1997) was concentrated on the administration of metronidazole and vancomycin; however, as of 2014, they additionally included fidaxomicin therapy[399]. In terms of resistance mechanisms, metronidazole, an antibiotic that is a member of the nitroimidazole family, works by preventing DNA's helical structure, which can cause strand breaks, reduce protein synthesis, and induce cell death[400]. Metronidazole-resistant *C. difficile* strains are sporadically isolated in clinical practice, but some strains seem to require a heme cofactor in order to be detectable[401, 402]. As evidenced by studies in other bacteria, *C. difficile* may utilize heme as a source of iron and a cofactor for redox-associated proteins during oxidative stress reactions to metronidazole[403, 404]. The high copy number plasmid pCD-METRO appears to be the mediator of this metronidazole resistance[405]. The alteration of pyruvate-ferredoxin/flavodoxin oxidoreductase (PFOR) catalytic domains, a protein encoded by the *nifJ* gene, is another genetic mechanism

[397] Freeman, J. et al.; "Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent *Clostridium Difficile* Ribotypes" in *Clinical Microbiology and Infection* 21, no. 3 (**2015**): 248.e9-248.e16.

[398] Imwattana et al., "A Species-Wide Genetic Atlas of Antimicrobial Resistance in Clostridioides Difficile."

[399] Chaar, A. & P. Feuerstadt; "Evolution of Clinical Guidelines for Antimicrobial Management of Clostridioides Difficile Infection" in *Therapeutic Advances in Gastroenterology* 14 (**2021**): 17562848211011953.

[400] Ibid.

[401] Boekhoud, I.M. et al.; "Haem Is Crucial for Medium-Dependent Metronidazole Resistance in Clinical Isolates of Clostridioides Difficile" in *Journal of Antimicrobial Chemotherapy* 76, no. 7 (**2021**): 1731–1740.

[402] Wu, X. et al.; "The Integrity of Heme Is Essential for Reproducible Detection of Metronidazole-Resistant Clostridioides Difficile by Agar Dilution Susceptibility Tests" in *Journal of Clinical Microbiology* 59, no. 9 (**2021**): 10.1128/jcm.00585-21.

[403] Giardina, G. et al.; "NO Sensing in *Pseudomonas Aeruginosa*: Structure of the Transcriptional Regulator DNR" in *Journal of Molecular Biology* 378, no. 5 (**2008**): 1002–1015.

[404] Wu et al., "The Integrity of Heme Is Essential for Reproducible Detection of Metronidazole-Resistant Clostridioides Difficile by Agar Dilution Susceptibility Tests."

[405] Boekhoud, I.M. et al.; "Plasmid-Mediated Metronidazole Resistance in Clostridioides Difficile" in *Nature Communications* 11, no. 1 (**2020**): 598.

implicated in metronidazole resistance in clinical isolates[406]. In terms of resistance mechanisms, strains of *C. difficile* have the ability to encode a vanG-type gene cluster (*vanGCd*), which provides resistance to the antibiotic vancomycin, a glycopeptide which prevents the formation of bacterial cell wall[407]. The creation of an alternative lipid II carrying a D-alanine-D-serine terminus that is seven times less bound by vancomycin than the D-alanine-D-alanine terminus specifically confers resistance[408]. It has been demonstrated that constitutive production of *vanGCd* occurs in both laboratory-generated mutants with mutations in the two-component VanSR system that regulates *vanGCd* and in vancomycin-resistant bacteria isolated in the clinical situation[409]. At last, fidaxomicin, a macrocyclic antibiotic with a restricted spectrum, inhibits the production of RNA. This medication inhibits bacterial RNA polymerase through its main component, lipiarmycin A3[410]. Thus, RNA polymerase mutations, particularly those affecting the β-subunit, provide resistance to fidaxomicin[411, 412]. Although antibiotic resistance is a burden regarding CDI, luckily, a pan-European study[413] found that the majority of countries reported metronidazole, vancomycin, and fidaxomicin resistance below 10% (with most reporting 0%), and only 15 out of over

[406] Deshpande, A. et al.; "Chromosomal Resistance to Metronidazole in Clostridioides Difficile Can Be Mediated by Epistasis between Iron Homeostasis and Oxidoreductases" in *Antimicrobial Agents and Chemotherapy* 64, no. 8 (**2020**): 10.1128/aac.00415-20.

[407] Chaar and Feuerstadt, "Evolution of Clinical Guidelines for Antimicrobial Management of Clostridioides Difficile Infection."

[408] Shen, W.-J. et al.; "Constitutive Expression of the Cryptic vanGCd Operon Promotes Vancomycin Resistance in Clostridioides Difficile Clinical Isolates" in *Journal of Antimicrobial Chemotherapy* 75, no. 4 (**2020**): 859–867.

[409] Ibid.

[410] Lin, W. et al.; "Structural Basis of Transcription Inhibition by Fidaxomicin (Lipiarmycin A3)" in *Molecular Cell* 70, no. 1 (**2018**): 60-71.e15.

[411] Babakhani, F. et al.; "Killing Kinetics of Fidaxomicin and Its Major Metabolite, OP-1118, against Clostridium Difficile" in *Journal of Medical Microbiology* 60, no. 8 (**2011**): 1213–1217.

[412] Babakhani, F., J. Seddon, & P. Sears; "Comparative Microbiological Studies of Transcription Inhibitors Fidaxomicin and the Rifamycins in Clostridium Difficile" in *Antimicrobial Agents and Chemotherapy* 58, no. 5 (**2014**): 2934–2937.

[413] Freeman et al., "Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent *Clostridium Difficile* Ribotypes."

10,000 strains tested in a species-wide genomic study[414] were found to have the pCD-METRO plasmid.

As previously mentioned in Chapter 1.3, FMT is a well-recognized therapy for CDI treatment[415]. Not only it has been effective in patients without any other disturbance[416], but also it has been reported to be an effective therapy in patients with inflammatory bowel disease [417], *i.e.*, Chron's disease and ulcerative colitis[418]. In the first place, this is due to the fact that, apart from treating and alleviating the symptoms of CDI, FMT is also able to restore a GM altered by these further complications. In addition, different FMT protocols have been proposed using different delivery methods and repeated administration. For instance, Baunwall et al. reported that in 24 studies analyzed the overall clinical effect was over 91% in repeated FMT treatments, while the efficacy was on 84% for a single FMT in 43 studies[419]. Besides the high efficacy rate, some agencies, such as FDA, have not approved the treatment owing to adverse effects[420]. Other CDI therapies have been proposed from the scientific community and have been FDA approved. For example, a phase 3 PUNCH CD3 clinical trial showed that a single dose of a live fecal microbiota biotherapeutic product (REBYOTA, Ferring Pharmaceuticals) demonstrated a major efficacy over placebo in

[414] Imwattana et al., "A Species-Wide Genetic Atlas of Antimicrobial Resistance in Clostridioides Difficile."

[415] Ianiro et al., "Efficacy of Different Faecal Microbiota Transplantation Protocols for Clostridium Difficile Infection."

[416] Baunwall, S.M.D. et al.; "Faecal Microbiota Transplantation for Recurrent *Clostridioides Difficile* Infection: An Updated Systematic Review and Meta-Analysis" in *EClinicalMedicine* 29–30 (**2020**): 100642.

[417] Porcari, S. et al.; "Fecal Microbiota Transplantation for Recurrent C. Difficile Infection in Patients with Inflammatory Bowel Disease: A Systematic Review and Meta-Analysis" in *Journal of Autoimmunity* 141, The emerging indications in fecal microbiota transplantation for inflammatory and autoimmune diseases (**2023**): 103036.

[418] Jaramillo, A.P. et al.; "Effectiveness of Fecal Microbiota Transplantation Treatment in Patients With Recurrent Clostridium Difficile Infection, Ulcerative Colitis, and Crohn's Disease: A Systematic Review" in *Cureus* 15, no. 7 (**2023**): e42120.

[419] Baunwall et al., "Faecal Microbiota Transplantation for Recurrent *Clostridioides Difficile* Infection."

[420] DeFilipp et al., "Drug-Resistant E. Coli Bacteremia Transmitted by Fecal Microbiota Transplant."

reducing CDI recurrence after antibiotic treatment[421]. Another example is given by a phase 3 ECOSPOR III clinical trial, which showed that oral administration of SER-109 (Seres Therapeutics), an orally active therapeutic with fecal microbiota spores, reduced the risk of recurrent CDI[422].

Another common alternative to antibiotics and FMT is rifaximin (**Figure 5**), an antimicrobial agent structurally analogous to rifampicin, obtained by the reaction between rifampicin O (an oxidized form of rifampicin B, from the Gram-positive bacterium *Amycolatopsis mediterranei*) and 2-amino-4-methylpyridine[423]. Rifaximin exerts its antibacterial activity by binding to the β-subunit of bacterial DNA-dependent RNA polymerase, thereby inhibiting bacterial RNA synthesis[424, 425]. *In vivo* animal and human studies have shown that systemic absorption of rifaximin after oral administration is negligible, being less than 0.4% of the administered dose. The discovery of the polymorphism of rifaximin in the early 2000s showed that the bioavailability of rifaximin is closely related to its polymorphic form, the α form (the one marketed) being one of the least bioavailable[426]. This allows it to act as a topical antimicrobial. In fact, it exerts its antibacterial activity against microorganisms that

[421] Khanna, S. et al.; "Efficacy and Safety of RBX2660 in PUNCH CD3, a Phase III, Randomized, Double-Blind, Placebo-Controlled Trial with a Bayesian Primary Analysis for the Prevention of Recurrent Clostridioides Difficile Infection" in *Drugs* 82, no. 15 (**2022**): 1527–1538.

[422] Feuerstadt, P. et al.; "SER-109, an Oral Microbiome Therapy for Recurrent Clostridioides Difficile Infection" in *The New England Journal of Medicine* 386, no. 3 (**2022**): 220–229.

[423] Sensi, P., A.M. Greco, & R. Ballotta; "Rifomycin. I. Isolation and Properties of Rifomycin B and Rifomycin Complex" in *Antibiotics Annual* 7 (**1959**): 262–270.

[424] Gillis, J.C. & R.N. Brogden; "Rifaximin. A Review of Its Antibacterial Activity, Pharmacokinetic Properties and Therapeutic Potential in Conditions Mediated by Gastrointestinal Bacteria" in *Drugs* 49, no. 3 (**1995**): 467–484.

[425] Jiang, Z.D. & H.L. DuPont; "Rifaximin: In Vitro and in Vivo Antibacterial Activity--a Review" in *Chemotherapy* 51 Suppl 1 (**2005**): 67–72.

[426] Viscomi, G.C. et al.; "Crystal Forms of Rifaximin and Their Effect on Pharmaceutical Properties" in *CrystEngComm* 10, no. 8 (**2008**): 1074–1081.

cause gastrointestinal infections, but not systemic infections, being excreted mainly in the feces as an unchanged drug[427, 428].

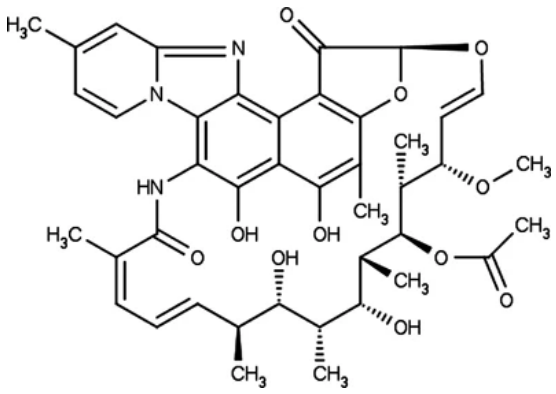

Figure 5. Chemical structure of rifaximin[427].

Repeated administration of rifaximin-α has been observed to exert only minimal and temporary effects on the composition of the fecal microbiota[429]. While rifaximin-α does not induce significant long-term alterations in the microbial composition at higher taxonomic levels such as phyla or orders[430], it has been found to encourage the growth of beneficial bacteria, including *Bifidobacterium* and *Lactobacillus* genera, supporting the restoration of mucosal integrity[431]. The drug's antimicrobial action is most potent in the bile-rich environment of the small intestine, which may account for its efficacy in treating conditions such as irritable bowel syndrome and small intestinal bacterial overgrowth[432]. Additionally,

[427] Jiang, Z.D. et al.; "In Vitro Activity and Fecal Concentration of Rifaximin after Oral Administration" in *Antimicrobial Agents and Chemotherapy* 44, no. 8 (**2000**): 2205–2206.

[428] Calanni, F. et al.; "Rifaximin: Beyond the Traditional Antibiotic Activity" in *The Journal of Antibiotics* 67, no. 9 (**2014**): 667–670.

[429] Fodor, A.A. et al.; "Rifaximin Is Associated with Modest, Transient Decreases in Multiple Taxa in the Gut Microbiota of Patients with Diarrhoea-Predominant Irritable Bowel Syndrome" in *Gut Microbes* 10, no. 1 (**2019**): 22–33.

[430] DuPont, H.L.; "Review Article: The Antimicrobial Effects of Rifaximin on the Gut Microbiota" in *Alimentary Pharmacology & Therapeutics* 43, no. S1 (**2016**): 3–10.

[431] Ponziani, F.R. et al.; "Eubiotic Properties of Rifaximin: Disruption of the Traditional Concepts in Gut Microbiota Modulation" in *World Journal of Gastroenterology* 23, no. 25 (**2017**): 4491–4499.

[432] DuPont, H.L.; "Therapeutic Effects and Mechanisms of Action of Rifaximin in Gastrointestinal Diseases" in *Mayo Clinic Proceedings* 90, no. 8 (**2015**): 1116–1124.

because rifaximin is nearly insoluble in water, only limited amounts reach the largely aqueous environment of the colon, meaning that only the small water-soluble fraction is available to interact with the colonic microbiota, yet this fraction is sufficient to produce biologically relevant effects[433].

Rifaximin-α has positive effects on host epithelial cells and can cause alteration on the pathogens' virulence[434, 435]. The effects of rifaximin on bacteria such as *E. coli* were primarily responsible for the decrease in bacterial adherence, whilst the drug's effects on epithelial cells were responsible for the decrease in bacterial internalization into cells[436, 437]. Furthermore, pre-treatment of epithelial cells with rifaximin changed the cell physiology in a number of ways to provide cytoprotection, such as down-regulating an apoptosis marker (indicating protection against bacterial induced apoptosis) and advantageous changes in the expression of proteins linked to the cytoskeleton and cellular integrity[438]. All the putative mechanisms of rifaximin-α on the gut and the GM are summarized in **Figure 6**.

[433] DuPont, H.L.; "The Potential for Development of Clinically Relevant Microbial Resistance to Rifaximin-α: A Narrative Review" in *Clinical Microbiology Reviews* 36, no. 4 (**2023**): e00039-23.

[434] Brown, E.L. et al.; "Pretreatment of Epithelial Cells with Rifaximin Alters Bacterial Attachment and Internalization Profiles" in *Antimicrobial Agents and Chemotherapy* 54, no. 1 (**2010**): 388–396.

[435] Jiang, Z.-D., S. Ke, & H.L. DuPont; "Rifaximin-Induced Alteration of Virulence of Diarrhoea-Producing *Escherichia Coli* and *Shigella Sonnei*" in *International Journal of Antimicrobial Agents* 35, no. 3 (**2010**): 278–281.

[436] Brown et al., "Pretreatment of Epithelial Cells with Rifaximin Alters Bacterial Attachment and Internalization Profiles."

[437] Dogan, B. et al.; "Rifaximin Decreases Virulence of Crohn's Disease-Associated Escherichia Coli and Epithelial Inflammatory Responses" in *The Journal of Antibiotics* 71, no. 5 (**2018**): 485–494.

[438] Schrodt, C. et al.; "Rifaximin-Mediated Changes to the Epithelial Cell Proteome: 2-D Gel Analysis" in *PLOS ONE* 8, no. 7 (**2013**): e68550.

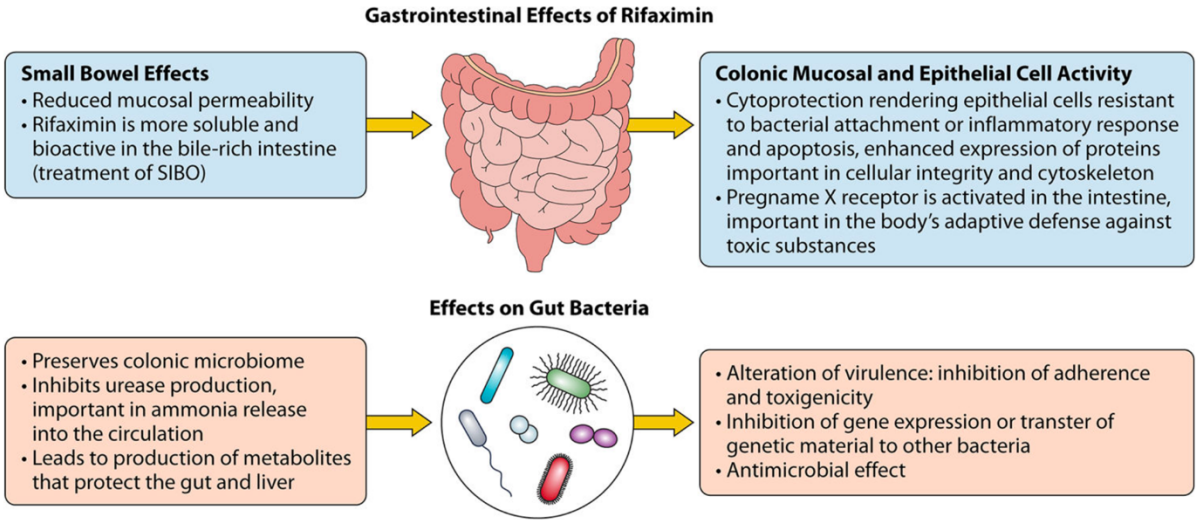

Figure 6. Putative mechanisms of rifaximin-α on the gut and GM[439].

The resistance to rifaximin-α is quite rare, nonetheless some mechanisms have been underlined. Generally speaking, the chromosomal one-step alteration in the gene encoding for bacterial DNA-dependent RNA polymerase (*rpoB* gene) is a common resistance mechanism[440]. Moreover, rifaximin, like rifampicin, can select for resistant bacterial strains in the gut but at low frequency (2.6×10^7 of spontaneous rifaximin-resistant clones[441]). Regarding *C. difficile* treatment, in a retrospective chart review, rifaximin resistance had been detected in 34.1% of patients with CDI overall and in 84.6% of those who had received rifaximin[442]. In conclusion, the prevalence of rifaximin-resistant *C. difficile* strains appears to differ across geographic regions. For instance, analysis of clinical isolates from patients with CDI showed a significantly higher rate of resistance in Italy compared to Canada (18.8% vs. 2.4%), along with a much higher resistance threshold (MIC_{90} > 16 μg/mL in Italy vs. 0.0008

[439] DuPont, "The Potential for Development of Clinically Relevant Microbial Resistance to Rifaximin-α."

[440] Jiang and DuPont, "Rifaximin."

[441] Debbia, E.A. et al.; "Effects of Rifaximin on Bacterial Virulence Mechanisms at Supra- and Sub-Inhibitory Concentrations" in *Journal of Chemotherapy* (**2008**).

[442] Reigadas, E. et al.; "Breakthrough Clostridium Difficile Infection in Cirrhotic Patients Receiving Rifaximin" in *Clinical Infectious Diseases* 66, no. 7 (**2018**): 1086–1091.

μg/mL in Canada)[443]. Indirect evidence from this study suggests that greater population-level exposure to rifaximin in Italy may have created selective pressure favoring rifamycin-resistant strains. At the time of the study, rifamycin-class antibiotics had been widely used in Italy for decades and they were approved for a variety of indications (*i.e.*, traveler's diarrhea, prophylaxis before gastrointestinal surgery etc.), while in Canada, these antibiotics were less commonly prescribed[444]. However, given the benefits of rifaximin, it remains a very crucial and essential tool to fight CDI as well as GM dysbiosis caused by other factors.

3.2 Materials, methods and assay optimization

3.2.1 Preliminary activities

Prior to carrying out the assay, the best performing selective medium for *C. difficile* was identified through literature research and *in vitro* tests, carried out starting from a pure culture of the *C. difficile* type strain (DSM 1296) (**Figure 7**) as well as from fecal samples spiked with different concentrations of *C. difficile*.

[443] Miller, M.A. et al.; "Divergent Rifamycin Susceptibilities of Clostridium Difficile Strains in Canada and Italy and Predictive Accuracy of Rifampin Etest for Rifamycin Resistance" in *Journal of Clinical Microbiology* 49, no. 12 (**2020**): 4319–4321.

[444] Ibid.

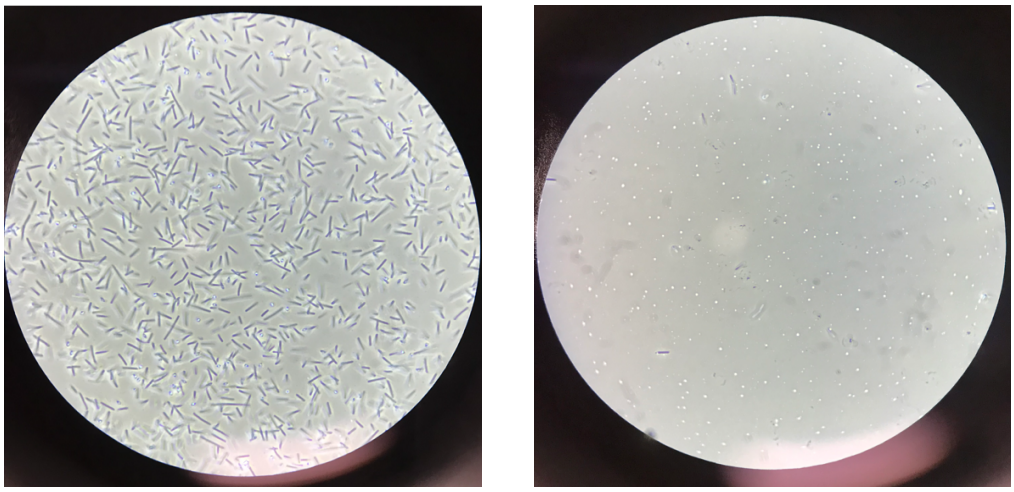

Figure 7. *C. difficile* type strain DSM 1296. Optical microscope, 100X zoom.

Growth of *C. difficile* was tested on: i) Fastidious Anaerobe Agar (FAA) medium with 10% horse defibrinate blood added; ii) mGAM medium, which was selected for Batch Gut Model Cultures based on available literature[445]; and iii) *Clostridium Difficile* Agar Base (spiked with Moxalactam, Norfloxacin and 7% horse defibrinate blood, total medium CDMN)[446, 447, 448] (**Figures 8**, **9** and **10**). The composition of the media is given in **Table 1**.

[445] Maier, L. et al.; "Extensive Impact of Non-Antibiotic Drugs on Human Gut Bacteria" in *Nature* 555, no. 7698 (**2018**): 623–628.

[446] Aspinall, S.T. & D.N. Hutchinson; "New Selective Medium for Isolating Clostridium Difficile from Faeces" in *Journal of Clinical Pathology* 45, no. 9 (**1992**): 812–814.

[447] Tkhawkho, L. et al.; "Comparison of Preliminary Treatment Methods and Selective Growth Media for Effective Isolation of Clostridium Difficile from Stool Samples.," **2016**.

[448] Wren, M.; "Clostridium Difficile Isolation and Culture Techniques" in *Methods in Molecular Biology (Clifton, N.J.)* 646 (**2010**): 39–52.

Table 1. Composition of media used for *C. difficile* growth.

Medium	Composition (g/L)		Company
Clostridium Difficile Agar Base	Proteose peptone	40.0	Oxoid
	Disodium hydrogen phosphate	5.0	
	Potassium dihydrogen phosphate	1.0	
	Magnesium sulphate	0.1	
	Sodium chloride	2.0	
	Fructose	6.0	
	Agar	15.0	
Fastidious Anaerobe Agar (FAA)	Peptone mix	23.0	LabM Ltd
	Sodium chloride	5.0	
	Soluble starch	1.0	
	Agar No. 2	12.0	
	Sodium bicarbonate	0.4	
	Glucose	1.0	
	Sodium pyruvate	1.0	
	Cysteine HCl monohydrate	0.5	
	Hemin	0.01	
	Vitamin K	0.001	
	L-arginine	1.0	
	Soluble pyrophosphate	0.3	
	Sodium succinate	0.5	
mGAM	Peptone	5.0	HyServe
	Soya peptone	3.0	
	Proteose peptone	5.0	
	Digested serum	10.0	
	Yeast extract	2.5	
	Meat extract	2.2	
	Liver extract	1.2	
	Dextrose	0.5	
	Soluble starch	5.0	
	L-tryptophan	0.2	
	L-cysteine hidrochloride	0.3	
	Sodium thioglycolate	0.3	
	L-arginine	1.0	
	Vitamin K1	0.005	
	Hemin	0.01	
	Potassium Dihydrogen phosphate	2.5	
	Sodium thioglycolate	0.3	
	L-arginine	1.0	

Vitamin K1	0.005
Hemin	0.01
Potassium Dihydrogen phosphate	2.5
Sodium chloride	3.0

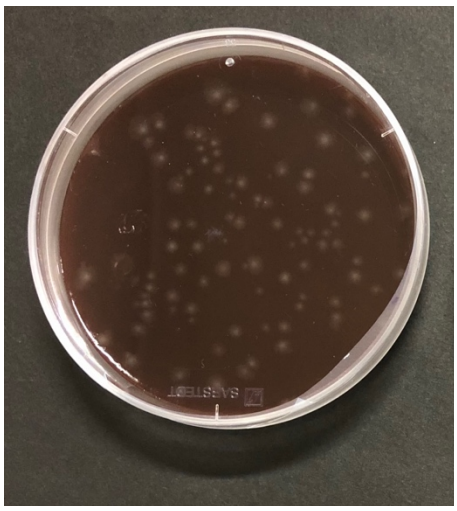

Figure 8. *C. difficile* DSM 1296 growth on Fastidious Anaerobe agar (FAA + 10% horse blood).

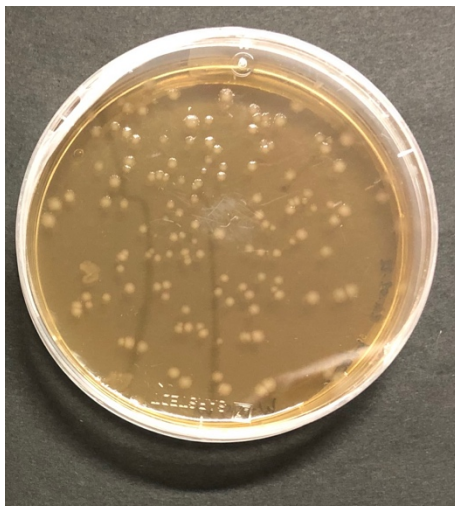

Figure 9. *C. difficile* DSM 1296 on mGAM medium.

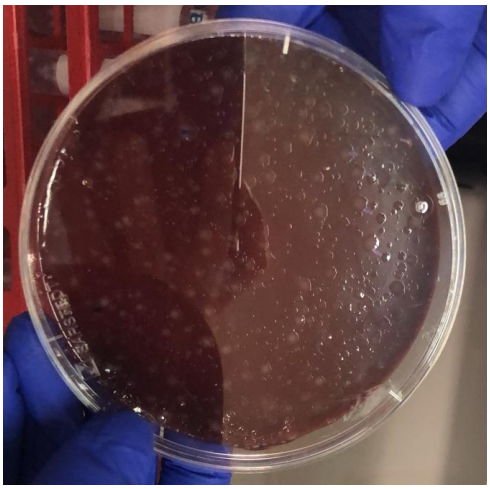

Figure 10. *C. difficile* DSM 1296 on CDMN medium.

For the performance assay of the selective medium, one fecal sample of a healthy subject was first used. Specifically, a slurry was prepared by resuspension of 1 g of feces in *Clostridium Difficile* Base medium (CDB, or CDMN medium in absence of the antibiotics Moxalactam and Norfloxacin and 7% of defibrinated horse blood). All the operations and the subsequent incubation of the plates were carried out in anaerobic hood (with a gas mixture

of 5% H_2, 10% CO_2, 85% N_2) at 37°C. At the end of the 24-hour incubation, as expected, no colonies with morphology comparable to *C. difficile* were found.

Subsequently, attempts were made to isolate *C. difficile* from fecal samples of 4 patients with CDI who had already been treated with antibiotics, but only from one sample it was possible to isolate the pathogen. This was probably because in the other samples *C. difficile* had been found to be inhibited by antibiotic therapy. So, considering the sporigenic nature of the pathogen, a step of reactivation of the spores was carried out by incubation of the fecal slurry at 80°C for 10 minutes and the results were compared with those obtained in the absence of this step. In the absence of the step of reactivation of the spores, on CDMN medium, a cell count of 1×10^3 CFU/g with morphology attributable to *C. difficile* was found. Following the spore reactivation step, a cell count of 8×10^3 CFU/g was found with morphology attributable to *C. difficile*. *C. difficile* identity was confirmed by Sanger sequencing of the DNA extracted from isolated colonies (percentage of homology > 98%). Each colony was also recovered for the preparation of glycerinates, as described in point 3.2.2. In parallel, research was carried out in the literature[449, 450, 451] to determine the fecal concentration of *C. difficile* in terms of CFU/g, as well as its relative abundance in the GM of patients with infection in progress. Based on the available results, the following range, 10^1 - 10^7 CFU/g of feces was identified. Next-generation sequencing studies of 16S rRNA gene and shotgun metagenomics showed a relative abundance in colonized patients of less than

[449] Crobach, M.J.T. et al.; "The Bacterial Gut Microbiota of Adult Patients Infected, Colonized or Noncolonized by Clostridioides Difficile" in *Microorganisms* 8, no. 5 (**2020**): 677.

[450] Kim et al., "Quantitative Characterization of Clostridioides Difficile Population in the Gut Microbiome of Patients with C. Difficile Infection and Their Association with Clinical Factors."

[451] Louie, T.J. et al.; "Differences of the Fecal Microflora With Clostridium Difficile Therapies" in *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 60 Suppl 2 (**2015**): S91-97.

3% and, in some cases, *undetectable*[452, 453, 454]. Based on this information, it was estimated the size of the inoculum, in terms of CFU/g, to be added to the fecal samples of patients with *C. difficile* AR infection, for the preparation of Batch Gut Model Cultures. Particularly, an inoculum of 10^7 CFU/g was chosen[455, 456, 457]. Prior to the final test, spikes with different concentrations of *C. difficile* (10^4, 10^5 and 10^6 CFU/mL) were carried out in fecal samples of healthy subjects to monitor the viable count of *C. difficile* under the same conditions as the assay. The count of *C. difficile* was performed on CDMN medium plates at three time points: at time 0 (T0), after 24 (T24) and 48 hours (T48). The results of the counts are summarized in the graph shown in **Figure 11**. In particular, the growth of *C. difficile* remained relatively stable until 24 hours, while it increased after 48 hours.

[452] Crobach et al., "The Bacterial Gut Microbiota of Adult Patients Infected, Colonized or Noncolonized by Clostridioides Difficile."

[453] Kim et al., "Quantitative Characterization of Clostridioides Difficile Population in the Gut Microbiome of Patients with C. Difficile Infection and Their Association with Clinical Factors."

[454] Louie et al., "Differences of the Fecal Microflora With Clostridium Difficile Therapies."

[455] Crobach et al., "The Bacterial Gut Microbiota of Adult Patients Infected, Colonized or Noncolonized by Clostridioides Difficile."

[456] Kim et al., "Quantitative Characterization of Clostridioides Difficile Population in the Gut Microbiome of Patients with C. Difficile Infection and Their Association with Clinical Factors."

[457] Louie et al., "Differences of the Fecal Microflora With Clostridium Difficile Therapies."

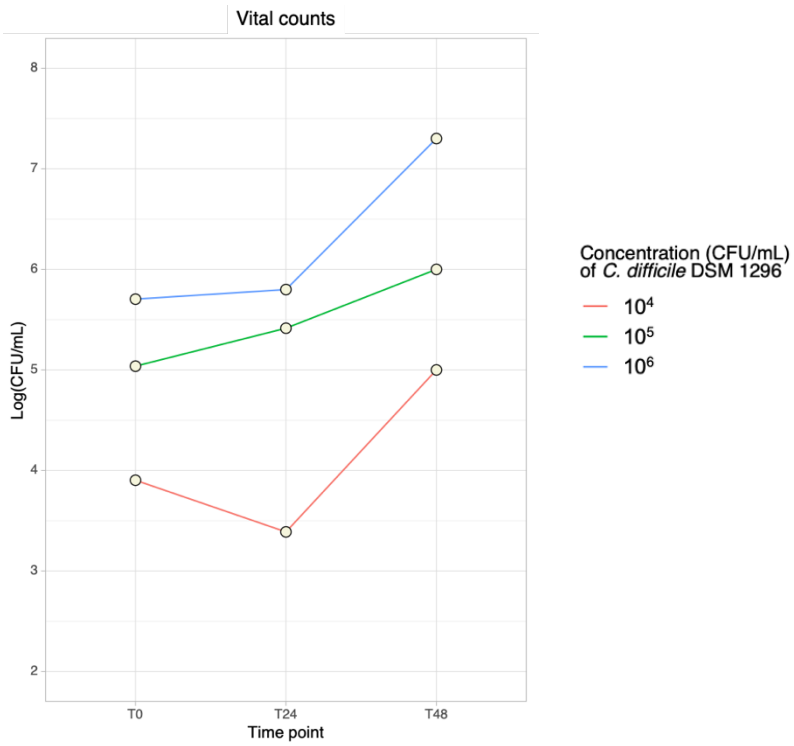

Figure 11. Results of vital counts in *C. difficile* plates after spike with different concentrations of *C. difficile* DSM 1296 (10^4, 10^5 and 10^6 CFU/mL) in the fecal sample of a healthy subject.

Finally, the OD/CFU conversion was determined by setting up an o/n culture of *C. difficile* DSM 1296, consistent with the literature[458]. In particular, the following ratio was identified: 1 OD = 2×10^8 CFU/mL. During these preliminary stages, the operating volumes, summarized in Chapter 3.3.4.1, were also determined.

3.2.2 Glycerol stock preparation

Following the growth of *C. difficile* on selective medium, glycerinates of *C. difficile* DSM 1296 were prepared from a single colony grown on FAA medium with 10% horse defibrinated blood and from a single colony grown on mGAM medium. Glycerinates of *C. difficile* AR isolates from patients were prepared from a single colony grown on CDMN medium. In short,

[458] Passmore, I.J. et al.; "Para-Cresol Production by Clostridium Difficile Affects Microbial Diversity and Membrane Integrity of Gram-Negative Bacteria" in *PLoS pathogens* 14, no. 9 (**2018**): e1007191.

colonies were put in liquid selective medium (CDB) and incubated in an anaerobic hood at 37°C o/n. The following day, bacterial cultures were centrifuged (5000 rpm for 10 minutes) and the pellet was resuspended in 1.5 mL of CDB selective medium with 10% glycerol. The suspensions were dispensed in sterile cryovials, which were immediately frozen and stored at -80°C.

3.2.3 Minimum Inhibitory Concentration of vancomycin against C. difficile

Analysis of the clinical history of patients with *C. difficile* AR infection in progress showed treatment with vancomycin (2 cycles lasting 7 days each, administered at a distance of one month before FMT) in one of the patients from whose fecal samples colonies of the pathogen were isolated. The Minimum Inhibitory Concentration (MIC) of vancomycin was therefore evaluated against the *C. difficile* strain isolated from the patient. Specifically, concentrations were tested in a range from 0.016 mg/mL to 32 mg/mL according to EUCAST data[459]. The MIC of vancomycin against *C. difficile* isolated from the patient was found to be 4 mg/mL. Based on the EUCAST reports[460], this strain was found to be resistant.

[459] "European Committee on Antimicrobial Susceptibility Testing. Data from the EUCAST MIC Distribution Website, Last Accessed 13 May 2024". https://www.eucast.org."

[460] Ibid.

3.2.4 Preparation of Batch Gut Model Cultures and subsequent analysis of fermentative samples

3.2.4.1 Set up of the in vitro assay

To assess the efficacy of the four compounds, named compound 1, compound 2, compound 3 and compound 4 on AR *C. difficile* and the human GM, a Batch Gut Model Cultures assay was set up using 2-mL 24-multiwell plates (**Figure 12**). The compounds to be tested were resuspended at the desired concentrations (summarized in **Table 2**) in a 1% methylcellulose suspension, which had been left in agitation o/n. The suspensions thus obtained were aliquoted in the wells while maintaining continuous agitation. Then, each well was added with mGAM medium and the fecal slurry with 10^7 CFU/mL of AR *C. difficile*. In total, three tests were carried out using dosages from 800 to 0.05 mg (**Table 2**).

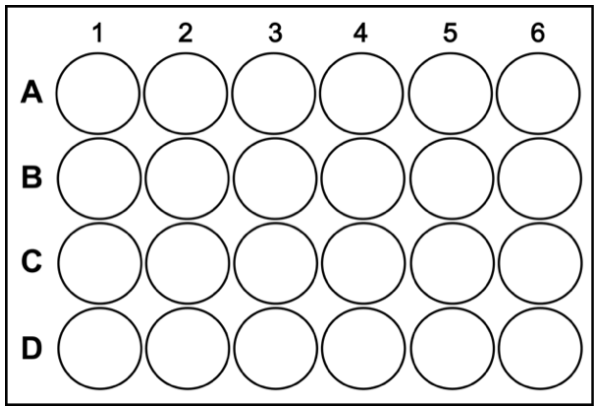

Figure 12. Diagram of the 24-multiwell plate used for the Batch Gut Model Cultures assays.

Table 2. Compounds dosages for each Batch Gut model Cultures assay.

	Compound 1	Compound 2	Compound 3	Compound 4
Assay 1	800 mg, 80 mg, 8 mg	800 mg, 80 mg, 8 mg	800 mg, 80 mg, 8 mg	800 mg, 80 mg, 8 mg
Assay 2	8 mg, 1.6 mg, 0.8 mg	8 mg, 1.6 mg, 0.8 mg	8 mg, 1.6 mg, 0.8 mg	8 mg, 1.6 mg, 0.8 mg
Assay 3	0.8 mg, 0.4 mg, 0.2 mg	0.8 mg, 0.4 mg, 0.2 mg, 0.1 mg, 0.05 mg	0.8 mg, 0.4 mg, 0.2 mg, 0.1 mg, 0.05 mg	/

Compounds were weighed considering the purity degree of each one as reported in the HPLC certificate of analysis, given by the company, namely:

- Compound 1: 99.5%
- Compound 2: 86.9%
- Compound 3: 98.2%
- Compound 4: 98.3%

Three controls were also prepared in the wells of the multiwell plate:

⇒ Control 1: compounds to be tested at the highest concentration, without the fecal slurry, to verify the absence of cross-contamination;

⇒ Control 2: mGAM medium, without further additions, to further verify the absence of cross-contamination;

⇒ Control 3: fecal slurry without compounds, to evaluate the interactions of AR *C. difficile* and the GM in the absence of the compounds.

Each condition and controls were tested in triplicate. The operating volumes used in each well of the Batch Gut Model Cultures assay are summarized in **Table 3**.

Table 3. Operating volumes for Batch Gut Model Cultures assays (final volume 2 mL).

	Compound	Slurry	mGAM
Wells with compounds	333 µL	200 µL	1467 µL
Wells with compounds at the highest concentration (control 1)	333 µL	/	1667 µL
Wells with mGAM medium (control 2)	/	/	2000 µL
Wells with slurry (control 3)	/	200 µL	1800 µL

From each well, at three time points (T0, T24 and T48), 500 µL aliquots were collected, stored at -80°C, to monitor the viable count of AR *C. difficile* and to assess the impact on the GM by sequencing the 16S rRNA gene (next-generation sequencing). For viable plate count, a pool from triplicates was prepared for each test condition, which was used to inoculate selective medium plates (CDMN). The next day the growth of *C. difficile* was evaluated.

3.2.4.2 Compounds MIC assay against C. difficile

Since all compounds at the concentrations given in **Table 2** were able to inhibit the growth of *C. difficile* already at T24, except compound 4 (see **Table 7-9** in Chapter 3.3.1), an assay was prepared using the same setup as the Batch Gut Model Cultures assay to identify the MIC of the compounds 1, 2 and 3. The determination of the MIC was performed with spikes (10^5 CFU/mL) of both the patient isolated AR *C. difficile* strain and *C. difficile* type strain DSM 1296 (for operating volumes, refer to **Table 3**, Chapter 3.2.4.1). In particular, for each compound, the following concentrations were used:

- 0.05 mg/mL
- 100 ng/mL
- 50 ng/mL

- 25 ng/mL
- 12.5 ng/mL
- 6.25 ng/mL
- 3.125 ng/mL
- 1.56 ng/mL
- 0.78 ng/mL
- 0.39 ng/mL

The 0.05 mg/mL concentration was tested again to obtain data similar to those observed in previous tests with Batch Gut Model Cultures.

For compound 3, the following concentrations were also tested:

- 0.05 mg/mL
- 0.025 mg/mL
- 0.0125 mg/mL
- 0.00625 mg/mL
- 0.003125 mg/mL
- 1562.5 ng/mL
- 781 ng/mL
- 391 ng/mL
- 195 ng/mL
- 100 ng/mL
- 50 ng/mL
- 25 ng/mL

For each compound, a stock solution was prepared at a concentration of 0.05 mg/mL in a final volume of 10 mL, considering the degree of purity reported in the HPLC certificate of analysis provided by the company, namely:

- Compound 1: 99.5% → 0.502 mg
- Compound 2: 86.9% → 0.575 mg
- Compound 3: 98.2% → 0.509 mg

To assess the presence of bacterial cells, OD_{600} was measured using a spectrophotometer. **Table 4** summarizes the MICs identified for each compound tested.

Table 4. MICs for the three compounds.

	Compound 1	**Compound 2**	**Compound 3**
Patient isolated AR *C. difficile* strain	12.5 ng/mL	12.5 ng/mL	781.3 ng/mL
C. difficile* type strain *DSM 1296	3.12 ng/mL	6.25 ng/mL	390.6 ng/mL

In addition, the Minimum Bactericidal Concentration (MBC) was assessed by plating an aliquot of the well corresponding to the MIC on agarized mGAM medium, as well as aliquots corresponding to three concentrations above and three concentrations below the MIC value, and observing growth after 24 hours. The results are summarized in **Table 5**. The choice of the agarized mGAM medium allowed the analysis of the MBC under the same conditions of the assay with Batch Gut Model Cultures.

Table 5. MBCs for the three compounds.

	Compound 1	Compound 2	Compound 3
Patient isolated AR *C. difficile* strain	25.0 ng/mL	25.0 ng/mL	1562.5 ng/mL
***C. difficile* type strain DSM 1296**	25.0 ng/mL	25.0 ng/mL	781.3 ng/mL

The identification of the MIC/MBC of each compound allowed to prepare the last assay with the Batch Gut Model Cultures model. Notably, as described in the next paragraph, two concentrations above and two concentrations below the MIC were selected, in order to assess any differences in compound response from both AR *C. difficile* isolated from the patient and *C. difficile* type strain DSM 1296.

3.2.4.3 Final Batch Gut Model Cultures Assay

The final Batch Gut Model Cultures assay was prepared according to the experimental conditions identified in the previous assays, testing for each compound the concentrations reported in **Table 6**.

Table 6. Final Batch Gut Model Cultures assay concentrations for the three compounds.

	Compound 1	Compound 2	Compound 3
Patient isolated AR *C. difficile* strain	50 ng/mL	50 ng/mL	3125.2 ng/mL
	25 ng/mL	25 ng/mL	1562.6 ng/mL
	12.5 ng/mL	12.5 ng/mL	781.3 ng/mL
	6.25 ng/mL	6.25 ng/mL	390.65 ng/mL
	3.125 ng/mL	3.125 ng/mL	195.33 ng/mL
***C. difficile* type strain DSM 1296**	12.5 ng/mL	25 ng/mL	1562.6 ng/mL
	6.25 ng/mL	12.5 ng/mL	781.3 ng/mL
	3.125 ng/mL	6.25 ng/mL	390.65 ng/mL
	1.56 ng/mL	3.125 ng/mL	195.33 ng/mL
	0.78 ng/mL	1.56 ng/mL	97.65 ng/mL

As indicated above, for each compound a stock solution was prepared at a concentration of 0.05 mg/mL in a volume of 10 mL, considering the degree of purity reported in the HPLC certificate of analysis, namely:

- Compound 1: 99.5% → 0.502 mg
- Compound 2: 86.9% → 0.575 mg
- Compound 3: 98.2% → 0.509 mg

3.2.5 Next-generation sequencing analysis

For each experimental condition of the Batch Gut Model Cultures (assay 1, 2, 3), 500 µL of each sample were collected for the subsequent microbial DNA extraction. The DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN), following the protocol described by Barone et al.[461]. Briefly, the microbial DNA was obtained from samples adding 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris−HCl pH 8, 50 mM EDTA, 4% [w/v] SDS), with 0.5 g of 0.1 mm zirconia beads (BioSpec Products) and four 3 mm glass beads, and homogenized using the FastPrep instrument (MP Biomedicals) with 3 × 1 minute bead-beating steps at 5.5 movements/s. After a 15-minutes incubation phase at 95°C, followed by a 5-minute 13,000 rpm centrifugation, the nucleic acids were precipitated by adding 260 µL of ammonium acetate and one volume of isopropanol. The pellets were then suspended in TE buffer. The following treatment with 2 µL of DNase-free RNase (10 mg/mL) at 37°C for 15 minutes and 15 µL of proteinase K at 70°C for 10 minutes allowed the removal of RNA and proteins. Ultimately, DNA purification was performed on silica columns following the manufacturer's instructions (QIAGEN). The purified DNA was measured with a NanoDrop ND-1000 spectophotometer (NanoDrop Technologies).

[461] Barone et al., "Searching for New Microbiome-Targeted Therapeutics through a Drug Repurposing Approach."

Library preparation was performed as described in the Illumina protocol "16S Metagenomic Sequencing Library Preparation" (Illumina). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 785R primers containing Illumina adapter overhang sequences as formerly reported[462]. KAPA HiFi HotStart ReadyMix (Roche) was then used for amplification, setting the thermocycle with the following protocol: 1 cycle of 3 minutes at 95°C, 25 cycles of 30 seconds at 95°C, 1 cycle of 30 seconds at 55°C, and 1 cycle at 30 seconds at 72°C, with a final step of 5 minutes at 72°C. The purification of the amplicons was performed with a magnetic bead-based clean-up system Agencourt AMPure XP (Beckman Coulter). Indexed libraries were prepared by limited-cycle PCR through the Nextera technology (Illumina), followed by a second clean-up step as aforementioned. An extraction control was processed along with the samples, as per good laboratory practices. After pooling the samples to an equimolar concentration of 4 nM, the resulting library was denatured and diluted to 5 pM using a 20% PhiX control. Following the manufacturer's instructions, sequencing was carried out on an Illumina MiSeq platform using a 2 × 250 bp paired-end technique.

Raw sequence data were analyzed using a pipeline combining PANDAseq[463] and QIIME 2[464]. All sequences were filtered for length (minimum = 350 bp; maximum = 550 bp) and quality (default parameters). Next, the DADA2 pipeline was used to bin the remaining reads into amplicon sequence variants (ASVs). Taxonomic classification was performed using the VSEARCH algorithm against the Greengenes database (May 2019 release). The resulting ASV tables were used for computing the intra-sample biodiversity (α-diversity) using

[462] Klindworth, A. et al.; "Evaluation of General 16S Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-Based Diversity Studies" in *Nucleic Acids Research* 41, no. 1 (**2013**): e1.

[463] Masella, A.P. et al.; "PANDAseq: Paired-End Assembler for Illumina Sequences" in *BMC Bioinformatics* 13, no. 1 (**2012**): 31.

[464] Bolyen et al., "Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2."

ecological indices such as the Shannon index, Faith's Phylogenetic Diversity and the Observed ASVs. The graph representing the viable counts in *C. difficile* plates after spike with different concentrations of *C. difficile* DSM 1296 in the fecal sample of a healthy subject (for the preliminary activities) and graphs representing α-diversity in the final assay were made using the "ggplot" function from the "ggplot2" package (https://cran.r-project.org/web/packages/ggplot2/index.html), while the taxonomic assignment of the identified ASVs, in order to assess differences in the composition of the intestinal microbial ecosystem in the presence of the tested compounds, was visualized with genus-level barplots built using the "barplot" function in the open source software R Studio (version 2023.06.2+561) and R (version 4.3.1).

3.3 Results

3.3.1 In vitro assay

Table 7, **8** and **9** summarize the plate count results of the three assay with Batch Gut Model Cultures, with different concentrations at T0, T24 and T48.

Table 7. Counts of assay 1 at three time points, for the four compounds at defined dosages (the numbers near the compounds indicate the dosage in mg). "/" corresponds to a value < 1E+03 CFU/mL.

T0

Experimental settings	CFU/mL
Slurry + spike	4.71E+07
Compound 1 800	/
Compound 1 80	2.00E+05
Compound 1 8	4.00E+06
Compound 2 800	/
Compound 2 80	/
Compound 2 8	3.40E+06

T24

Experimental settings	CFU/mL
Slurry + spike	1E+09
Compound 1 800	/
Compound 1 80	/
Compound 1 8	/
Compound 2 800	/
Compound 2 80	/
Compound 2 8	/

T48

Experimental settings	CFU/mL
Slurry + spike	8.76E+08
Compound 1 800	/
Compound 1 80	/
Compound 1 8	/
Compound 2 800	/
Compound 2 80	/
Compound 2 8	/

T0

Experimental settings	CFU/mL
Slurry + spike	2.81E+07
Compound 3 800	2.00E+05
Compound 3 80	1.50E+06
Compound 3 8	7.80E+06
Compound 4 800	/
Compound 4 80	/
Compound 4 8	1.70E+06

T24

Experimental settings	CFU/mL
Slurry + spike	7.12E+08
Compound 3 800	/
Compound 3 80	/
Compound 3 8	/
Compound 4 800	/
Compound 4 80	/
Compound 4 8	/

T48

Experimental settings	CFU/mL
Slurry + spike	1.51E+09
Compound 3 800	/
Compound 3 80	/
Compound 3 8	/
Compound 4 800	/
Compound 4 80	/
Compound 4 8	/

Table 8. Counts of assay 2 at three time points, for the four compounds at defined dosages (the numbers near the compounds indicate the dosage in mg). " /" corresponds to a value < 1E+03 CFU/mL.

T0

Experimental settings	CFU/mL
Slurry+spike	9.64E+07
Compound 1 8	3.42E+07
Compound 1 1.6	8.24E+07
Compound 1 0.8	9.10E+07
Compound 2 8	8.40E+06
Compound 2 1.6	3.21E+07
Compound 2 0.8	8.94E+07

T24

Experimental settings	CFU/mL
Slurry+spike	>1.00E+09
Compound 1 8	/
Compound 1 1.6	/
Compound 1 0.8	6.00E+05
Compound 2 8	/
Compound 2 1.6	/
Compound 2 0.8	/

T48

Experimental settings	CFU/mL
Slurry+spike	>1.00E+09
Compound 1 8	/
Compound 1 1.6	/
Compound 1 0.8	/
Compound 2 8	/
Compound 2 1.6	/
Compound 2 0.8	/

T0

Experimental settings	CFU/mL
Slurry+spike	6.16E+07
Compound 3 8	2.90E+07
Compound 3 1.6	2.39E+07
Compound 3 0.8	6.07E+07
Compound 4 8	5.56E+07
Compound 4 1.6	6.82E+07
Compound 4 0.8	9.56E+07

T24

Experimental settings	CFU/mL
Slurry+spike	>1.00E+09
Compound 3 8	/
Compound 3 1.6	/
Compound 3 0.8	/
Compound 4 8	/
Compound 4 1.6	>1.00E+09
Compound 4 0.8	>1.00E+09

T48

Experimental settings	CFU/mL
Slurry+spike	>1.00E+09
Compound 3 8	/
Compound 3 1.6	/
Compound 3 0.8	/
Compound 4 8	/
Compound 4 1.6	>1.00E+09
Compound 4 0.8	>1.00E+09

Table 9. Counts of assay 3 at three time points, for the four compounds at defined dosages (the numbers near the compounds indicate the dosage in mg). "/" corresponds to a value < 1E+03 CFU/mL.

T0

Experimental settings	CFU/mL
Slurry+spike	9.80E+07
Compound 1 0.8	7.49E+07
Compound 1 0.4	7.82E+07
Compound 1 0.2	3.04E+07
Compound 2 0.8	5.82E+07
Compound 2 0.4	9.54E+07
Compound 2 0.2	7.68E+07
Compound 2 0.1	2.84E+07
Compound 2 0.05	6.68E+07
Compound 3 0.8	4.96E+07
Compound 3 0.4	1.05E+08
Compound 3 0.2	3.02E+07
Compound 3 0.1	8.04E+07
Compound 3 0.05	8.42E+07

T24

Experimental settings	CFU/mL
Slurry+spike	1.64E+07
Compound 1 0.8	/
Compound 1 0.4	/
Compound 1 0.2	/
Compound 2 0.8	/
Compound 2 0.4	/
Compound 2 0.2	/
Compound 2 0.1	/
Compound 2 0.05	/
Compound 3 0.8	/
Compound 3 0.4	/
Compound 3 0.2	/
Compound 3 0.1	/
Compound 3 0.05	/

T48

Experimental settings	CFU/mL
Slurry+spike	>1.00E+09
Compound 1 0.8	/
Compound 1 0.4	/
Compound 1 0.2	/
Compound 2 0.8	/
Compound 2 0.4	/
Compound 2 0.2	/
Compound 2 0.1	/
Compound 2 0.05	/
Compound 3 0.8	/
Compound 3 0.4	/
Compound 3 0.2	/
Compound 3 0.1	/
Compound 3 0.05	/

Table 10 shows the results of the MIC performed for compound 1, 2 and 3 against both the patient isolated AR *C. difficile* strain and *C. difficile* type strain DSM 1296.

Table 10. Final assay counts at three time points for the three compounds at defined concentrations. In red is indicated the MIC for the AR *C. difficile* strain isolated by the patient, in blue the MIC for *C. difficile* type strain DSM 1296. Red background cells indicate the results of the concentrations of the compounds tested on AR *C. difficile* isolated by the patient, blue background cells indicate the results of the concentrations of the compounds tested on *C. difficile* type strain DSM 1296. "/" corresponds to a value < 1E+03 CFU/mL, "-" indicates that the concentration was not tested.

T0

Experimental settings (ng/mL)	CFU/mL	
	C. difficile isolated from patient	Type strain DSM 1296
Slurry	5.14E+07	4.71E+07
Compound 1 50	4.68E+07	-
Compound 1 25	5.63E+07	-
Compound 1 **12.5**	3.88E+07	4.42E+07
Compound 1 6.25	6.70E+07	3.90E+07
Compound 1 **3.125**	4.52E+07	6.62E+07
Compound 1 1.56	-	3.77E+07
Compound 1 0.78	-	6.58E+07
Compound 2 50	5.45E+07	-
Compound 2 25	3.07E+07	4.56E+07
Compound 2 **12.5**	3.32E+07	2.66E+07
Compound 2 **6.25**	4.73E+07	5.32E+07
Compound 2 3.125	3.10E+07	4.02E+07
Compound 2 1.56	-	4.86E+07
Compound 3 3125.2	3.60E+07	-
Compound 3 1562.6	3.58E+07	5.72E+07
Compound 3 **781.3**	2.80E+07	4.34E+07
Compound 3 **390.65**	5.02E+07	5.70E+07
Compound 3 195.33	4.90E+07	5.80E+07
Compound 3 97.65	-	2.41E+08

T24

Experimental settings (ng/mL)	CFU/mL	
	C. difficile isolated from patient	Type strain DSM 1296
Slurry	>1.00E9	>1.00E9
Compound 1 50	/	-
Compound 1 25	/	-
Compound 1 **12.5**	/	/
Compound 1 6.25	/	/
Compound 1 **3.125**	/	/
Compound 1 1.56	-	>1.00E9
Compound 1 0.78	-	5.18E+07
Compound 2 50	/	-
Compound 2 25	/	/
Compound 2 **12.5**	/	/
Compound 2 **6.25**	/	/
Compound 2 3.125	7.72E+07	7.96E+07
Compound 2 1.56	-	>1.00E9
Compound 3 3125.2	/	-
Compound 3 1562.6	/	/
Compound 3 **781.3**	/	/
Compound 3 **390.65**	/	/
Compound 3 195.33	4.60E+06	/
Compound 3 97.6	-	1.60E+06

T48

Experimental settings (ng/mL)	CFU/mL	
	C. difficile isolated from patient	Type strain DSM 1296
Slurry	>1.00E9	>1.00E9
Compound 1 50	/	-
Compound 1 25	/	-
Compound 1 **12.5**	/	/
Compound 1 6.25	/	/
Compound 1 **3.125**	1.20E+06	/
Compound 1 1.56	-	>1.00E9
Compound 1 0.78	-	>1.00E9
Compound 2 50	/	-
Compound 2 25	/	/
Compound 2 **12.5**	/	/
Compound 2 **6.25**	/	/
Compound 2 3.125	1.01E+08	/
Compound 2 1.56	-	>1.00E9
Compound 3 3125.2	/	-
Compound 3 1562.6	/	/
Compound 3 **781.3**	/	/
Compound 3 **390.65**	3.90E+07	2.00E+06
Compound 3 195.33	>1.00E9	>1.00E9
Compound 3 97.6	-	>1.00E9

3.3.2 Next-generation sequencing analysis

Regarding the taxonomic composition of the GM under different test conditions, relative abundance data were analyzed at genus level for dosages 800 mg, 80 mg, 8 mg (assay 1), for 0.8 mg, 1.6 mg and 0.8 mg (assay 2) and for the final assay concentrations summarized in **Table 6**.

Regarding assay 1 (**Figure 13**), it should be noted that the percentage of *Clostridium* (red) increased over time in the slurry while zeroing in the presence of the compounds 1, 2 and 3, at each of the concentrations tested. In contrast, in the case of compound 4, *Clostridium* abundance remains high both after 24 and 48 hours at all dosages. At the same time, for all compounds, a clear prevalence of *Enterococcus* was observed over time.

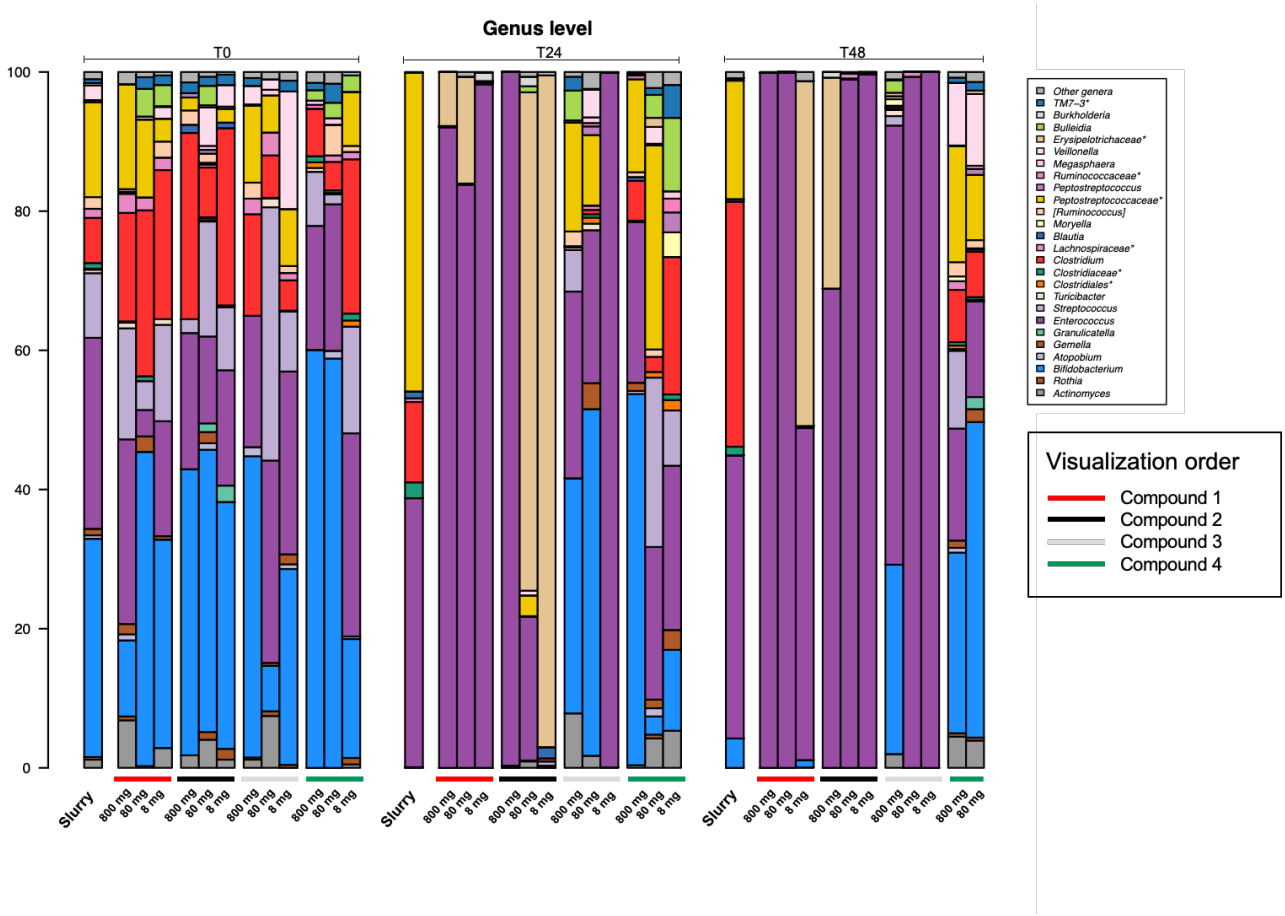

Figure 13. Genus relative abundance profiles of the gut microbiota for each condition of Batch Gut Model Cultures in assay 1. Barplots report the data on slurry and in the presence of compounds 1, 2, 3 and 4 at dosages 800 mg, 80 mg and 8 mg at time points T0, T24 and T48.

Similar results were obtained for assay 2 (**Figure 14**): the abundance of *Clostridium* genus increased over time in the slurry, while it was resetting with compounds 1, 2 and 3, but not with compound 4. Moreover, as already seen in assay 1, for each compound, at each concentration, over time the biodiversity tended to decrease, with a prevalence of *Enterococcus*.

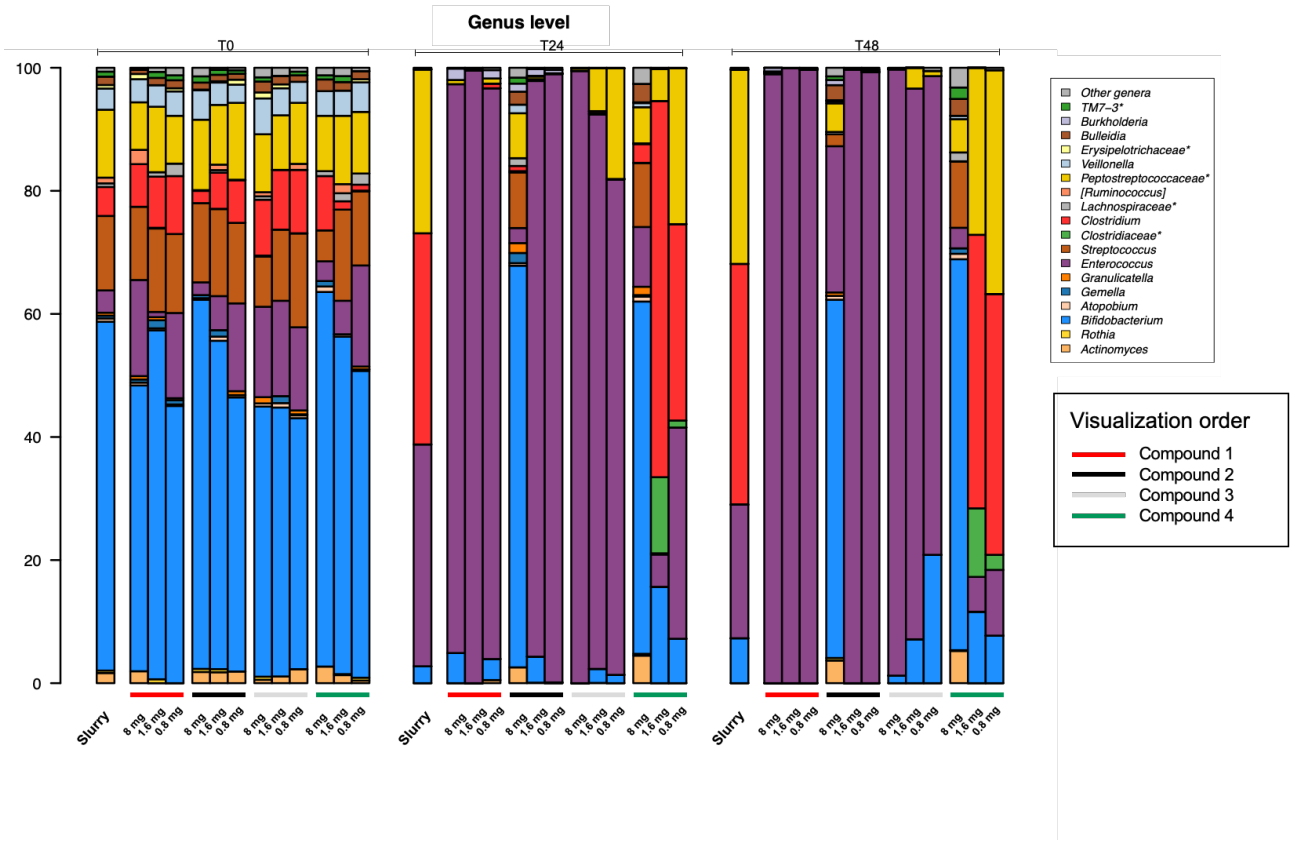

Figure 14. Genus relative abundance profiles of the gut microbiota for each condition of Batch Gut Model Cultures in assay 2. Barplots report the data on slurry and in the presence of compounds 1, 2, 3 and 4 at dosages 8 mg, 1.6 mg and 0.8 mg at time points T0, T24 and T48.

For the last assay, α-diversity data, both for AR *C. difficile* isolated from the patient and *C. difficile* type strain DSM 1296, were also analyzed and are shown respectively in **Figure 15** and **16**. In particular, for the samples spiked with AR *C. difficile* isolated from the patient, it is possible to note that α-diversity values in all the three compounds were quite similar. Especially, for compound 1 α-diversity values were slightly higher while for compound 3 they were a little lower. Notably, the lowest concentration in compound 1 (3.125 ng/mL) resulted in the highest α-diversity at T24. For compound 3, there was a similar pattern for the

Phylogenetic diversity and Observed ASVs metrics, while for the Shannon index at the lowest concentration (195.33 ng/mL) diversity markedly decreased. Generally, for all the three compounds, there was a decrease in the biodiversity at T24 compared to T0, with an increase at T48. Nonetheless, at T48, the biodiversity values of the slurry (control) were definitely higher compared to the treatments with all the metrics used.

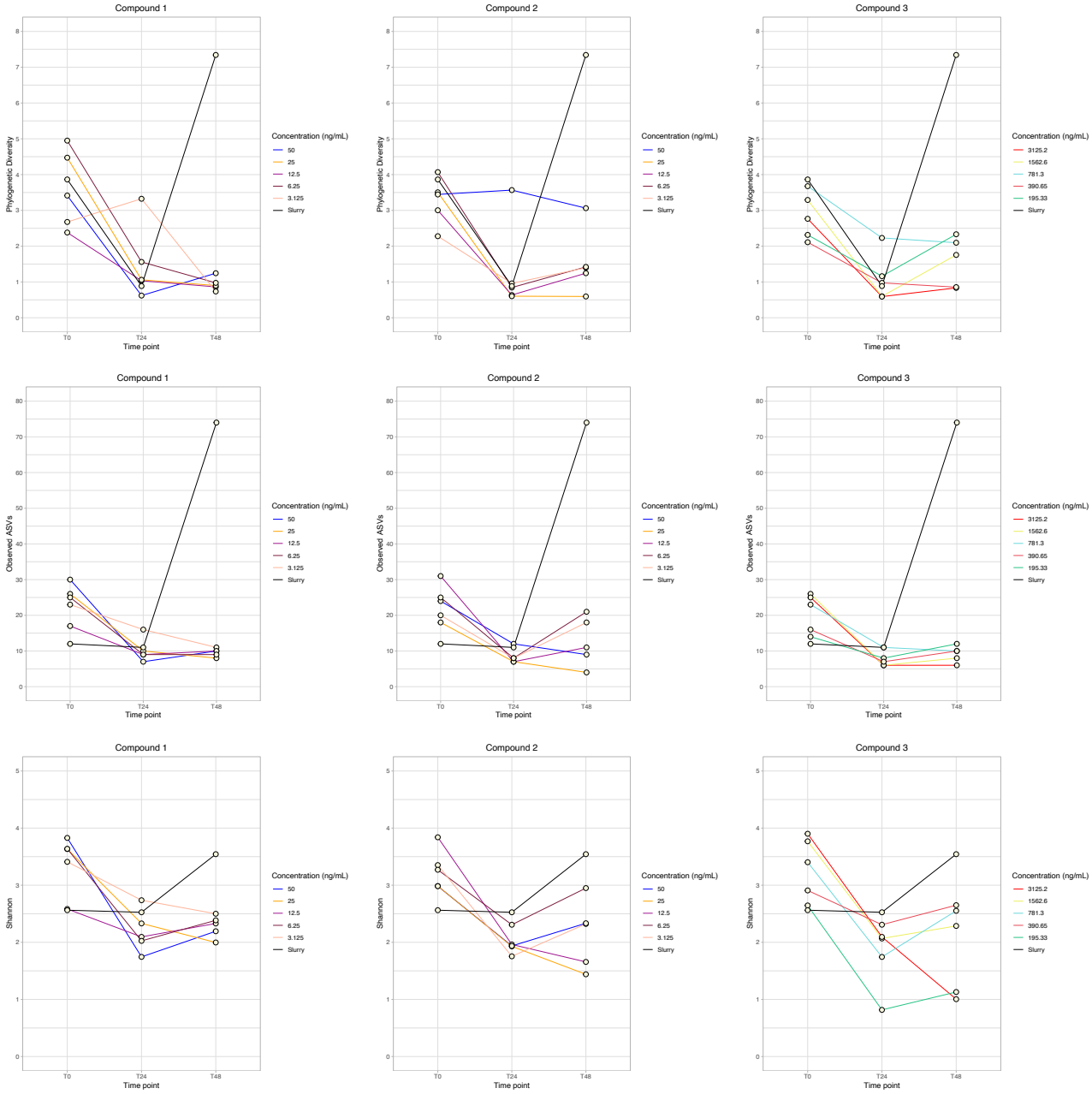

Figure 15. Analysis of the α-diversity, measured by Phylogenetic Diversity, Observed ASVs and Shannon indexes, for compounds 1, 2, 3 at T0, T24 and T48 time points, compared to fecal slurry (Slurry). The samples analyzed correspond to those in the last assay with Batch Gut Model Cultures spiked with AR *C. difficile* isolated from the patient.

For samples spiked with *C. difficile* type strain DSM 1296 (**Figure 16**), the layout seems a bit different. Generally, for all the compounds with the three metrics, no big differences were found but, compared to the samples spiked with AR *C. difficile* isolated from the patient, there was not a general decrease from T0 to T24. For compound 1, at 12.5 ng/mL and 0.78 ng/mL, respectively the highest and the lowest concentrations, the α-diversity values were the highest in Phylogenetic diversity and Observed ASVs metrics. This pattern was also visible for compound 2, where for the 12.5 ng/mL concentration there was a peak in biodiversity at T24 with all the three metrics. The compound 3 showed a different behavior based on concentration: at T24 it showed the same trend for 1562.5 ng/mL and 195.33 ng/mL, with α-diversity reducing and then raising again over time. This behavior differed from that of other concentrations, where the diversity raised at T24. At T48, the concentration 390.65 ng/mL showed a peak with all the three metrics. Compared to the results of the samples spiked with AR *C. difficile*, it seems that the concentration of the compounds has a major effect on the diversity, despite the fact that here the α-diversity values were lower.

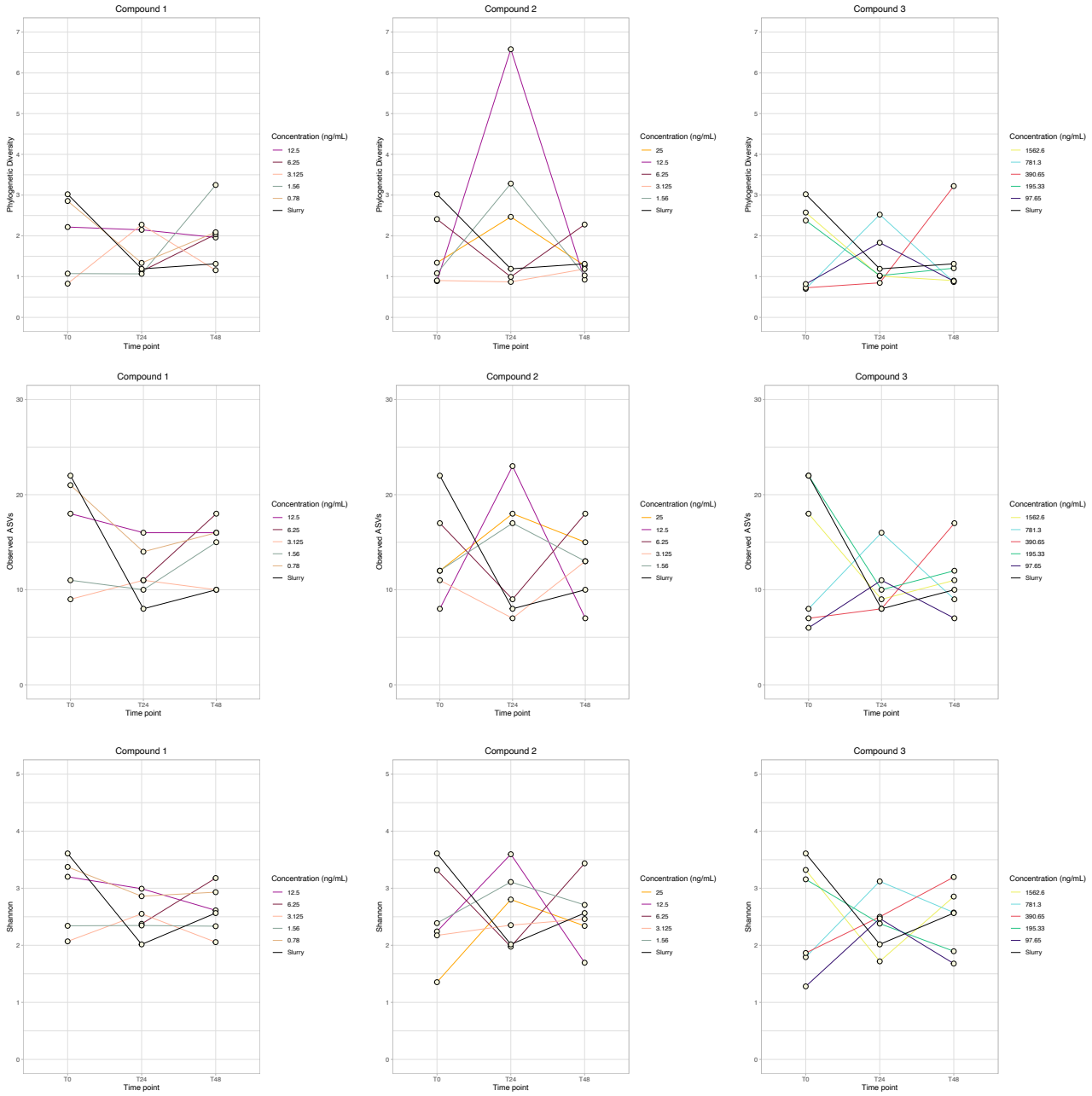

Figure 16. Analysis of the α-diversity, measured by Phylogenetic Diversity, Observed ASVs and Shannon indexes, for compounds 1, 2, 3 at T0, T24 and T48 time points, compared to fecal slurry (Slurry). The samples analyzed correspond to those in the last assay with Batch Gut Model Cultures spiked with *C. difficile* type strain DSM 1296.

From the taxonomic standpoint (**Figures 17** and **18**), the abundance of *Clostridium* increased over time in the slurry while decreasing to zero at concentrations above the MIC. Moreover, for each compound, at each concentration, over time there was a tendency towards reduced biodiversity, with a prevalence of *Enterococcus* in samples with AR *C. difficile* isolated from the patient, in line with previous analyses. Instead, in samples spiked

with *C. difficile* type strain DSM 1296, ecosystem diversity remained higher. Regardless of the origin of the *C. difficile* strain, an increase in *Bifidobacterium* at low concentrations of compounds was noted.

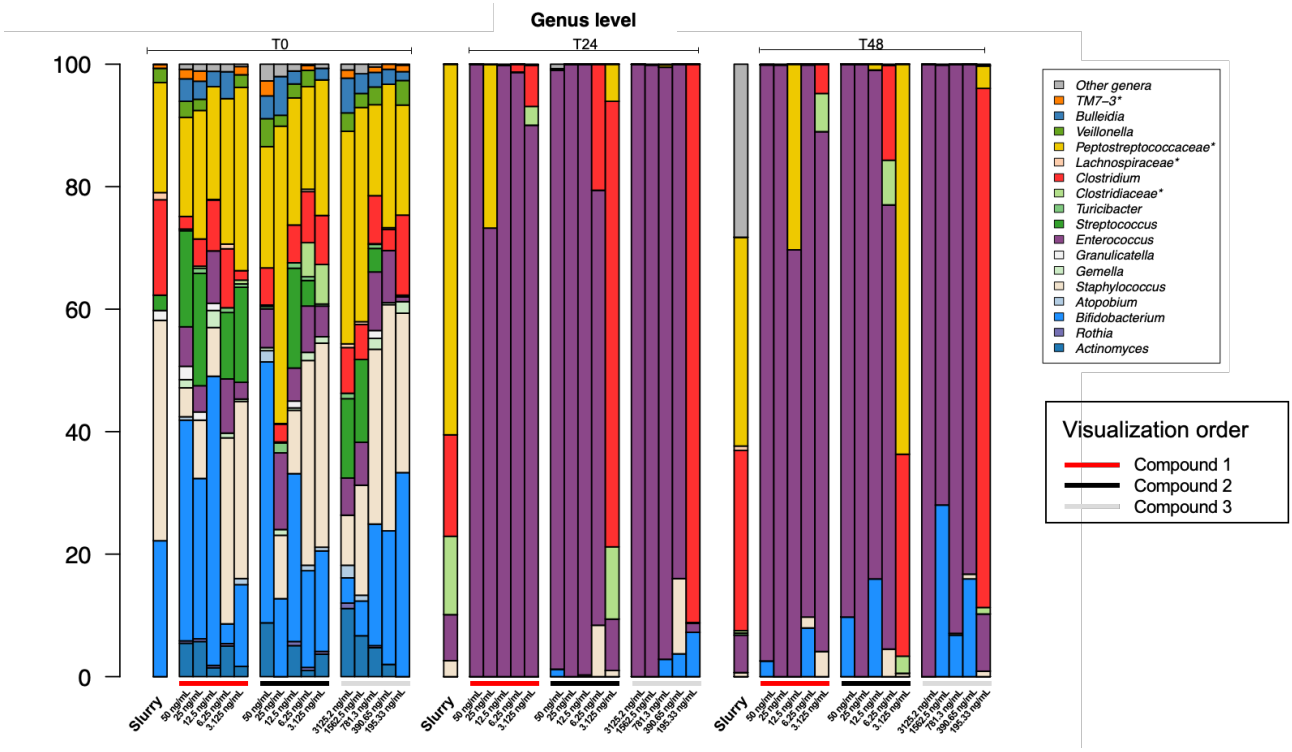

Figure 17. Genus relative abundance profiles of the gut microbiota for each condition of Batch Gut Model Cultures in the final assay, in which the slurry was added with AR *C. difficile* isolated from the patient. Barplots report the data on slurry and in the presence of compounds 1, 2, 3 and 4 at the concentrations reported in Table 9 at time points T0, T24 and T48.

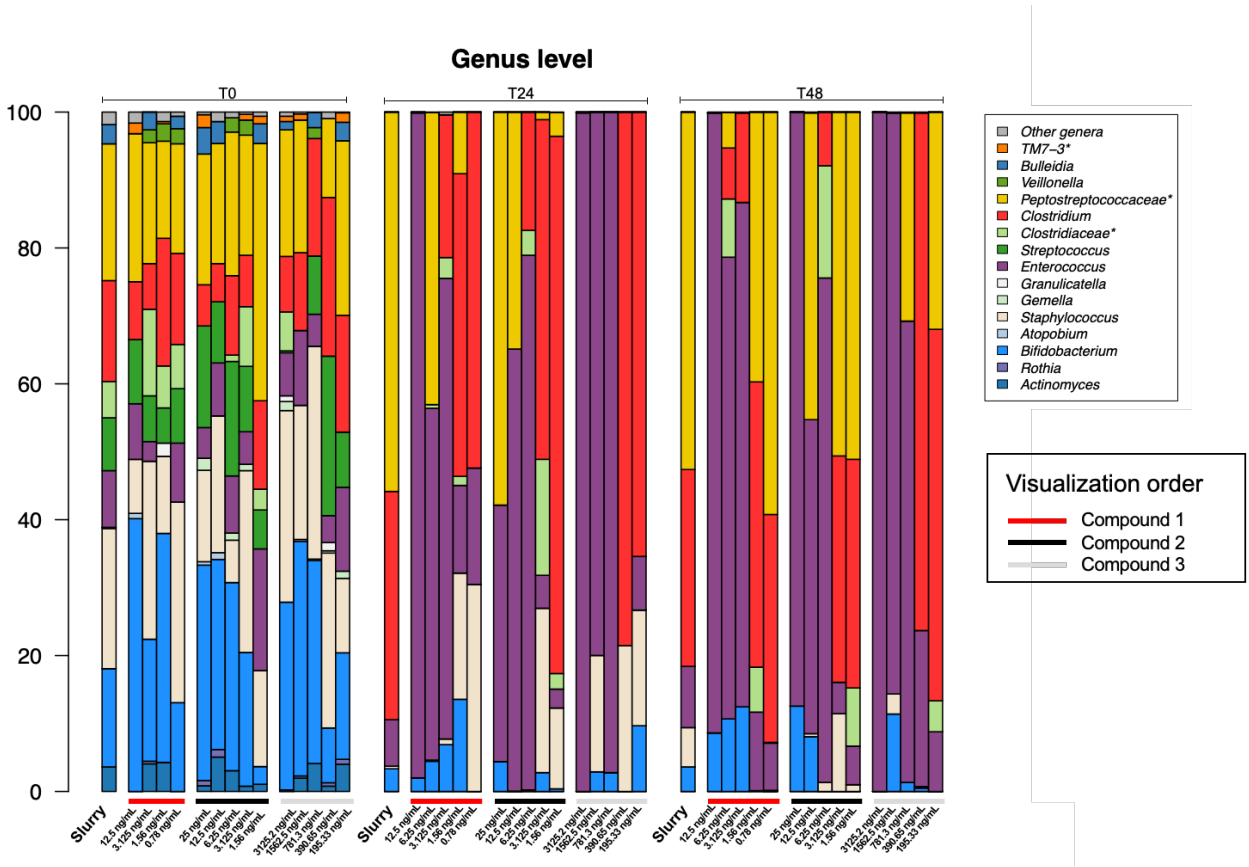

Figure 18. Genus relative abundance profiles of the gut microbiota for each condition of Batch Gut Model Cultures in the final assay, in which the slurry was added with *C. difficile* type strain DSM 1296. Barplots report the data on slurry and in the presence of compounds 1, 2, 3 and 4 at the concentrations reported in Table 9 at time points T0, T24 and T48.

3.4 Discussion

The preparation of the *in vitro* assays with Batch Gut Model Cultures model allowed to evaluate the efficacy of four different novel compounds in counteracting the infection with *C. difficile* and its impact on the intestinal microbiota ecosystem. Particularly, this type of tests allowed to evaluate well the impact of new compounds without using more sophisticated *in vivo* models (although they are a necessary next step), giving the opportunity to refine some parameters (*i.e.* compound concentrations) and being less time- and resources-consuming compared to their *in vivo* counterparts. However, in this type of assays, since at one condition corresponds just one sample, no statistical analysis was done.

Concerning the compounds tested in these assays, compound 1 and 2 exhibited a similar bactericidal action against both the *C. difficile* strain isolated from the patient (vancomycin resistant) and *C. difficile* type strain DSM 1296. This observation was confirmed by both CDMN selective culture analysis and molecular analysis for the 800 mg to 0.8 mg dosages and the concentrations tested in the final test. In contrast, compounds 3 and 4 had a lower bactericidal action. In particular, the MBC of compound 3 was 1562.6 ng/mL (for AR *C. difficile* isolated from the patient) and 781.3 ng/mL (for *C. difficile* type strain DSM 1296). Compound 4, on the other hand, showed no activity below the dosage of 8 mg. Regarding the final assay, the compounds led to increased α-diversity in samples spiked with AR *C. difficile* compared to the samples spiked with *C. difficile* type strain DSM 1296, showing that these new compounds could have positive effects on the GM of patients with a *C. difficile* AR infection.

With regard to the impact on a taxonomy composition level, the compounds 1, 2 and 3 induced a decrease in biodiversity (**Figure 17** and **18**), reducing the percentage of *Clostridium* but allowing at the same time an expansion of *Enterococcus*. In particular, in samples spiked with AR *C. difficile*, all the compounds except compounds 2 and 3 at the

lowest concentrations allowed an almost complete expansion of *Enterococcus* genus, with also a little percentage of *Clostridium* and *Bifidobacterium*. On the other hand, in samples spiked with *C. difficile* type strain DSM 1296, *Enterococcus* prevalence was less notable at both T24 and T48, with a major expansion also of *Peptostreptococcaceae* family and *Staphylococcus* genus. The *Enterococcus* expansion could be related to the fact that the fecal sample was derived from a patient with CDI who underwent pharmacological treatment with vancomycin. Several studies have found a correlation between CDI and vancomycin-resistant *Enterococcus* (VRE) colonization[465, 466], as well as other studies have demonstrated the correlation between VRE colonization and the use of rifaximin for the treatment of other conditions. For instance, in a study of patients with cirrhosis, the treatment with rifaximin was associated with acquisition of VRE infection[467]. Also, in another study, the administration of antibiotics including rifaximin was associated with an increased rate of pre-transplant VRE acquisition in patients undergoing liver transplantation[468].

The optimization of the Batch Gut Model Cultures for testing various compounds has yielded positive outcomes regarding both their efficacy against *C. difficile* and their modulation of the entire gut ecosystem. Further in-depth studies will certainly be required to better understand the mechanisms of action of these compounds, as well as their role in a complex system as the human gut. Nevertheless, these preliminary assays are valuable for gaining a broad understanding of the effects that compounds exert on the target microorganism and

[465] Fujitani, S. et al.; "Implications for Vancomycin-Resistant Enterococcus Colonization Associated with Clostridium Difficile Infections" in *American Journal of Infection Control* 39, no. 3 (**2011**): 188–193.

[466] Özsoy, S. & A. İlki; "Detection of Vancomycin-Resistant Enterococci (VRE) in Stool Specimens Submitted for Clostridium Difficile Toxin Testing" in *Brazilian Journal of Microbiology: [publication of the Brazilian Society for Microbiology]* 48, no. 3 (**2017**): 489–492.

[467] Barger, M. et al.; "VRE in Cirrhotic Patients" in *BMC Infectious Diseases* 19, no. 1 (**2019**): 711.

[468] Banach, D.B. et al.; "The Clinical and Molecular Epidemiology of Pre-Transplant Vancomycin-Resistant Enterococci Colonization among Liver Transplant Recipients" in *Clinical Transplantation* 30, no. 3 (**2016**): 306–311.

the GM. Refining these assays can provide a more precise approach, facilitating further targeted investigations in *in vivo* animal models.

Chapter 4: NUTRITIONAL INTERVENTION IN A MURINE MODEL OF EARLY WEANING FOR THE RESTORATION OF GUT MICROBIOME DYSBIOSIS

4.1 Brief introduction

In mammals, weaning is a critical phase in the passage to adulthood. Weaning is thus not simply an event, but a gradual process that is furthermore subject to numerous environmental or social influences. In fact, this phase describes the total and complete dependence of the mother to nutritional and social independence and is accompanied by evident changes in the physiology and the behavior of both mother and progeny[469, 470]. Weaning has therefore been recognized as a major life-history variable that is vital to both sexual and reproductive strategies from an ecological point of view[471, 472, 473]. The perinatal period is described by intense ontogenetic plasticity due to increased epigenetic machinery activity[474]. Then, the exposure to stress during this period may change the epigenome by promoting individual adaptation to early environmental conditions. However, if the environmental conditions are changed throughout life, the subject becomes maladapted and susceptible to the development of NCDs and metabolic disorders[475]. With these

[469] Counsilman, J.J. & L.M. Lim; "The Definition of Weaning" in *Animal Behaviour* 33, no. 3 (**1985**): 1023–1024.

[470] Martin, P.; "Weaning: A Reply to Counsilman & Lim" in *Animal Behaviour* 33, no. 3 (**1985**): 1024–1026.

[471] Lee, P.C.; "The Meanings of Weaning: Growth, Lactation, and Life History" in *Evolutionary Anthropology: Issues, News, and Reviews* 5, no. 3 (**1996**): 87–98.

[472] Richter, S.H. et al.; "A Time to Wean? Impact of Weaning Age on Anxiety-Like Behaviour and Stability of Behavioural Traits in Full Adulthood" in *PloS One* 11, no. 12 (**2016**): e0167652.

[473] Trivers, R.L.; "Parent-Offspring Conflict" in *American Zoologist* 14, no. 1 (**1974**): 249–264.

[474] Gluckman, P.D., M.A. Hanson, & T. Buklijas; "A Conceptual Framework for the Developmental Origins of Health and Disease" in *Journal of Developmental Origins of Health and Disease* 1, no. 1 (**2010**): 6–18.

[475] Hanson, M.A. & P.D. Gluckman; "Early Developmental Conditioning of Later Health and Disease: Physiology or Pathophysiology?" in *Physiological Reviews* 94, no. 4 (**2014**): 1027–1076.

assumptions, it becomes clear that anticipating the weaning period could have deleterious effects on progeny.

Generally, the GM composition in early life, as seen in Chapter 1.1.1, is very fluctuating, with an ecosystem dominated by bifidobacteria in the first months, when the baby is on a milk-based diet. The gut microbial ecosystem moves to one that is dominated by Firmicutes and Bacteroidota when solid food is introduced[476], with the latter able to metabolize starches present in a more complex dietary pattern[477]. Actually, human milk contains oligosaccharides and other bioactive compounds which are beneficial to infants, as they promote better growth and also strengthen the immune system of the baby, reducing the risk of diarrhea and consequent dehydration, protecting against allergies and metabolic disorders (e.g., obesity). Of course, breastfeeding can also prevent alterations in GM composition, which likely contribute to the development of autoimmune and NCDs later in life[478]. Indeed, the introduction of solid food with the weaning process changes the infant's microbial gut structure with significant shifts in taxonomic groups, increasing the GM diversity towards a stable adult composition[479]. Plenty of longitudinal studies have indicated that microbial composition changes significantly around the shift period of introduction to solid foods and

[476] Quercia et al., "From Lifetime to Evolution."

[477] Moore, R.E. & S.D. Townsend; "Temporal Development of the Infant Gut Microbiome" in *Open Biology* 9, no. 9 (**2019**): 190128.

[478] Ho, N.T. et al.; "Meta-Analysis of Effects of Exclusive Breastfeeding on Infant Gut Microbiota across Populations" in *Nature Communications* 9, no. 1 (**2018**): 4169.

[479] Thompson, A.L. et al.; "Milk- and Solid-Feeding Practices and Daycare Attendance Are Associated with Differences in Bacterial Diversity, Predominant Communities, and Metabolic and Immune Function of the Infant Gut Microbiome" in *Frontiers in Cellular and Infection Microbiology* 5 (**2015**).

cessation of breastfeeding and/or formula feeding[480, 481, 482, 483, 484, 485]. In particular, *Lactobacillaceae*, *Bifidobacteriaceae*, *Enterococcaceae*, *Clostridiaceae* and *Enterobacteriaceae* abundance decreased while *Lachnospiraceae*, *Ruminococcaceae* and *Bacteroidaceae* abundance increased during the period from 9 to 18 months (*i.e.*, during the period characterized by transition from milk-based feeding to family diet)[486] regardless of geographic differences, use of antibiotics, mode of delivery and milk feeding practices[487]. These shifts are shown in **Figure 19**.

[480] Amarri, S. et al.; "Changes of Gut Microbiota and Immune Markers During the Complementary Feeding Period in Healthy Breast-Fed Infants" in *Journal of Pediatric Gastroenterology and Nutrition* 42, no. 5 (**2006**): 488–495.

[481] Favier, C.F. et al.; "Molecular Monitoring of Succession of Bacterial Communities in Human Neonates" in *Applied and Environmental Microbiology* 68, no. 1 (**2002**): 219–226.

[482] Koenig, J.E. et al.; "Succession of Microbial Consortia in the Developing Infant Gut Microbiome" in *Proceedings of the National Academy of Sciences* 108, no. supplement_1 (**2011**): 4578–4585.

[483] Roger, L.C. & A.L. McCartney; "Longitudinal Investigation of the Faecal Microbiota of Healthy Full-Term Infants Using Fluorescence in Situ Hybridization and Denaturing Gradient Gel Electrophoresis" in *Microbiology* 156, no. 11 (**2010**): 3317–3328.

[484] Thompson et al., "Milk- and Solid-Feeding Practices and Daycare Attendance Are Associated with Differences in Bacterial Diversity, Predominant Communities, and Metabolic and Immune Function of the Infant Gut Microbiome."

[485] Wang, M. et al.; "T-RFLP Combined with Principal Component Analysis and 16S rRNA Gene Sequencing: An Effective Strategy for Comparison of Fecal Microbiota in Infants of Different Ages" in *Journal of Microbiological Methods* 59, no. 1 (**2004**): 53–69.

[486] Bergström, A. et al.; "Establishment of Intestinal Microbiota during Early Life: A Longitudinal, Explorative Study of a Large Cohort of Danish Infants" in *Applied and Environmental Microbiology* 80, no. 9 (**2014**): 2889–2900.

[487] Fallani, M. et al.; "Determinants of the Human Infant Intestinal Microbiota after the Introduction of First Complementary Foods in Infant Samples from Five European Centres" in *Microbiology* 157, no. 5 (**2011**): 1385–1392.

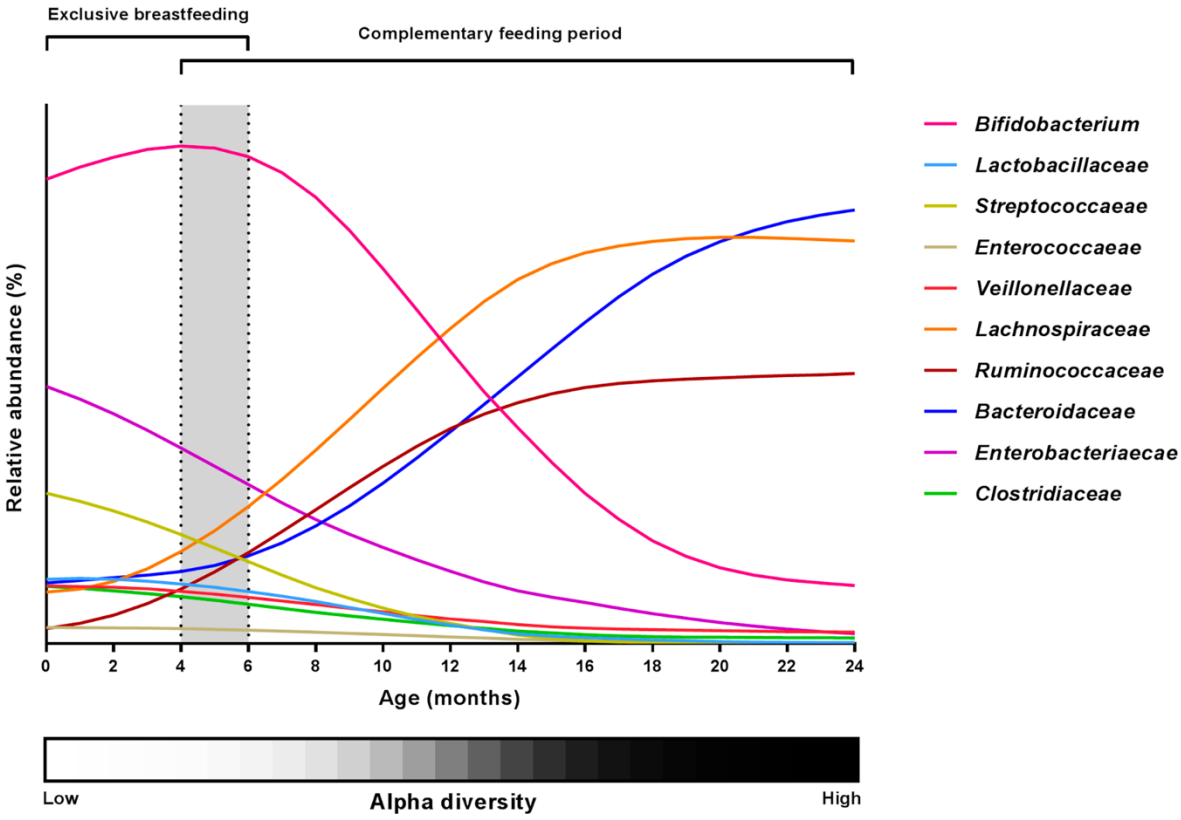

Figure 19. Changes in the major gut microbial taxa during the first 24 months of life[488].

To better understand these changes, an elegant study from Nabhani et al. in mice explored the so-called “weaning reaction”, a term coined by authors which refers to the immune reaction to the expanding GM at weaning[489]. This reaction is crucial for the development of the immune system after birth. It is partly regulated by milk-derived factors and cannot occur either before or after weaning. In the absence or prevention of the weaning reaction, mice exhibit a "pathological imprinting" that results in increased vulnerability to multiple inflammatory diseases later in life. The existence of a critical time window for microbiota exposure, necessary to prevent pathological imprinting, has been demonstrated in the

[488] Laursen, M.F. et al.; “First Foods and Gut Microbes” in *Frontiers in Microbiology* 8 (**2017**): 356.

[489] Nabhani, Z.A. et al.; “A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult” in *Immunity* 50, no. 5 (**2019**): 1276-1288.e5.

intestine, skin, and lungs[490, 491, 492]. Knoop et al. identified that this "window of opportunity" opens 10 days post-birth and closes approximately 3 to 4 weeks after birth, shortly after weaning[493]. All the changes regarding the GM and immune system are shown in **Figure 20**.

[490] Gollwitzer, E.S. et al.; "Lung Microbiota Promotes Tolerance to Allergens in Neonates via PD-L1" in *Nature Medicine* 20, no. 6 (**2014**): 642–647.

[491] Olszak, T. et al.; "Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function" in *Science* 336, no. 6080 (**2012**): 489–493.

[492] Russell, S.L. et al.; "Early Life Antibiotic-driven Changes in Microbiota Enhance Susceptibility to Allergic Asthma" in *EMBO reports* 13, no. 5 (**2012**): 440–447.

[493] Knoop, K.A. et al.; "Microbial Antigen Encounter during a Preweaning Interval Is Critical for Tolerance to Gut Bacteria" in *Science Immunology* 2, no. 18 (**2017**).

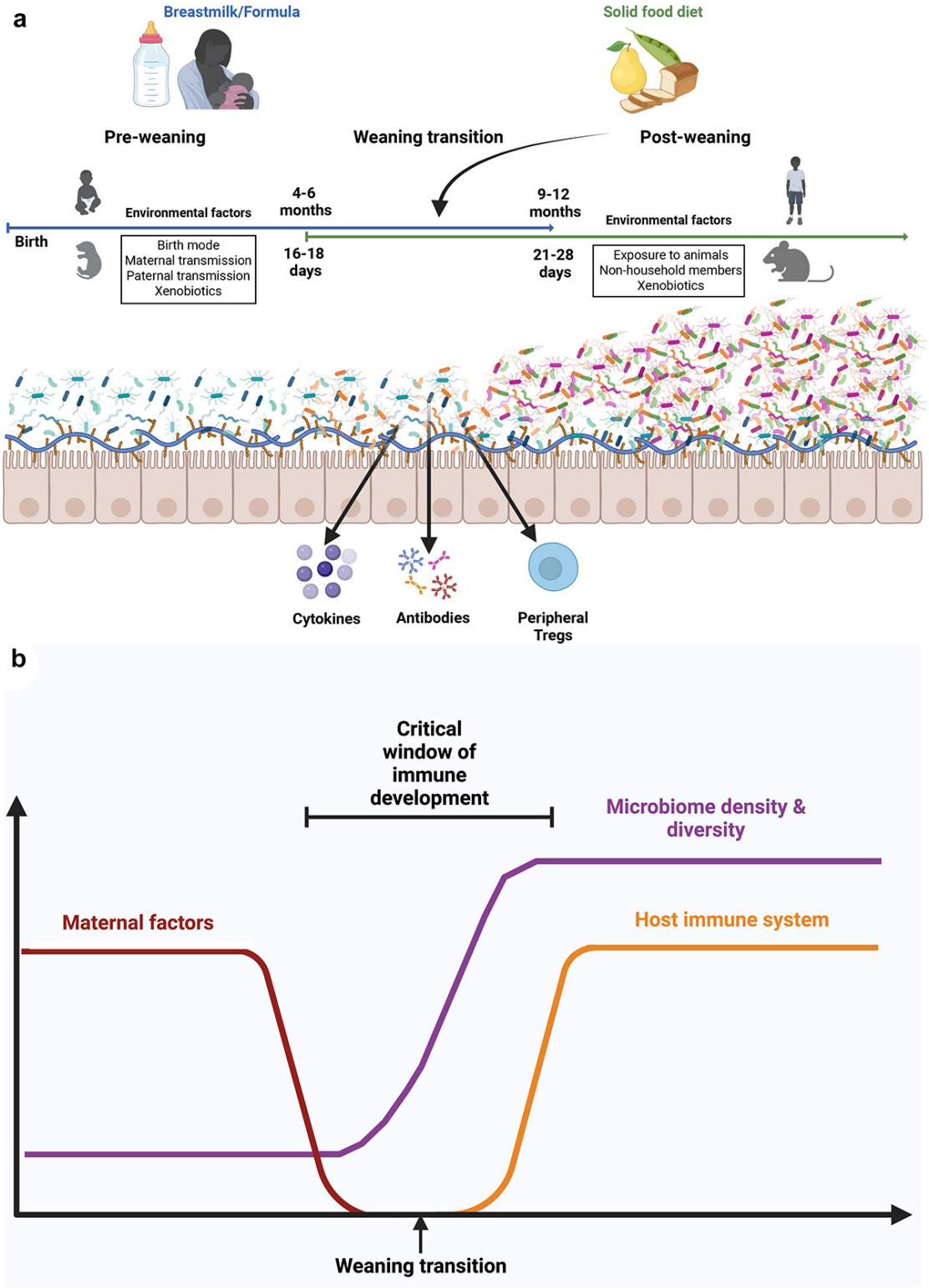

Figure 20. Weaning-associated microbiome and immune system development. a) Model showing that the dietary transition from milk to solid food at weaning leads to significant changes in the composition, density, and diversity of the intestinal microbiota. b) Schematic showing the loss of maternal factors and the increase in microbiome density and diversity at weaning, which mark a window of opportunity[494].

[494] Flores, J.N., J.-B. Lubin, & M.A. Silverman; "The Case for Microbial Intervention at Weaning" in *Gut Microbes* 16, no. 1 (**2024**): 2414798.

Nonetheless, the specific effects of early weaning (EW) on human GM have not been explored yet. Many of the studies on the changes that EW brings on GM have been conducted in mice and farm animals, such as pigs, cows or calves, since it is a relevant topic especially in animal production systems[495, 496]. Swine production, in particular, represents a critical component of the global livestock and agricultural industries. Commercial swine production typically follows a four-phase cycle: nursing, nursery, growing, and finishing. In this context, weaning marks the transition from the nursing to the nursery phase and is notably more traumatic and stressful in swine production compared to other livestock systems, due to a numerous of simultaneous changes without gradual adaptation. Specifically, young pigs, which have immature gastrointestinal system, are abruptly separated from the sow, shifted from a milk-based diet to solid feed, and introduced to new social dynamics resulting from the mixing of different litters[497, 498]. On the contrary, other mammalian livestock species have the opportunity to gradually adapt to solid feed. Consequently, newly weaned piglets present unique challenges for swine producers[499].

While studies in pigs focus on the issue directly, studies in mice address the effects of EW by serving as a useful model for humans. Shortly, three EW models have been settled up

[495] Mateos, G.G. et al.; "- Invited Review - Pig Meat Production in the European Union-27: Current Status, Challenges, and Future Trends" in *Animal Bioscience* 37, no. 4 (**2024**): 755–774.

[496] See, M.T.; "Current Status and Future Trends for Pork Production in the United States of America and Canada" in *Animal Bioscience* 37, no. 4 (**2024**): 775.

[497] Campbell, J.M., J.D. Crenshaw, & J. Polo; "The Biological Stress of Early Weaned Piglets" in *Journal of Animal Science and Biotechnology* 4, no. 1 (**2013**): 19.

[498] Sutherland, M.A., B.L. Backus, & J.J. McGlone; "Effects of Transport at Weaning on the Behavior, Physiology and Performance of Pigs" in *Animals* 4, no. 4 (**2014**): 657–669.

[499] Collins, C.L. et al.; "Post-Weaning and Whole-of-Life Performance of Pigs Is Determined by Live Weight at Weaning and the Complexity of the Diet Fed after Weaning" in *Animal Nutrition* 3, no. 4 (**2017**): 372–379.

over time (**Figure 21**). Maternal deprivation is one of them, consisting in the separation of the dam from her litter. Since in rodents "standard weaning" occurs on postnatal day (PND)

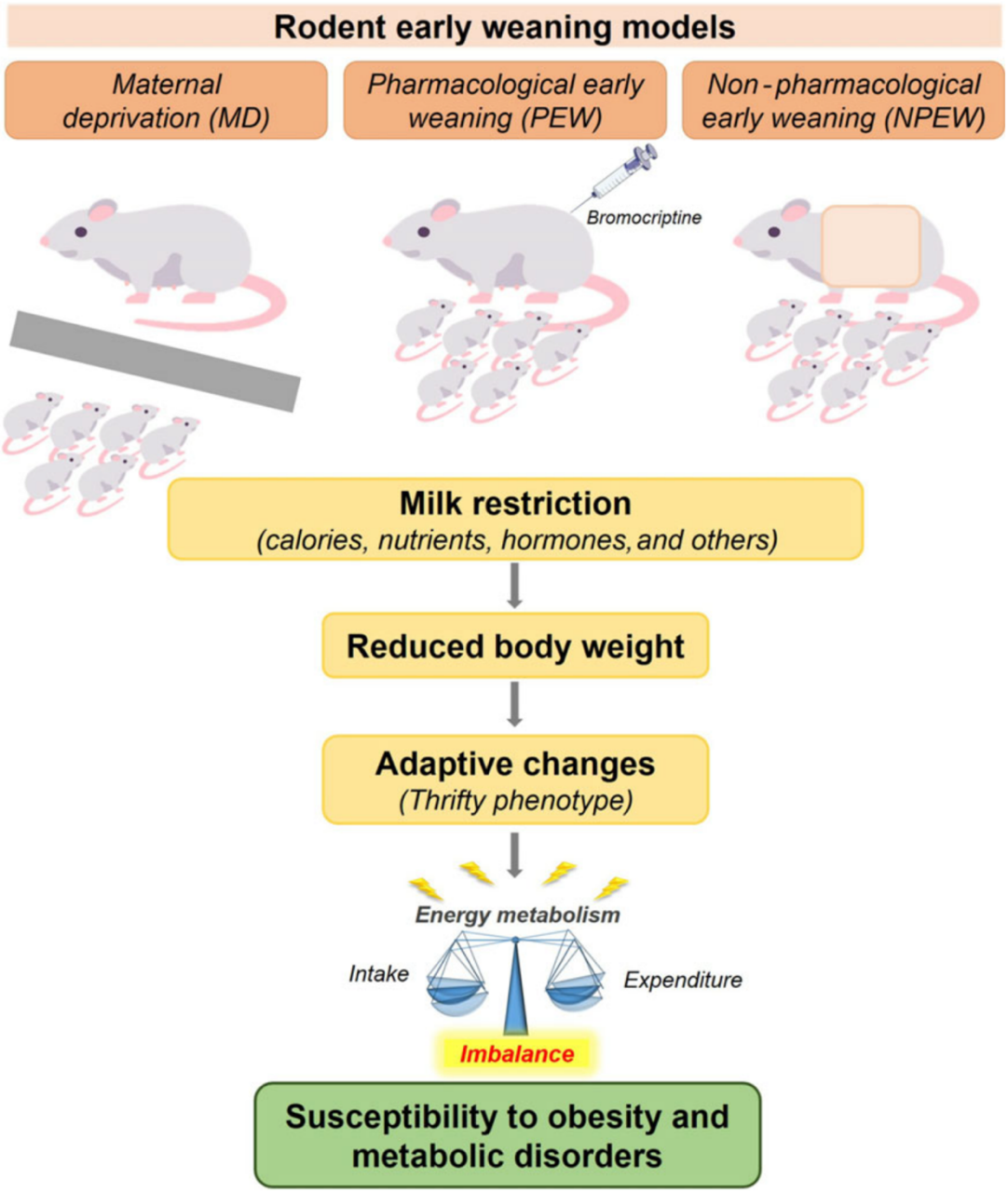

Figure 21. Three mice models to study EW and offspring metabolic outcomes. Maternal deprivation (MD), pharmacological early weaning (PEW), and nonpharmacological early weaning (NPEW)[500].

[500] Souza, L.L., E.G. de Moura, & P.C. Lisboa; "Does Early Weaning Shape Future Endocrine and Metabolic Disorders? Lessons from Animal Models" in *Journal of Developmental Origins of Health and Disease* 11, no. 5 (**2020**): 441–451.

21[501, 502], the separation of a mother and her litter before PND21 constitutes a model of EW[503]. This model influences offspring metabolism and behavior across their lifespan[504, 505, 506, 507, 508] and is characterized by restricted access to maternal milk and care, thereby inducing perinatal stress[509]. Consequently, the observed outcomes in offspring may represent adaptive responses to both nutritional alterations and emotional stress. This model provides a valuable framework for studying EW, as it closely replicates the real-life conditions experienced by children abandoned by their mothers. In rodent studies, repeated disruptions in mother-litter interactions serve as an analogue for early-life neglect, a significant social and public health issue. Since maternal care restriction alone impacts offspring development, rodent models of EW that exclude maternal separation may help mitigate emotional stress, allowing researchers to isolate the effects of breastfeeding

[501] Curley, J.P. et al.; "The Meaning of Weaning: Influence of the Weaning Period on Behavioral Development in Mice" in *Developmental Neuroscience* 31, no. 4 (**2009**): 318–331.

[502] Sengupta, P.; "The Laboratory Rat: Relating Its Age With Human's" in *International Journal of Preventive Medicine* 4, no. 6 (**2013**): 624–630.

[503] Souza, Moura, and Lisboa, "Does Early Weaning Shape Future Endocrine and Metabolic Disorders?"

[504] Back, D.W. & J.F. Angel; "Effects of Premature Weaning on the Metabolic Response to Dietary Sucrose in Adult Rats" in *The Journal of Nutrition* 112, no. 5 (**1982**): 978–985.

[505] Crispel, Y. et al.; "Effects of Breastfeeding on Body Composition and Maturational Tempo in the Rat" in *BMC Medicine* 11, no. 1 (**2013**): 114.

[506] dos Santos Oliveira, L. et al.; "Early Weaning Programs Rats to Have a Dietary Preference for Fat and Palatable Foods in Adulthood" in *Behavioural Processes* 86, no. 1 (**2011**): 75–80.

[507] Kikusui, T. et al.; "Early Weaning Augments Neuroendocrine Stress Responses in Mice" in *Behavioural Brain Research* 175, no. 1 (**2006**): 96–103.

[508] Nakagaki, B.N. et al.; "Immune and Metabolic Shifts during Neonatal Development Reprogram Liver Identity and Function" in *Journal of Hepatology* 69, no. 6 (**2018**): 1294–1307.

[509] Ghizoni, H. et al.; "Regulation of Corticosterone Function during Early Weaning and Effects on Gastric Cell Proliferation" in *Nutrition (Burbank, Los Angeles County, Calif.)* 30, no. 3 (**2014**): 343–349.

limitation on developmental outcomes[510]. Another model of early-life stress[511, 512] is the pharmacological EW. Certain drugs, such as bromocriptine (a dopamine-2 receptor agonist) are well known for their rapid suppression of prolactin production at the pituitary level[513], effectively reducing maternal milk synthesis. Clinically, bromocriptine is primarily used as a therapeutic agent for managing prolactin-secreting tumors[514]. So, the pharmacological EW model also simulates early-life exposure to bromocriptine, and the observed outcomes in offspring may reflect both the drug's direct effects and the impact of milk restriction. Interestingly, offspring treated directly with bromocriptine from PND 11 to PND 20 exhibited different outcomes compared to pharmacological EW offspring (whose mothers received bromocriptine), the latter displaying hyperphagia and hyperthyroidism in adulthood[515]. Thus, while milk restriction plays a significant role in shaping the lifelong outcomes of pharmacological EW offspring, the direct effects of bromocriptine cannot be entirely excluded. Indeed, an EW model without pharmacological intervention, while preserving maternal care, could provide more precise insights into the consequences of early maternal milk restriction, closely mirroring typical human EW scenarios[516]. Finally, a third mouse model of EW is the non-pharmacological EW model, which actually more closely resembles typical human EW scenarios and avoids confounding factors such as heightened stress or

[510] Souza, Moura, and Lisboa, "Does Early Weaning Shape Future Endocrine and Metabolic Disorders?"

[511] de Moura, E.G. et al.; "Maternal Prolactin Inhibition during Lactation Programs for Metabolic Syndrome in Adult Progeny" in *The Journal of Physiology* 587, no. Pt 20 (**2009**): 4919–4929.

[512] Fraga, M.C. et al.; "Maternal Prolactin Inhibition at the End of Lactation Affects Learning/Memory and Anxiety-like Behaviors but Not Novelty-Seeking in Adult Rat Progeny" in *Pharmacology Biochemistry and Behavior* 100, no. 1 (**2011**): 165–173.

[513] Ben-Jonathan, N. & R. Hnasko; "Dopamine as a Prolactin (PRL) Inhibitor" in *Endocrine Reviews* 22, no. 6 (**2001**): 724–763.

[514] Schlechte, J.A.; "Long-Term Management of Prolactinomas" in *The Journal of Clinical Endocrinology and Metabolism* 92, no. 8 (**2007**): 2861–2865.

[515] Carvalho, J.C. et al.; "Effects of Postnatal Bromocriptine Injection on Thyroid Function and Prolactinemia of Rats at Adulthood" in *Neuropeptides* 59 (**2016**): 71–81.

[516] Souza, Moura, and Lisboa, "Does Early Weaning Shape Future Endocrine and Metabolic Disorders?"

drug-related side effects. The non-pharmacological EW model is performed during the final 3 days of lactation by using a physical barrier (e.g., breast bandage) to prevent nipple suction[517]. Unlike other EW models, this model does not induce acute stress in offspring (in fact, the basal serum corticosterone level at PND21 and in adulthood is found to be unchanged[518]) reinforcing the isolated impact of maternal milk restriction during late lactation on offspring outcome.

Although these models are widely used, rodent models of early-life stress (including EW) have only recently been applied to understanding how stress exposure in early life may affect the GM, while in the literature the EW effect on the GM has not yet been explored enough. In fact, just a few studies are available, where the differences highlighted regarded the taxonomic composition and the intra- and inter-sample biodiversity. For instance, in a recent study conducted with an early-life stress model using germ-free mice, 16S rRNA gene sequencing analysis revealed that, at 8 weeks of age, the abundance of *Erysipelotrichaceae* in early weaned mice was lower than that in normal weaned mice and bacterial richness and evenness were higher in normal weaned mice than in early weaned mice. Principal coordinate analysis (PCoA) based on weighted UniFrac distances depicted age-related changes in GM composition implying an immature microbiota in the adult EW mice[519]. Similarly, another study with a mouse model of maternal separation and EW (MSEW) highlighted that the GM of mice exposed to early-life stress was enriched in ASVs belonging

[517] Lima, N. da S. et al.; "Early Weaning Causes Undernutrition for a Short Period and Programmes Some Metabolic Syndrome Components and Leptin Resistance in Adult Rat Offspring" in *British Journal of Nutrition* 105, no. 9 (**2011**): 1405–1413.

[518] Lima, N.S. et al.; "Developmental Plasticity of Endocrine Disorders in Obesity Model Primed by Early Weaning in Dams" in *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones Et Metabolisme* 45, no. 1 (**2013**): 22–30.

[519] Kamimura, I. et al.; "Modulation of Gut Microbiota Composition Due to Early Weaning Stress Induces Depressive Behavior during the Juvenile Period in Mice" in *Animal Microbiome* 6, no. 1 (**2024**): 33.

to the genera *Clostridium senso stricto 1* and *Lachnoclostridium*, which may affect the inflammatory state of the host[520]. Authors showed that MSEW mice also had a greater abundance of *Peptostreptococcaceae*, a family of strict anaerobes that is associated, for instance, with CRC[521, 522], together with a reduction in α-diversity levels[523].

Compared to its effects on GM, the long-term effects of EW on health have been much more studied, highlighting that EW is one of the causes leading to the onset of NCDs. For example, not only EW promotes early malnutrition by improperly introducing food that babies cannot yet consume, but, compared to breastmilk, commercial infant formulas contain larger energy and protein contents, which may account for formula-fed newborns' quick body weight gain[524]. Furthermore, the higher protein level may promote the release of insulin, which would increase body fat[525]. Also, shortening the lactation period may influence the availability of bioactive compounds in breast milk such as leptin, which is thought to be a critical component in the programming of a healthy phenotype and necessary for the proper development of neuroendocrine pathways regulating energy metabolism[526]. Furthermore,

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[520] Banaszkiewicz, A. et al.; "Enterotoxigenic Clostridium Perfringens Infection and Pediatric Patients with Inflammatory Bowel Disease☆" in *Journal of Crohn's and Colitis* 8, no. 4 (**2014**): 276–281.

[521] Ahn, J. et al.; "Human Gut Microbiome and Risk for Colorectal Cancer" in *JNCI: Journal of the National Cancer Institute* 105, no. 24 (**2013**): 1907–1911.

[522] Tsoi, H. et al.; "Peptostreptococcus Anaerobius Induces Intracellular Cholesterol Biosynthesis in Colon Cells to Induce Proliferation and Causes Dysplasia in Mice" in *Gastroenterology* 152, no. 6 (**2017**): 1419-1433.e5.

[523] Kemp, K.M. et al.; "Early Life Stress in Mice Alters Gut Microbiota Independent of Maternal Microbiota Inheritance" in *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 320, no. 5 (**2021**): R663–R674.

[524] Thompson, A.L.; "Developmental Origins of Obesity: Early Feeding Environments, Infant Growth, and the Intestinal Microbiome" in *American Journal of Human Biology: The Official Journal of the Human Biology Council* 24, no. 3 (**2012**): 350–360.

[525] Lucas, A. et al.; "Metabolic and Endocrine Responses to a Milk Feed in Six-Day-Old Term Infants: Differences between Breast and Cow's Milk Formula Feeding" in *Acta Paediatrica Scandinavica* 70, no. 2 (**1981**): 195–200.

[526] Palou, M., C. Picó, & A. Palou; "Leptin as a Breast Milk Component for the Prevention of Obesity" in *Nutrition Reviews* 76, no. 12 (**2018**): 875–892.

epidemiological studies suggest a dose-dependent association between a longer breastfeeding period and a decreased risk of obesity and overweight[527, 528]. In a recent work of Sun et al.[529], authors found hyperinsulinemia in early-weaned rats, a factor that promotes fat storage and body growth. Hyperinsulinemia presence is also associated with adult-onset obesity in rats[530] and can be mechanistically upstream of the development of obesity[531, 532]. Authors also found that early-weaned rats had increased orexigenic neuropeptide Y levels in the hypothalamus and increased food intake preceded obesity. The increased energy intake from palatable foods[533] and with aging[534], through effects on central pathways regulating energy balance could be programmed by EW. Lastly, liver transcriptomic studies showed that a set of lipid metabolism-related genes was altered in the liver of early-weaned rats long before these animals exhibited signs of excess body weight/adiposity and metabolic dysfunction[535, 536].

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[527] Harder, T. et al.; "Duration of Breastfeeding and Risk of Overweight: A Meta-Analysis" in *American Journal of Epidemiology* 162, no. 5 (**2005**): 397–403.

[528] Rito, A.I. et al.; "Association between Characteristics at Birth, Breastfeeding and Obesity in 22 Countries: The WHO European Childhood Obesity Surveillance Initiative – COSI 2015/2017" in *Obesity Facts* 12, no. 2 (**2019**): 226–243.

[529] Sun, Y. et al.; "Leucine Supplementation Ameliorates Early-Life Programming of Obesity in Rats" in *Diabetes* 72, no. 10 (**2023**): 1409–1423.

[530] Patel, M.S. & M. Srinivasan; "Metabolic Programming in the Immediate Postnatal Life" in *Annals of Nutrition & Metabolism* 58 Suppl 2, no. Suppl 2 (**2011**): 18–28.

[531] Erion, K.A. & B.E. Corkey; "Hyperinsulinemia: A Cause of Obesity?" in *Current Obesity Reports* 6, no. 2 (**2017**): 178–186.

[532] Kopp, W.; "Development of Obesity: The Driver and the Passenger" in *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 13 (**2020**): 4631–4642.

[533] dos Santos Oliveira et al., "Early Weaning Programs Rats to Have a Dietary Preference for Fat and Palatable Foods in Adulthood."

[534] Sun et al., "Leucine Supplementation Ameliorates Early-Life Programming of Obesity in Rats."

[535] Bonet, M.L., J. Ribot, & C. Picó; "Decoding the Mechanisms Behind Early Weaning–Driven Obesity and the Leucine 'Solution'" in *Diabetes* 72, no. 10 (**2023**): 1347–1349.

[536] Sun et al., "Leucine Supplementation Ameliorates Early-Life Programming of Obesity in Rats."

Also, it has been shown that EW can program for some metabolic syndrome components, a condition characterized by the co-occurrence of three major risk factors for cardiovascular disease - hyperglycaemia, high blood pressure, and dyslipidaemia (decreased high-density lipoprotein cholesterol or raised triglycerides)[537]. An animal study conducted in rats showed that at 21 days of life, EW caused lower body weight, length, visceral fat mass, total body fat, hypoglycaemia and hypoinsulinaemia; all changes were linked to malnutrition status. However, at 180 days of life, the animals displayed overweight, higher total and visceral adiposity, higher serum levels of triacylglycerols and glucose as well as a higher insulin resistance index, which are components of the metabolic syndrome. Serum insulin levels were inappropriately unchanged, also suggesting a pancreatic β-cell failure[538]. After 21 days of life, EW offspring showed decreased serum leptin concentration. Authors associated this with a possible imprinting for central leptin resistance and obesity[539, 540, 541]. Additionally, postnatal overfeeding caused by litter size reduction led to the same mechanism in adult animals from a molecular point of view, also suggesting central leptin resistance[542]. At least, regarding the lipid profile, aside from the lack of significant changes in total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, or very-low-density lipoprotein cholesterol, the presence of hypertriglyceridemia in adult offspring born to energy-restricted mothers may point to an increased risk of atherogenesis, suggesting a

[537] Han, T.S. & M.E.J. Lean; "Metabolic Syndrome" in *Medicine* 43, no. 2, Nutrition (**2015**): 80–87.

[538] Lima et al., "Early Weaning Causes Undernutrition for a Short Period and Programmes Some Metabolic Syndrome Components and Leptin Resistance in Adult Rat Offspring."

[539] Banks, W.A.; "Leptin Transport across the Blood-Brain Barrier: Implications for the Cause and Treatment of Obesity" in *Current Pharmaceutical Design* 7, no. 2 (**2001**): 125–133.

[540] Burguera, B. et al.; "Obesity Is Associated with a Decreased Leptin Transport across the Blood-Brain Barrier in Rats" in *Diabetes* 49, no. 7 (**2000**): 1219–1223.

[541] Lima et al., "Early Weaning Causes Undernutrition for a Short Period and Programmes Some Metabolic Syndrome Components and Leptin Resistance in Adult Rat Offspring."

[542] Rodrigues, A.L. et al.; "Postnatal Early Overfeeding Induces Hypothalamic Higher SOCS3 Expression and Lower STAT3 Activity in Adult Rats" in *The Journal of Nutritional Biochemistry* 22, no. 2 (**2011**): 109–117.

heightened susceptibility to cardiovascular disease[543, 544]. Indeed, there is evidence linking low birth weight to dyslipidemia and hypertension in later life[545].

Not only EW lays the foundation for obesity and leptin resistance in adults, but it appears to be involved in the onset of T2D[546]. T2D is marked by persistent hyperglycemia and is closely linked to insulin resistance, a condition that arises due to impairments in insulin secretion and/or its functional activity[547]. Insulin operates through the tyrosine kinase receptor, which triggers the phosphorylation of insulin receptor substrates (IRS1). Research on the glycemic homeostasis of animals subjected to EW is limited and these studies have shown that males subjected to EW exhibit insufficient plasma insulin levels[548, 549]. Increased insulin secretion in response to different glucose concentrations was observed in male rats subjected to EW at 45 days of life. However, in adulthood, these males demonstrated impaired insulin secretion when exposed to high glucose levels, along with alterations in protein expression related to the secretory processes of pancreatic β-cells and insulin resistance in skeletal muscle. Rodent pancreatic β-cells, like those in humans, can increase in mass and insulin secretion to maintain normoglycemia through mechanisms such as β-cell proliferation and

[543] Després, J.P.; "Lipoprotein Metabolism in Visceral Obesity" in *International Journal of Obesity* 15 Suppl 2 (**1991**): 45–52.

[544] Lima et al., "Early Weaning Causes Undernutrition for a Short Period and Programmes Some Metabolic Syndrome Components and Leptin Resistance in Adult Rat Offspring."

[545] Fall, C.H. et al.; "Fetal and Infant Growth and Cardiovascular Risk Factors in Women" in *BMJ (Clinical research ed.)* 310, no. 6977 (**1995**): 428–432.

[546] Pietrobon, C.B. et al.; "Early Weaning Induces Short- and Long-Term Effects on Pancreatic Islets in Wistar Rats of Both Sexes" in *The Journal of Physiology* 598, no. 3 (**2020**): 489–502.

[547] International Diabetes Federation; *IDF Diabetes Atlas*, 10th edition., **2021**.

[548] de Moura et al., "Maternal Prolactin Inhibition during Lactation Programs for Metabolic Syndrome in Adult Progeny."

[549] dos Santos Oliveira et al., "Early Weaning Programs Rats to Have a Dietary Preference for Fat and Palatable Foods in Adulthood."

hypertrophy[550]. Previous research on nutritional programming suggests that EW and protein restriction during lactation lead to reduced insulin sensitivity in β-cells[551], [552] which results in lower insulin secretion and disrupted insulin signaling pathways, involving proteins such as GLUT4, IR-β, IRS, PI3K, and AKT. This dysregulation contributes to the development of insulin resistance[553]. Prolactin also plays a crucial role in maintaining glycemic balance by promoting β-cell proliferation and enhancing insulin secretion[554]. Lower prolactin levels in early-weaned males, which have been observed at 180 days of age, may contribute to the reduced expression of glucokinase in pancreatic islets, ultimately leading to impaired insulin secretion and insulin resistance in skeletal muscle[555, 556].

To conclude, EW, like other examples of maternal neglect, could impact the behavior of mammalian infants, since they heavily depend on their mothers[557]. In rodent studies, daily maternal separation during the first two weeks after birth leads to long-term effects, including

[550] Mezza, T. et al.; "Insulin Resistance Alters Islet Morphology in Nondiabetic Humans" in *Diabetes* 63, no. 3 (**2014**): 994–1007.

[551] de Oliveira, J.C. et al.; "Metabolic Imprinting by Maternal Protein Malnourishment Impairs Vagal Activity in Adult Rats" in *Journal of Neuroendocrinology* 23, no. 2 (**2011**): 148–157.

[552] Ferreira, F. et al.; "Decreased Cholinergic Stimulation of Insulin Secretion by Islets from Rats Fed a Low Protein Diet Is Associated with Reduced Protein Kinase Calpha Expression" in *The Journal of Nutrition* 133, no. 3 (**2003**): 695–699.

[553] Deshmukh, A.S.; "Insulin-Stimulated Glucose Uptake in Healthy and Insulin-Resistant Skeletal Muscle" in *Hormone Molecular Biology and Clinical Investigation* 26, no. 1 (**2016**): 13–24.

[554] Huang, P.L.; "A Comprehensive Definition for Metabolic Syndrome" in *Disease Models & Mechanisms* 2, no. 5–6 (**2009**): 231–237.

[555] de Moura et al., "Maternal Prolactin Inhibition during Lactation Programs for Metabolic Syndrome in Adult Progeny."

[556] Lima et al., "Early Weaning Causes Undernutrition for a Short Period and Programmes Some Metabolic Syndrome Components and Leptin Resistance in Adult Rat Offspring."

[557] Heim, C. et al.; "The Role of Early Adverse Experience and Adulthood Stress in the Prediction of Neuroendocrine Stress Reactivity in Women: A Multiple Regression Analysis" in *Depression and Anxiety* 15, no. 3 (**2002**): 117–125.

heightened anxiety and amplified neuroendocrine stress responses[558, 559, 560]. Separation in late-suckling periods similarly disrupts neurobehavioral development[561, 562]. EW consistently results in adult mice with increased anxiety, aggression, delayed fear extinction, and reduced empathy[563, 564, 565, 566]. These mice also display heightened activity in the hypothalamic-pituitary-adrenal axis, reflecting elevated stress responses to mild stressors and novel environments[567, 568]. These findings suggest that the developing brain is highly sensitive to stress during late suckling and that early social experiences have lasting behavioral impacts. From a molecular point of view, EW in rodents induces heightened hypothalamic-pituitary-adrenal axis activity causing high levels of anxiety. Early-weaned

[558] Levine, S.; "Maternal and Environmental Influences on the Adrenocortical Response to Stress in Weanling Rats" in *Science* 156, no. 3772 (**1967**): 258–260.

[559] Liu, D. et al.; "Maternal Care, Hippocampal Glucocorticoid Receptors, and Hypothalamic-Pituitary-Adrenal Responses to Stress" in *Science (New York, N.Y.)* 277, no. 5332 (**1997**): 1659–1662.

[560] Plotsky, P.M. et al.; "Long-Term Consequences of Neonatal Rearing on Central Corticotropin-Releasing Factor Systems in Adult Male Rat Offspring" in *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology* 30, no. 12 (**2005**): 2192–2204.

[561] Kikusui, T. & Y. Mori; "Behavioural and Neurochemical Consequences of Early Weaning in Rodents" in *Journal of Neuroendocrinology* 21, no. 4 (**2009**): 427–431.

[562] Mogi, K., M. Nagasawa, & T. Kikusui; "Developmental Consequences and Biological Significance of Mother-Infant Bonding" in *Progress in Neuro-Psychopharmacology & Biological Psychiatry* 35, no. 5 (**2011**): 1232–1241.

[563] Kanari, K. et al.; "Multidimensional Structure of Anxiety-Related Behavior in Early-Weaned Rats" in *Behavioural Brain Research* 156, no. 1 (**2005**): 45–52.

[564] Kikusui, T., Y. Takeuchi, & Y. Mori; "Early Weaning Induces Anxiety and Aggression in Adult Mice" in *Physiology & Behavior* 81, no. 1 (**2004**): 37–42.

[565] Mogi, K. et al.; "Early Weaning Impairs Fear Extinction and Decreases Brain-Derived Neurotrophic Factor Expression in the Prefrontal Cortex of Adult Male C57BL/6 Mice" in *Developmental Psychobiology* 58, no. 8 (**2016**): 1034–1042.

[566] Nakamura, K. et al.; "Changes in Social Instigation- and Food Restriction-Induced Aggressive Behaviors and Hippocampal 5HT1B mRNA Receptor Expression in Male Mice from Early Weaning" in *Behavioural Brain Research* 187, no. 2 (**2008**): 442–448.

[567] Ito, A. et al.; "Effects of Early Weaning on Anxiety and Autonomic Responses to Stress in Rats" in *Behavioural Brain Research* 171, no. 1 (**2006**): 87–93.

[568] Kikusui et al., "Early Weaning Augments Neuroendocrine Stress Responses in Mice."

male mice show higher basal corticosterone levels than the normally weaned mice, as well as altered hippocampal glucocorticoid receptor mRNA expression[569].

4.2 Materials and Methods

4.2.1 Animals, experimental design and project workflow

Based on the results from the previous part of the MicroWean project, a model consisting of three groups of RjOrl: SWISS mice was developed[570]. In the first group, the individuals were weaned early at 2 weeks (EW = Early Weaning); in the second group, individuals were weaned early at 2 weeks and were given the microbial supplement during the first week of life (EW + B = Early Weaning + Bacteria). Finally, in the third group, the individuals were kept as a control, with weaning performed at 3 weeks (NW = Normal Weaning). To evaluate the effects of EW and the microbial supplement over time, two trials were performed, using a total of 60 mice; 30 mice were sacrificed at 3 weeks of age, while the others were sacrificed at 6 weeks of age. For each trial, a total of 15 females and 15 males were used (4 cages/experimental group, n = 10 mice per group; 3 cages for the control group). The litters were born after few days the mother's arrival at the animal facilities of the French National Institute of Agricultural Research (INRAE Jouy-en-Josas, France). Before the beginning of the weaning period, the litters were put in the same cage as the respective mothers. Mice were maintained at 21°C and housed in cages of 3 or 4. They were given food and water *ad libitum* and experienced a 12:12 hours light-dark cycle. The groups and the study workflow

[569] Ibid.

[570] Barone, M. et al.; "Gut Barrier-Microbiota Imbalances in Early Life Lead to Higher Sensitivity to Inflammation in a Murine Model of C-Section Delivery" in *Microbiome* 11, no. 1 (**2023**): 140.

are represented in **Figures 22** and **23**. The weight was registered the last day, before the sacrifice.

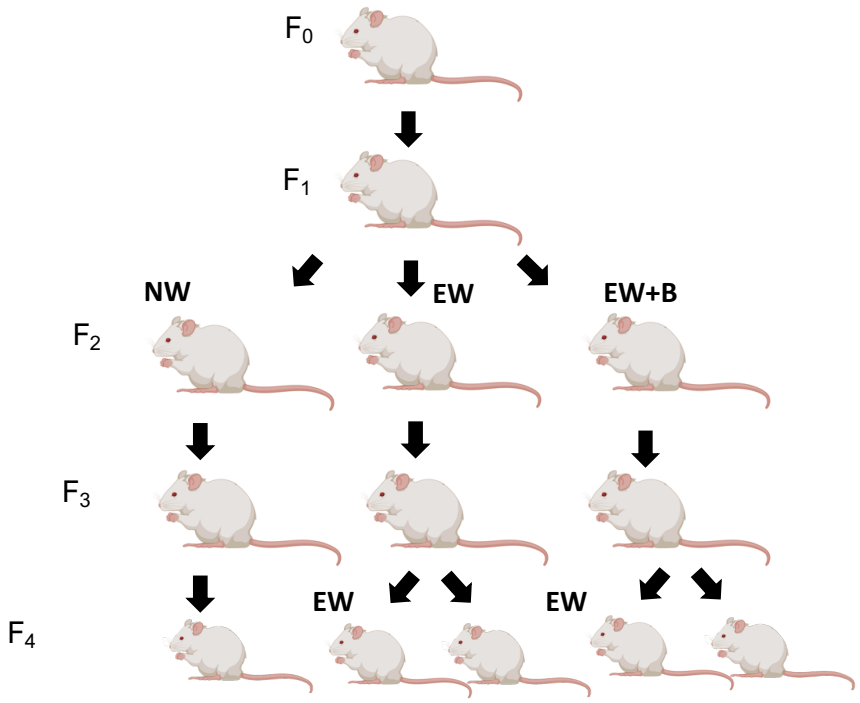

Figure 22. Mice groups used in the experiment. NW = normal weaning; EW = early weaning; EW + B = early weaning with bacterial supplementation. This experiment focused on the generation F_2.

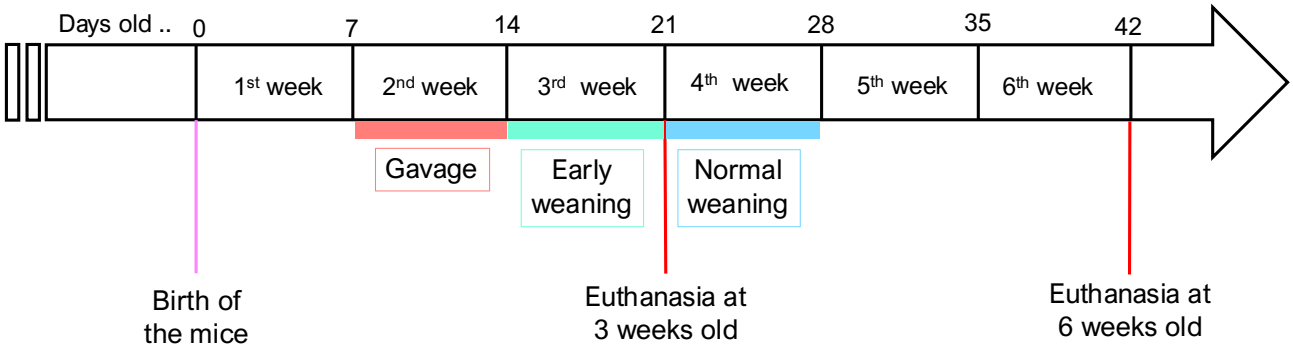

Figure 23. Study workflow over time.

4.2.2 Determination and preparation of the bacterial consortium

Based on previous results from a prior part of the same MicroWean study, which underlined the loss of some SCFAs, the bacterial consortium composition was chosen. In particular, previous data (not published yet) showed a decrease in lactate, acetate, propionate, butyrate and succinate in mice weaned at 2 weeks instead of 3 weeks. So, to improve the production of lactate and acetate[571], two strains of *Bifidobacterium* were chosen (*B. breve* and *B. longum*), along with *Anaerostipes caccae* and *Eubacterium limosum* for the production of both butyrate and propionate[572, 573, 574, 575] as well as *Alistipes shahii* for succinate, acetate and propionate production[576]. Basically, these bacteria were grown in anaerobic atmosphere (85% N_2, 10% CO_2, 5% H_2) in an anaerobic chamber in De Man-Rogosa-Sharpe (MRS) medium with cysteine for *Bifidobacterium* strains and in Brain-Hearth Infusion (BHI) Broth added with hemin, vitamins K1 and K3, cellobiose, maltose and cysteine for the remaining microorganisms. The taxonomic assignment of microorganisms was confirmed by Sanger sequencing. Briefly, a PCR mix was prepared by adding 0.5 µL of J4 primer (5'-ACGGCTACCTTGTTACGACTT-3'), 0.5 µL of J7 primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 0.5 µL of dNTPs, 0.1 µL of DreamTaq, 2.5 µL of Green

[571] Sharma, S. et al.; "Isomaltooligosaccharides Utilization and Genomic Characterization of Human Infant Anti-Inflammatory Bifidobacterium Longum and Bifidobacterium Breve Strains" in *3 Biotech* 12, no. 4 (**2022**): 89.

[572] Bui, T.P.N. et al.; "Conversion of Dietary Inositol into Propionate and Acetate by Commensal Anaerostipes Associates with Host Health" in *Nature Communications* 12, no. 1 (**2021**): 4798.

[573] Kanauchi, O. et al.; "Eubacterium Limosum Ameliorates Experimental Colitis and Metabolite of Microbe Attenuates Colonic Inflammatory Action with Increase of Mucosal Integrity" in *World Journal of Gastroenterology : WJG* 12, no. 7 (**2006**): 1071–1077.

[574] Litty, D. & V. Müller; "Butyrate Production in the Acetogen Eubacterium Limosum Is Dependent on the Carbon and Energy Source" in *Microbial Biotechnology* 14, no. 6 (**2021**): 2686–2692.

[575] Schwiertz, A. et al.; "*Anaerostipes Caccae* Gen. Nov., Sp. Nov., a New Saccharolytic, Acetate-Utilising, Butyrate-Producing Bacterium from Human Faeces" in *Systematic and Applied Microbiology* 25, no. 1 (**2002**): 46–51.

[576] Song, Y. et al.; "Alistipes Onderdonkii Sp. Nov. and Alistipes Shahii Sp. Nov., of Human Origin" in *International Journal of Systematic and Evolutionary Microbiology* 56, no. Pt 8 (**2006**): 1985–1990.

Buffer 10x and 1 µL of DNA, for a final volume of 25 µL. For DNA preparation, one colony of microorganisms was dissolved into 10 µL of sterile water. The thermocycler was set with a protocol that included 15 minutes at 95°C, 30 seconds at 95°C, 30 seconds at 52°C and 1 minute and 30 seconds at 72°C, repeated 38 times, and a final step of 10 minutes at 72°C. The generation of amplicons was confirmed by electrophoresis on 1% agarose gel. The samples were then sent to an external service (Eurofins Genomics) for Sanger sequencing. After Sanger confirmation, the correlation between CFU/mL and OD was established using MRS or BHI agar plates and a spectrophotometer (OD600 DiluPhotometer™, Implen). Finally, the bacterial solution was prepared using PBS with 10^{10} CFU/mL of the above species. Briefly, to concentrate the bacterial cultures to a final concentration of 10^{10} CFU/mL, a volume of each culture was centrifugated and the supernatant was discarded. Then, the pellet was resuspended in 1 mL of PBS, to allow a concentration of 100x. The obtained bacterial solution was then divided into 2 mL aliquots in cryotubes and put at -80°C for the following administration to the EW+B group (see paragraph 4.2.4).

4.2.3 Milk bowls preparation

To sustain the growth of mice in the early weaned groups, bowls of milk for rodents (Beaphar) were prepared. This choice was made since, from previous results of an early part of the MicroWean project, the EW group showed a low survival ratio, compared to the EW group with a formula feeding supplement. For the preparation of 12 bowls (half bowl par cage), 576 mL of water were boiled in a beaker and 7.2 g of agar were added to it. Then, maintaining the stirring and the heating, 330 g of powdered milk were added to the agar solution. When all the components were dissolved and well homogenized, the solution was heated until boiling. This last procedure was repeated twice. Finally, the agarized milk was put in a silicon tray and let rest overnight at +4°C.

4.2.4 Nutritional intervention and samples collection

After the arrival of the mothers and the birth of the pups, during their second week of life, in the EW + B group the nutritional intervention was performed by giving to the newborns some drops of the bacterial solution during the entire week, every two days. During the third week of life, the weaning was performed in the EW and EW + B groups. Finally, euthanasia was performed when the mice were 3 and 6 weeks old, respectively, to evaluate the effects of EW and the microbial supplement at these two time points. The same day the weight of every mouse was recorded and colon, ileum, caecum, jejunum, duodenum, mesenteric lymphoid, spleen and liver, as well as blood samples were collected from each individual. All the samples were collected in tubes and stored at -80°C, while spleen and liver were put into histological cassettes and immediately into Carnoy fixative and paraformaldehyde (PAF) fixative. The histological cassettes and the blood samples were stored at +4°C.

4.2.5 In vitro tests

4.2.5.1 Processing of blood samples

Serum samples were collected in lithium heparin separation tubes, which permits the separation of plasma and blood cells. The tubes were then centrifuged at 2,000 $\times$ *g* at +4°C for 10 minutes. The supernatant (serum) was then collected and put into a 96 deep well plate, with one sample for each well, and stored at -80°C.

4.2.5.2 Processing of colon samples

To extract proteins for subsequent analysis via ELISA assays, colon samples were processed to 0.5 mL of 1.4 mm diameter ceramic beads and 3 2.8 mm diameter ceramic beads were added to each colon sample, with 500 µL of PBS. Samples were then treated with a homogenizer (Precellys, Bertin Technologies) set with 3 cycles of 30 seconds, at 8,000 rpm; liquid nitrogen was added to guarantee a temperature of +4°C. Samples were after vortexed and then centrifugated at 14,000 × *g*, for 5 minutes at +4°C. Supernatants were then collected and put in a new 96 deepwell plate and stored at -80°C.

4.2.5.3 ELISA assays

ELISA assays were performed for the quantitative determination of mouse IgA, IgG, IgM and Lipocalin-2 in serum and colon samples from 3- and 6-week-old mice. For immunoglobulins, the ELISA Flex kits (Mabtech) were used and the protocol was carried out following manufacturer's instructions. Briefly, the capture antibodies for IgA, IgG and IgM were diluted and used to coat a 96-well half-area plates overnight at +4°C. The next day, the plate was emptied and washed for three times with PBS containing 0.05% Tween 20, then the incubation buffer (PBS with 0.05% Tween 20 and 0.1% BSA) was added to block the plate. After 1 hour incubation at room temperature (RT), the plate was emptied and washed again and both the standards and the samples were added, after being diluted in a proper volume of incubation buffer and then incubated for 2 hours at RT. After washing 5 times, the detection antibody, conjugated with alkaline phenyl phosphatase (ALP) was added to the plates. All the plates were incubated 1 hour at RT. After a 5-times wash, a para-Nitrophenylphosphate (pNPP), a non-proteinaceous chromogenic substrate, was added for IgA and IgG detection and, in approximately 60 minutes, was able to produce phenolate,

which gives a yellow bright color to each well according to the concentration of immunoglobulin of interest. Lastly, the optical density at 405 nm (the maximum absorption of phenolate) was recorded using a spectrophotometer (Infinite® 200 PRO, Tecan). For IgM, before adding the detection antibody, another 1 hour incubation was performed after adding a biotinylated antibody, which will be linked to the ALP antibody after 1 hour incubation.

For Lipocalin-2 and soluble CD14 (sCD14), the DuoSet ELISA kit (R&D System) was used, following the protocol given. Briefly, the capture antibody was diluted to the working concentration and used to coat a 96-well plate that was then incubated overnight at RT. The next day, the plate was emptied and washed 3 times, adding then the reagent diluent (PBS with 1% BSA) to block the plate and incubated at RT for 1 hour. After repeating the washing, standards and samples were added to the wells and let them incubate for 2 hours at RT. Again, after washing other 3 times, the detection antibody was added and another 2 hours incubation was performed. At the end of the incubation, after washing, a streptavidin conjugated to the enzyme horseradish peroxidase (streptavidin-HRP B) antibody was added to each well. This enzyme is used as a detection reagent for biotin conjugated primary antibodies. The plate was then incubated for 20 minutes at RT, avoiding direct light. Then, a Substrate Solution (provided by the manufacturer) was added and the plate was incubated again 20 minutes at RT, avoiding direct light. After this, the stop solution (2 N H_2SO_4) was added to each sample to block the Substrate Solution and the optical density was determined at 450 nm using a spectrophotometer (Infinite® 200 PRO, Tecan). The instrument gave raw data outputs that were analyzed using Microsoft Excel (version 16.69.1). Shortly, the blank value was subtracted to the standard and the samples values. After that, a standard curve was made linking the known concentration of the standards (explicated in the protocol) and the corresponding optical density. Sample values were correlated with these values and multiplied by dilution factor.

4.2.5.4 Total Antioxidant Capacity assay (TAC)

The Total non-enzymatic Antioxidant Capacity (TAC) is a parameter indicative of the sample ability to counteract oxidative stress induced in cells and allows to measure the concentration of the combination of small molecules and protein antioxidants of a sample. To perform this assay in serum samples, the Total Antioxidant Capacity Assay Kit (Sigma-Aldrich) was used, following the manufacturer's instructions. This kit uses the Cu^{2+} ion which, thanks to antioxidant molecules in the sample, is converted into Cu^{+}. Cu^{+} ion chelates with a colorimetric probe with an absorption peak at 600 nm. Briefly, standards given by the kit and samples were put in a 96-well plate with water to bring them at a proper dilution. The Cu^{2+} Working Solution was added to each well, and incubated for 90 minutes at RT. The absorbance was read with a spectrophotometer (Infinite® 200 PRO, Tecan) at 570 nm. Since the absorbance is proportional to TAC, the raw data were then analyzed as described in the previous paragraph. Shortly, the raw data outputs given by the instrument were analyzed using Microsoft Excel (version 16.69.1). The blank value was subtracted to the standard and the samples values and a standard curve was made linking the known concentration of the standards (explicated in the protocol) and the corresponding optical density. Sample values were correlated with these values and multiplied by dilution factor.

4.2.5.5 Next-Generation Sequencing Analysis of 16S rRNA

The colon content of the samples of 3- and 6-week-old mice were processed by INRAE for microbial DNA extraction, using the QIAamp power fecal Pro DNA kit (QIAGEN), following the manufacturer's instructions. Briefly, 250 mg of each sample were added to 800 µL of Solution CD1 in a PowerBead Pro Tube. Samples were then homogenized using Precellys homogenizer (Bertin Technologies) with 2 × 45 seconds steps at 6,800 rpm, and centrifuged

at 15,000 × *g* for 1 minute. Supernatants were then transferred to a clean 2 mL Microcentrifuge Tube with 200 µL of Solution CD2 and vortexed for 5 seconds. After centrifugation at 15,000 × *g* for 1 minute, 700 µL of supernatant were transferred to a clean 2 mL Microcentrifuge tube. A volume of 600 µL of Solution CD3 were added to each sample and then vortexed for 5 seconds. After, 650 µL of the lysate were then loaded onto an MB Spin Column and centrifuged at 15,000 × *g* for 1 minute. After discarding the flow-through, this step was repeated. The MB Spin Column was then placed into a clean 2 mL Collection Tube and 500 µL of Solution EA were added to the MB Spin Column, followed by centrifugation at 15,000 × *g* for 1 minute. After discarding the flow-through, 500 µL of Solution C5 were added to the MB Spin Column and a centrifugation step at 15,000 × *g* for 1 minute was performed. After discarding the flow-through, samples were centrifuged at 16,000 × *g* for 2 minutes and the MB Spin Column was placed into a new 1.5 mL Elution Tube. The DNA was then eluted using 100 µL of Solution C6 and centrifuging at 15,000 × *g* for 1 minute. The library preparation was performed as described in the Chapter 3.2.5.

4.2.5.6 Bioinformatics and statistical analysis

All analysis except 16S results were carried out using GraphPad Prism version 10.2.1 for statistical analyses and plotting boxplots. For comparisons among groups, nonparametric Kruskal-Wallis tests followed by post hoc Dunn's tests were used. For colon content microbiota, all statistical analyses were performed with open-source software R Studio (version 2023.06.2+561) and R (version 4.3.1). PCoA plots were generated using the "vegan" package (v. 2.6-4, https://cran.r-project.org/web/packages/vegan/index.html), and data separation was tested using a permutation test with pseudo-F ratio ("Adonis" function in "vegan"). The Kruskal-Wallis test, followed by post-hoc Wilcoxon tests, was used to

assess group differences in α-diversity and relative taxon abundances. Genus-level barplots and boxplots were built using the “barplot” function and the “boxplot” functions, respectively, in the open source software R Studio (version 2023.06.2+561) and R (version 4.3.1). Genus-level heatmaps were generated using the “made4” package (https://bioconductor.org/packages/release/bioc/html/made4.html) and the “heatmap.2” function from the “gplots” package (https://cran.r-project.org/web/packages/gplots/index.html). Differences were considered significant at *p* value ≤ 0.05.

4.3 Results

In this section, the bigger graphs in the figures represent the results of this experiment. For weight and *in vitro* tests, smaller graphs at the top right of the bigger ones represents the results (not published yet) of the first part of the MicroWean project. The aim of that previous part of the project was to develop a murine model of early weaning that would be useful for both human and pig hosts. In the first part, the model was made of 3 groups of mice: one group with early weaned mice, one group with early weaned mice plus formula feeding and a last group of normal weaned mice (weaning at 3 weeks). So, the group with early weaned mice plus formula feeding and the normal weaned mice group are comparable with the EW and NW groups of this experiment, since their condition in the two experiments were the same.

4.3.1 Systemic inflammation

The registered weight is shown in **Figure 24**. At 3 weeks, it is possible to note that the NW group had the same weight as in the previous experiment, while differences occurred between the NW and EW + B groups (*p* value = 0.012), as well as with EW group (*p* value = 0.041). After 6 weeks, in both EW (*p* value = 0.02) and EW + B groups (*p* value = 0.009), the weight of the mice was higher compared to the NW group, both in this experiment and in the previous one. This could probably be explained by the fact that, both in the EW and EW + B group, mice received formula feeding.

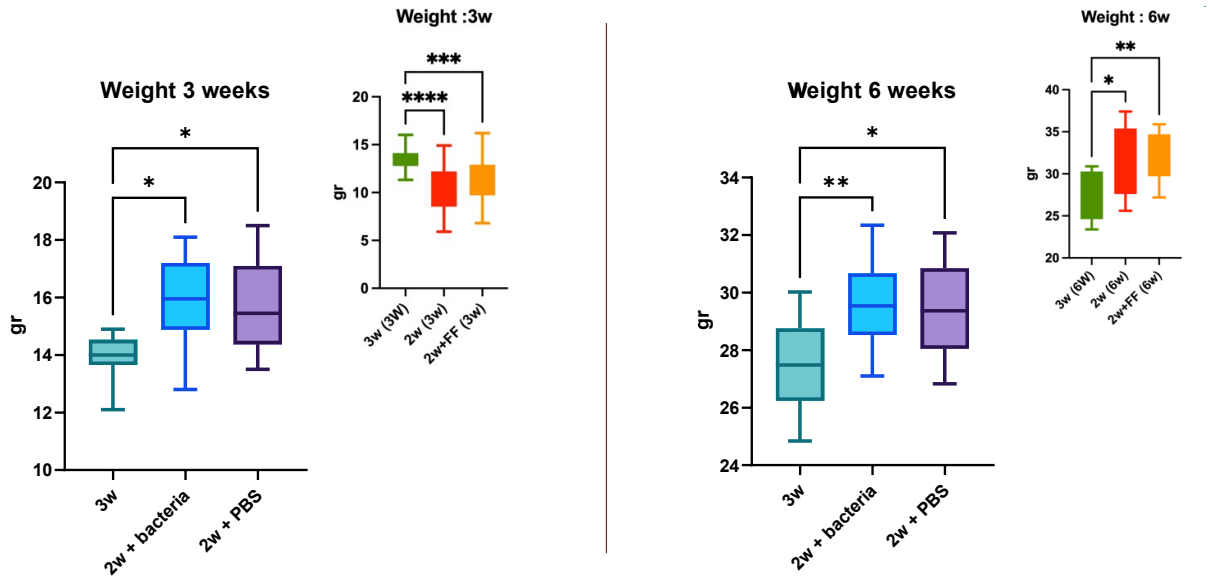

Figure 24. Boxplots representing the weight, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. For bigger graphs (current experiment): 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. For smaller graphs (previous experiment): 3w (3W) = normal weaning group at 3 weeks; 2w (3w) = early weaned group at 3 weeks; 2w+FF (3w) = early weaned group + formula feeding at 3 weeks; 3w (6W) = normal weaning group at 6 weeks; 2w (6w) = early weaned group at 6 weeks; 2w+FF (6w) = early weaned group + formula feeding at 6 weeks. Kruskal-Wallis test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01.

Immunoglobulin levels resulting from serum samples are shown in **Figure 25** for the 3- and 6-week-old mouse groups. For 3-week-old mice, the IgA levels increased over time, while in 6-week-old mice it was possible to note a reduction in the group with the nutritional intervention, making it more similar to the NW group. Also, in 6-week-old mice, there was a significant difference between EW + B and EW groups (*p* value = 0.02), but not between

EW + B and NW groups, indicating a possible restoration of this parameter by the nutritional intervention. IgG levels also increased over time, with significant differences between the EW and NW groups (*p* value = 0.03), but no differences occurred between the 6-week-old mouse groups. Finally, for IgM, higher levels were found in the EW + B group, probably due to the nutritional intervention, both in 3-week-old (EW + B vs NW, *p* value = 0.006; EW + B vs EW, *p* value = 0.01) and 6-week-old (EW + B vs NW, *p* value = 0.016) mouse groups. Compared with the previous experiment, levels were higher in all the 3- and 6-week-old mice groups.

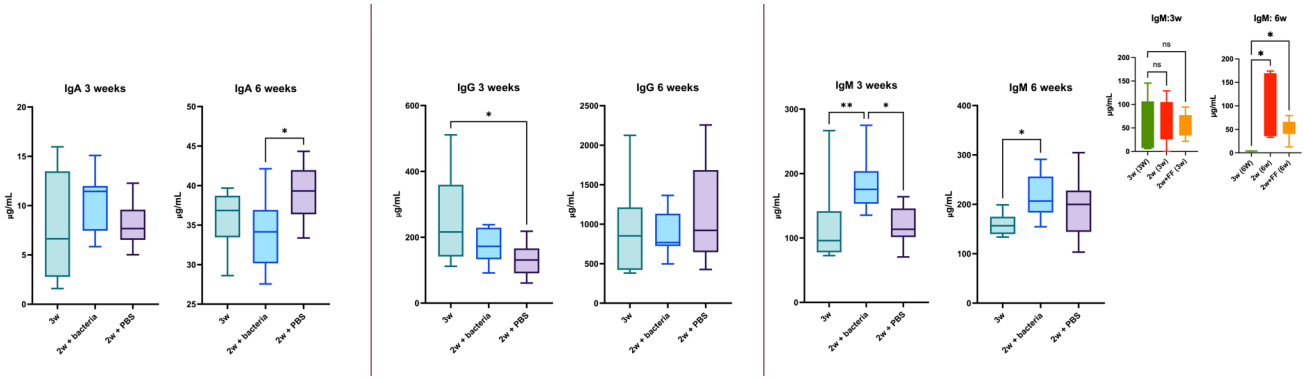

Figure 25. Boxplots representing IgA, IgG and IgM levels in serum samples, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. For bigger graphs (current experiment): 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. For smaller graphs (previous experiment): 3w (3W) = normal weaning group at 3 weeks; 2w (3w) = early weaned group at 3 weeks; 2w+FF (3w) = early weaned group + formula feeding at 3 weeks; 3w (6W) = normal weaning group at 6 weeks; 2w (6w) = early weaned group at 6 weeks; 2w+FF (6w) = early weaned group + formula feeding at 6 weeks. Kruskal-Wallis test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01.

For Lipocalin-2 levels (**Figure 26**), a similar pattern to the previous experiment was observed in 3- and 6-week-old mouse groups. Also, the levels were definitely higher in the last experiment compared to the previous one. Both EW groups (with and without nutritional intervention) showed differences compared to NW at 3 weeks (EW + B vs NW, *p* value = 0.004; EW vs NW, *p* value = 0.04) however, these differences disappeared by 6 weeks.

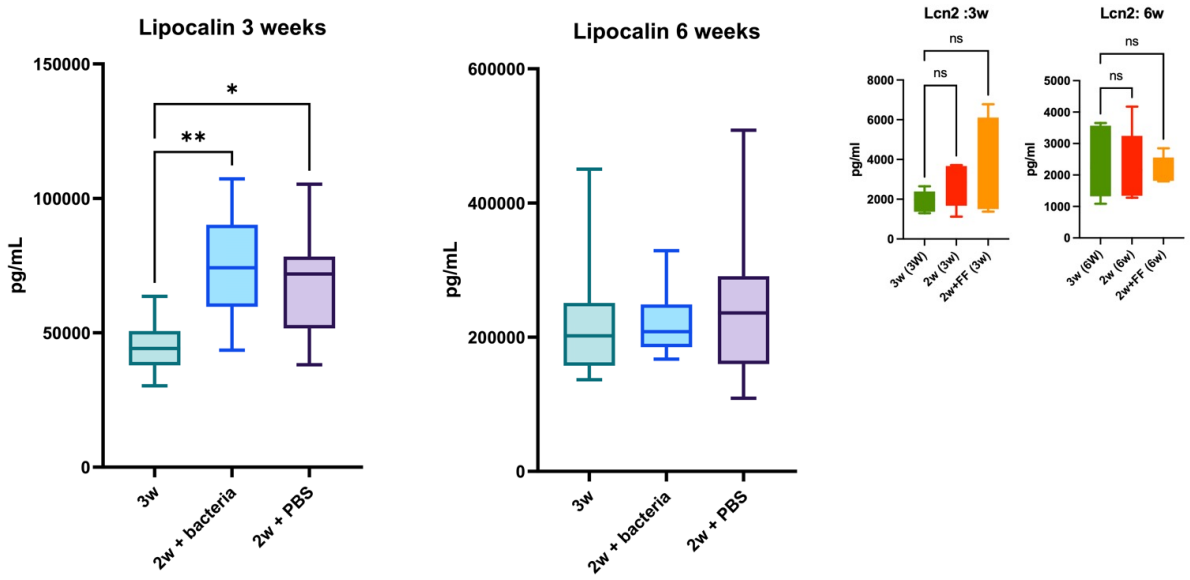

Figure 26. Boxplot representing Lipocalin-2 levels in serum samples, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. For bigger graphs (current experiment): 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. For smaller graphs (previous experiment): 3w (3W) = normal weaning group at 3 weeks; 2w (3w) = early weaned group at 3 weeks; 2w+FF (3w) = early weaned group + formula feeding at 3 weeks; 3w (6W) = normal weaning group at 6 weeks; 2w (6w) = early weaned group at 6 weeks; 2w+FF (6w) = early weaned group + formula feeding at 6 weeks. Kruskal-Wallis test, * p value ≤ 0.05; ** p value ≤ 0.01.

For sCD14 (**Figure 27**), no differences occurred both in 3- and 6-week-old mouse groups, unlike the previous experiment. Also, the levels were quite different; in this experiment in fact levels seemed to be higher compared to the previous one, with the 6-week-old mouse groups levels slightly higher compared to the 3-week-old mouse groups.

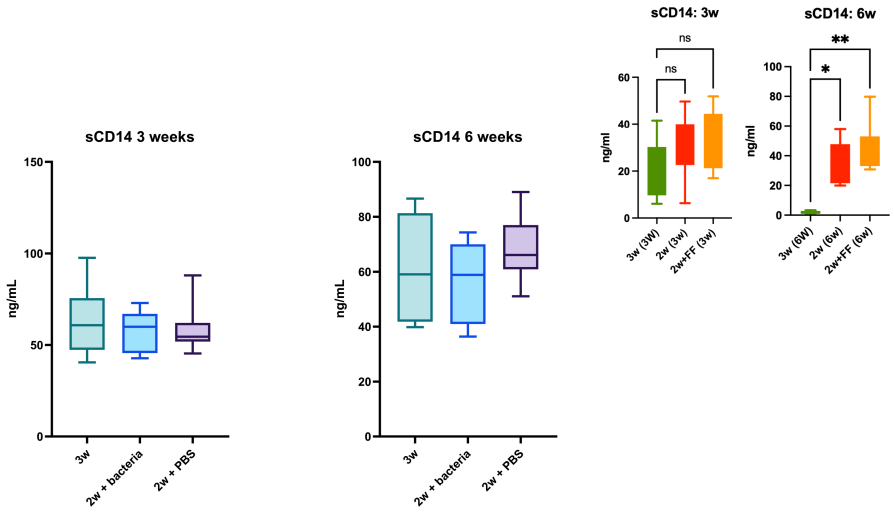

Figure 27. Boxplot representing sCD14 levels in serum samples, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. For bigger graphs (current experiment): 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. For smaller graphs (previous experiment): 3w (3W) = normal weaning group at 3 weeks; 2w (3w) = early weaned group at 3 weeks; 2w+FF (3w) = early weaned group + formula feeding at 3 weeks; 3w (6W) = normal weaning group at 6 weeks; 2w (6w) = early weaned group at 6 weeks; 2w+FF (6w) = early weaned group + formula feeding at 6 weeks. Kruskal-Wallis test, * p value ≤ 0.05; ** p value ≤ 0.01.

Regarding the TAC analysis (**Figure 28**), there were no differences between the 3 groups at 3 weeks, but it was possible to note that the same differences of the previous experiment come out at 6 weeks (EW + B vs NW, p value = 0.04; EW vs NW, p value < 0.0001). Also, at 6 weeks TAC values increased a bit, but in the previous experiment they were a bit higher.

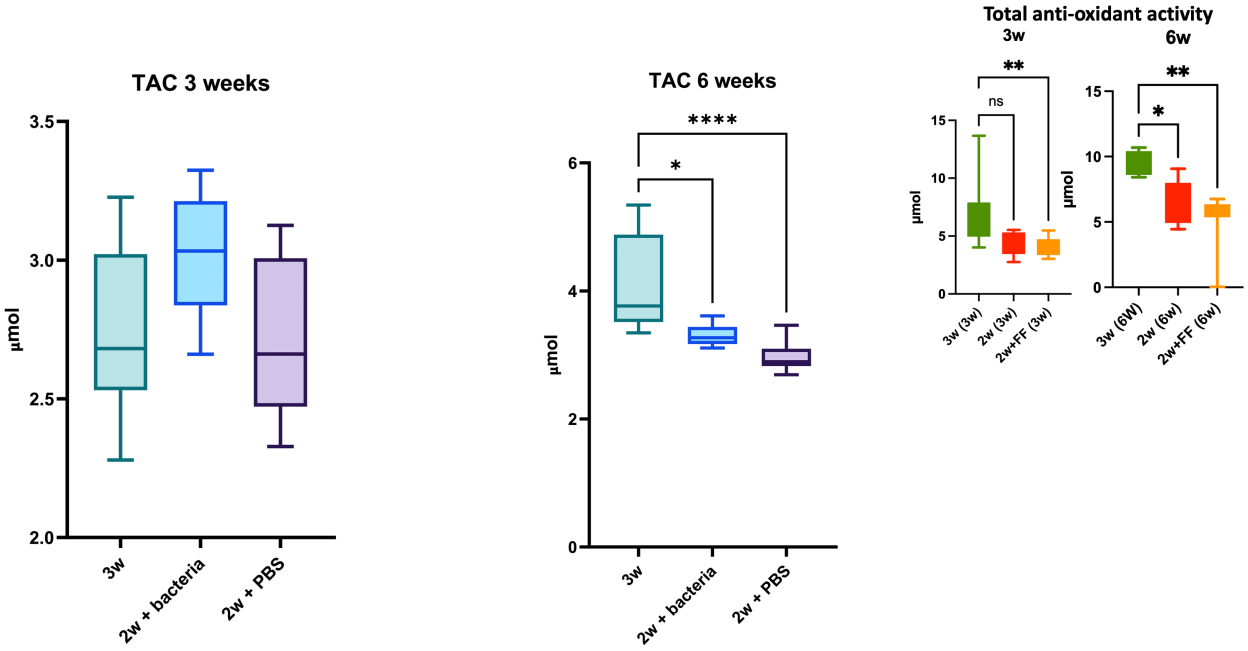

Figure 28. Boxplot representing TAC levels in serum samples, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. For bigger graphs (current experiment): 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. For smaller graphs (previous experiment): 3w (3W) = normal weaning group at 3 weeks; 2w (3w) = early weaned group at 3 weeks; 2w+FF (3w) = early weaned group + formula feeding at 3 weeks; 3w (6W) = normal weaning group at 6 weeks; 2w (6w) = early weaned group at 6 weeks; 2w+FF (6w) = early weaned group + formula feeding at 6 weeks. Kruskal-Wallis test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01; **** *p* value ≤ 0.0001.

4.3.2 Local inflammation

To assess local inflammation, levels of IgA, IgG, IgM and Lipocalin-2 were measured using colon samples. IgA levels increased over time in the 6-week-old mouse groups, while remaining similar in the 3-week-old mouse groups. At 6 weeks, there was a reduction in the EW + B group (*p* value = 0.007), as well as differences between EW and NW groups (*p* value = 0.02) but no differences occurred between the EW + B and NW groups, indicating a potential restoration to normal IgA levels by the nutritional intervention. The same results were also highlighted in the serum samples. IgG levels increased over time, but no differences occurred between the 3- and 6-week-old mouse groups. No differences were also shown in IgM levels between groups (**Figure 29**).

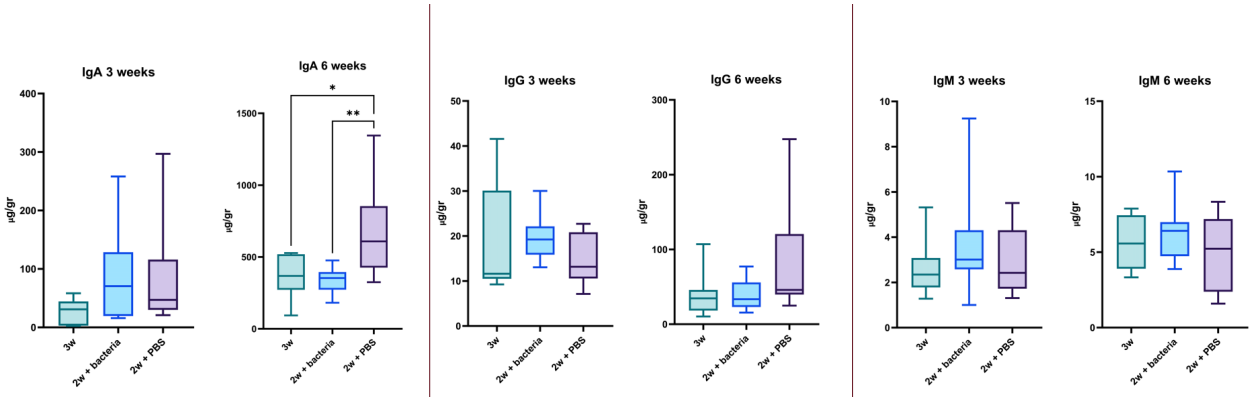

Figure 29. Boxplot representing IgA, IgG and IgM levels in colon samples, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. Kruskal-Wallis test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01.

Lipocalin-2 levels in the early weaning groups, with and without nutritional intervention, were generally low, without significant differences in the 3-week-old mouse groups. On the other hand, at 6 weeks, levels were lower compared to those registered at 3 weeks, with differences between NW and EW + B (*p* value = 0.04). In the latter group, Lipocalin-2 levels were markedly lower compared to the NW group (**Figure 30**).

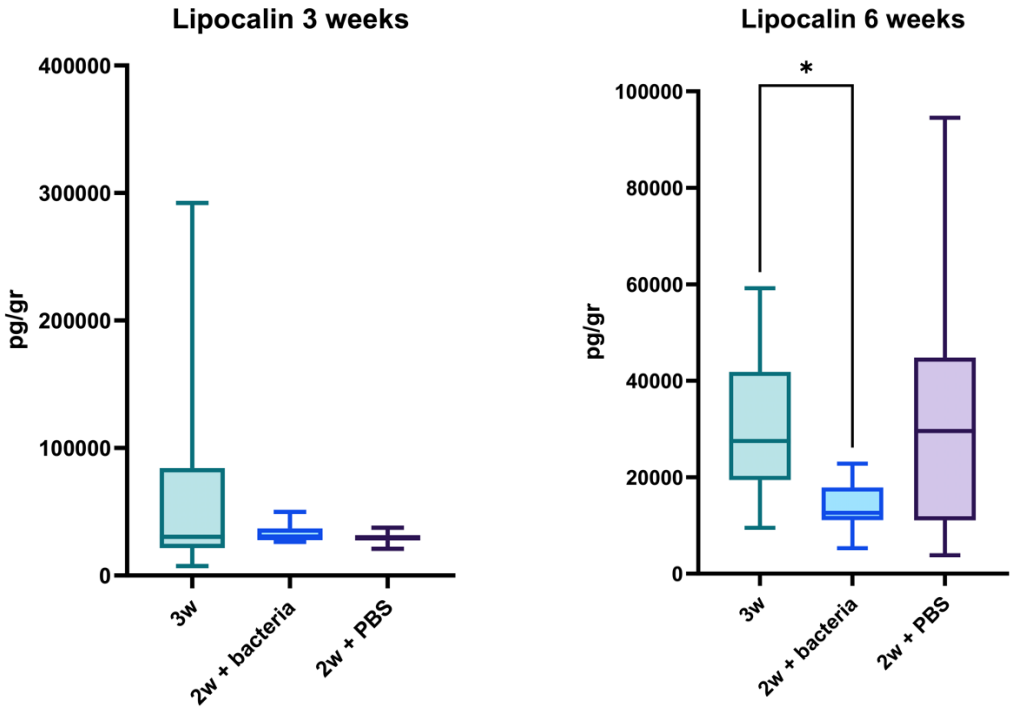

Figure 30. Boxplot representing Lipocalin-2 levels in colon samples, in 3- and 6-week-old mice. Darker lines inside the boxplots represent the median values. 3w = normal weaning group; 2w + bacteria = early weaned group + formula feeding + bacterial supplementation; 2w + PBS = early weaned group + formula feeding. Kruskal-Wallis test, * *p* value ≤ 0.05.

4.3.3 16S rRNA analysis of the mouse gut microbiome

16S rRNA amplicon sequencing outputs showed some differences for intra- and inter-sample diversity, as well as in the taxonomical composition. For α-diversity, differences occurred between groups with two of the three metrics analyzed (**Figure 31 A**). In particular, at 3 weeks, early weaning led to a loss of biodiversity (according to the Faith's phylogenetic diversity metric; *p* value = 0.02), which was partly restored with the nutritional intervention. At 6 weeks, the biodiversity levels of the EW and NW groups differed from the EW + B group, whose biodiversity was lower, according to the Faith's phylogenetic diversity (EW + B vs. EW *p* value = 0.01; EW + B vs NW *p* value = 0.04) and the number of observed ASVs metrics (EW + B vs. EW, *p* value = 0.01; EW + B vs NW, *p* value = 0.03). A significant segregation between groups was also observed at both 3 and 6 weeks in the weighted and unweighted

UniFrac-based PCoA (**Figure 31 B**). In particular, at 3 weeks, significant differences occurred between EW and NW groups with both metrics (weighted UniFrac *p* value = 0.04; unweighted UniFrac *p* value = 0.001), and between EW and EW + B with the unweighted UniFrac metric (*p* value = 0.001). At 6 weeks, EW and EW + B significantly segregated in both PCoAs (weighted UniFrac *p* value = 0.04; unweighted UniFrac *p* value = 0.009).

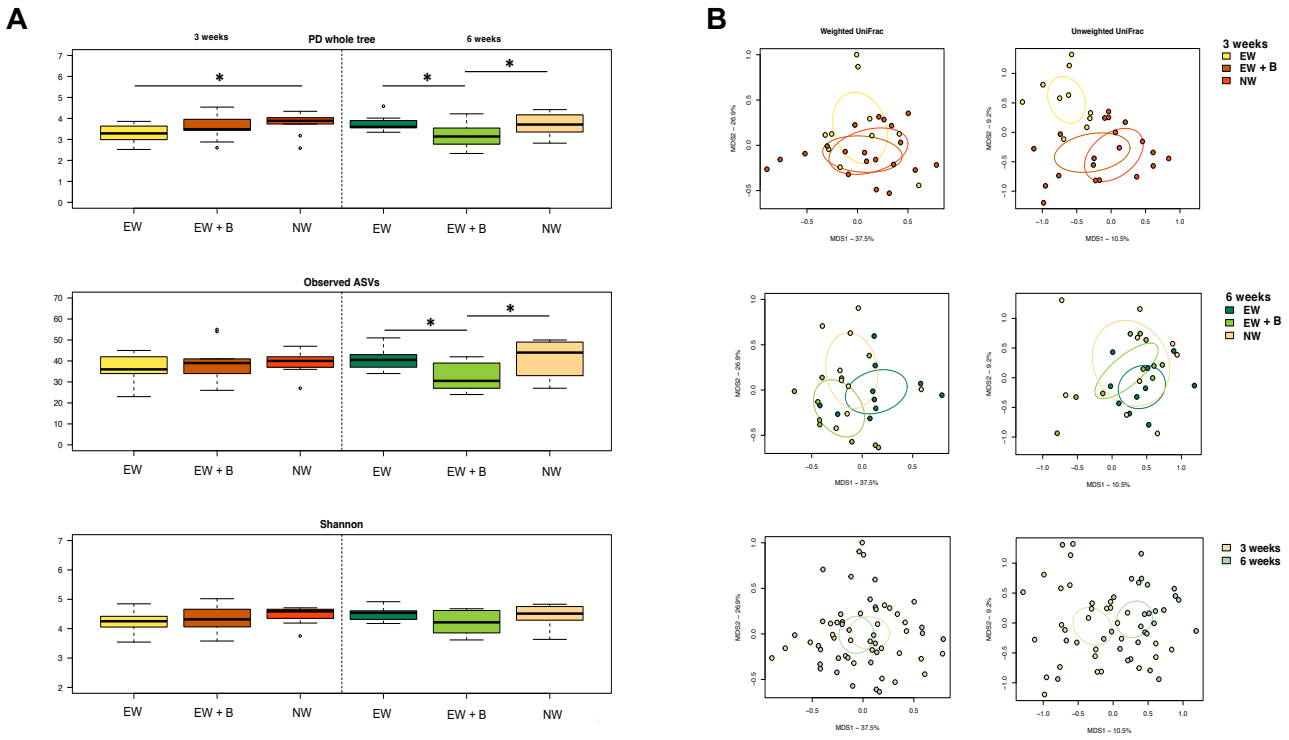

Figure 31. GM dynamics in 3- and 6-week-old mice. **A**, boxplots showing the distribution of α-diversity, estimated with Faith's phylogenetic diversity (PD whole tree), the number of observed ASVs and the Shannon index, in the three groups at both 3 weeks and 6 weeks. Wilcoxon test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01. **B**, Principal Coordinates Analysis (PCoA) based on weighted and unweighted UniFrac distances between the microbial profiles of the study groups. At 3 weeks, a significant segregation was observed between EW and NW groups (*p* value = 0.04 for weighted UniFrac, *p* value = 0.001 for unweighted UniFrac) and between EW and EW + B groups for unweighted UniFrac (*p* value = 0.001). At 6 weeks, a significant segregation was observed between EW and EW + B groups (*p* value = 0.04 for weighted UniFrac, *p* value = 0.009 for unweighted UniFrac). The 3 weeks and 6 weeks groups were found to be significantly different in both PCoAs (*p* value = 0.005 for weighted Unifrac, *p* value = 0.001 for unweighted Unifrac).

Taxonomically, the three groups differed in the relative abundance of several taxa, with remarkable differences even at phylum level. **Figure 32 A** and **B** show the phylum-level relative abundances of the six groups, with an increase of Bacteroidota in EW group (*p* value = 0.03) and Firmicutes (*p* value = 0.05) in NW group at 6 weeks. Similar abundances were observed between EW + B and NW groups.

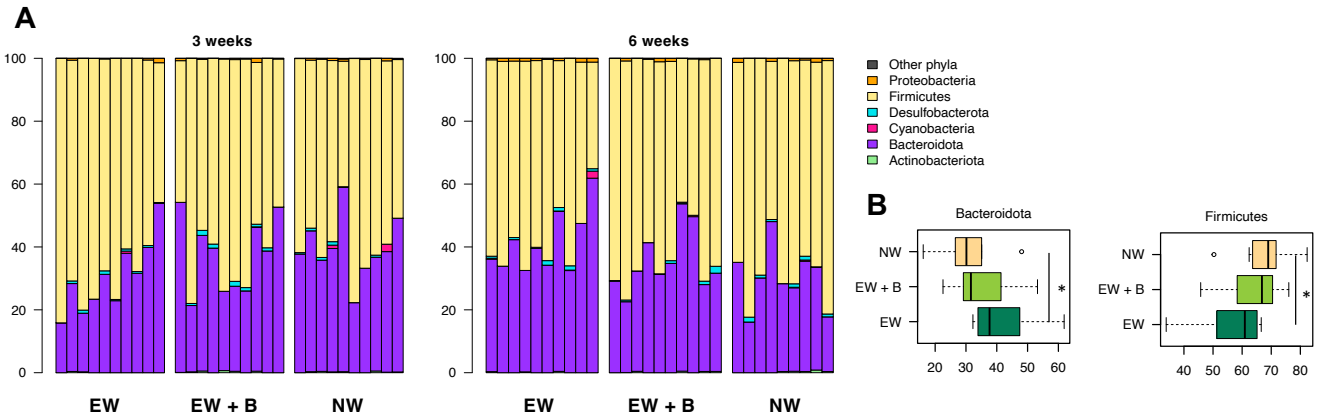

Figure 32. Phylum-level compositional structure of 3- and 6-week-old mice. **A**, barplots showing the relative abundance profiles of the mice's colon content, at 3 and 6 weeks, divided by the three groups of study. **B**, boxplots showing the relative abundance distribution of phyla in the three groups at 6 weeks. Wilcoxon test, * *p* value ≤ 0.05.

More differences were found when considering relative abundances at the family level, visible in both barplot and heatmap representations (**Figure 33 A** and **B**). In particular, a higher relative abundance of *Bacteroidaceae* (*p* value = 0.02) and *Prevotellaceae* (*p* value = 0.02) was found in the 3-week-old mice, while *Muribaculaceae* (*p* value = 0.046) and *Erysipelotrichaceae* (*p* value = 0.016) were overrepresented in the 6-week-old mice (**Figure 34 A**).

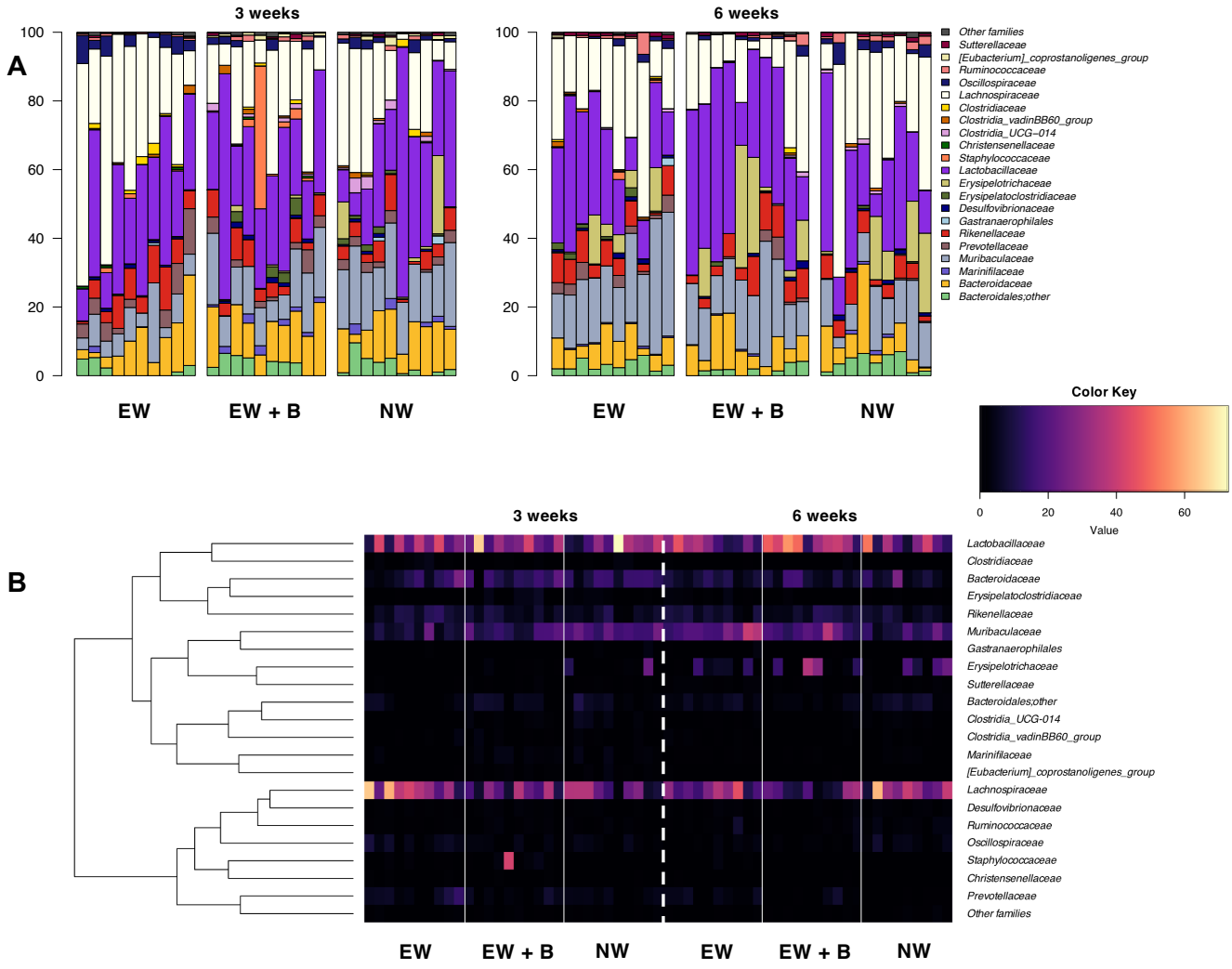

Figure 33. Family-level compositional structure of 3- and 6-week-old mice. **A**, barplots showing the relative abundance profiles of the mice's colon content, at 3 and 6 weeks, divided by the three groups of study. **B**, heatmap showing the relative abundance profiles of the mice's colon content, at 3 and 6 weeks, divided by the three groups of study.

When exploring differences between groups at 3 weeks (**Figure 34 B**), we found that early weaning led to depletion of *Marinifilaceae* (*p* value = 0.009) , while the intervention was able to restore its abundance to the NW levels, as well as that of *Muribaculaceae* (*p* value = 0.03). Interesting differences also occurred for *Lachnospiraceae* (*p* value = 0.03) and *Oscillospiraceae* (*p* value = 0.04) families, which showed higher levels in the EW group compared to the EW + B group. Significant differences were also observed for the *Muribaculaceae* (*p* value = 0.01) and *Lachnospiraceae* (*p* value = 0.03) families at 6 weeks (**Figure 34 C**). Specifically, for the *Muribaculaceae* family, the abundances displayed a completely opposite trend compared to what was observed at 3 weeks. In contrast, for the

Lachnospiraceae family, the abundances in the EW + B group were significantly lower than those in the NW group. The nutritional intervention also appeared to have an effect on the *Prevotellaceae* family, whose levels were much higher in mice undergoing early weaning (*p* value = 0.01). Finally, the nutritional intervention had an interesting effect on *Lactobacillaceae*, increasing their levels at 6 weeks (*p* value = 0.045).

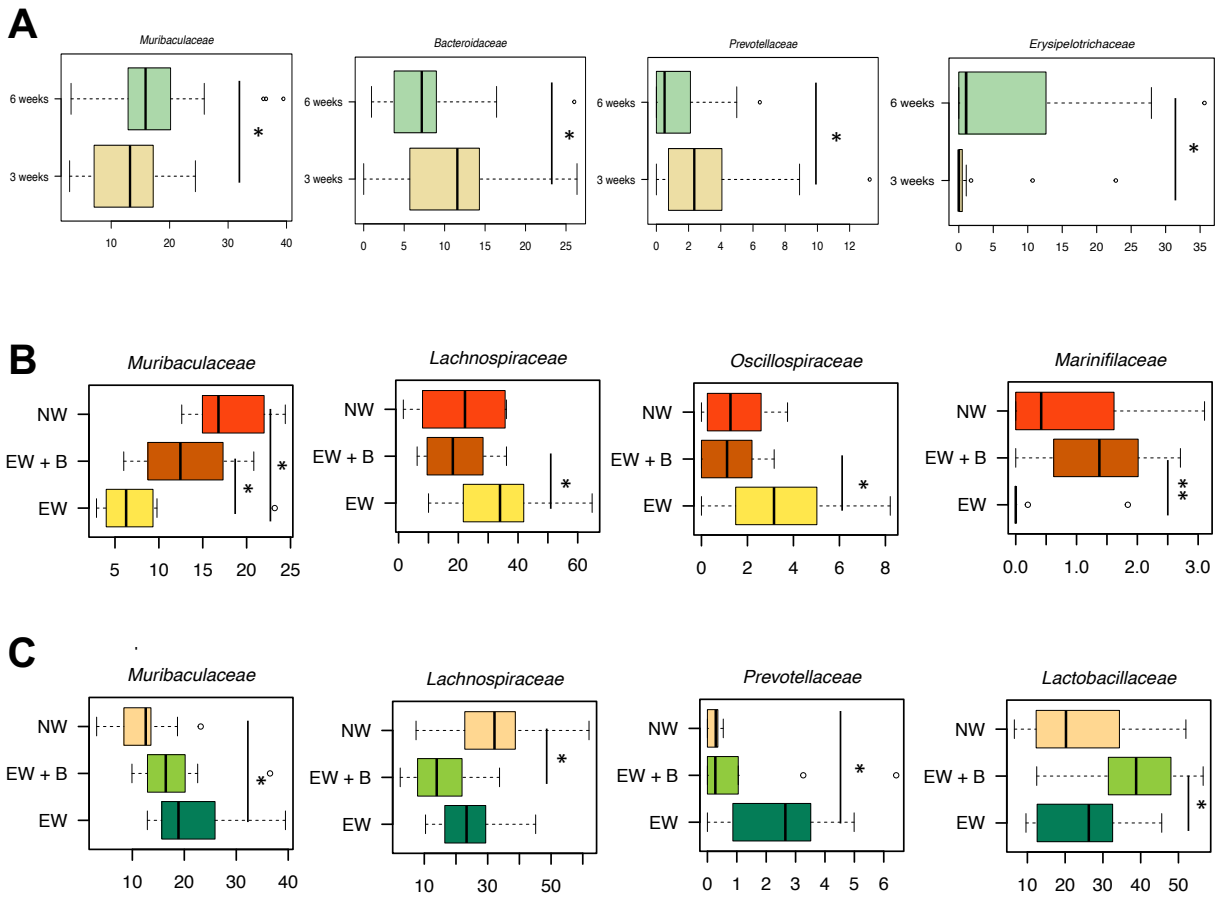

Figure 34. Family-level relative abundance profiles differentially represented in 3- and 6-week-old mice. **A**, boxplots showing the relative abundance distribution of families in the three groups between 3- and 6-week-old mice. **B**, boxplots showing the relative abundance distribution of families in the three groups at 3 weeks. **C**, boxplots showing the relative abundance distribution of families in the three groups at 6 weeks. Wilcoxon test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01.

Finally, **Figure 35 A** and **B** show the taxonomic composition at the genus level with both barplot and heatmap representations. Overall, *Bacteroides*, *Lactobacillus* and *Lachnospiraceae;other* were the three genera most represented at both 3 and 6 weeks, with *Odoribacter* and *Alloprevotella* more represented at 3 weeks, even if their abundances were

quite low. In the heatmap it was possible to appreciate the abundances of *Duboisiella* that came out at 6 weeks, maintaining very low abundances in the first 3 weeks. Significant differences between 3 and 6 weeks were observed for *Bacteroides* (*p* value = 0.02) and *Alloprevotella* (*p* value = 0.007) (**Figure 35 C**), both of which were overrepresented at 3 weeks.

Between the three groups at 3 weeks (**Figure 36 A**), early weaning led to an enrichment of *Alloprevotella* (*p* value = 0.034) and a depletion of *Odoribacter* (*p* value = 0.009), while the nutritional intervention partly restored the latter. These differences were even more evident at 6 weeks (**Figure 36 B**). Differences were also found for *Lachnospiraceae;other* between EW and NW groups (*p* value = 0.009) (and between EW + B and NW at 6 weeks (*p* value = 0.045)) and for *Lachnospiraceae;uncultured* between EW and EW + B (*p* value = 0.029) groups. At 6 weeks, the nutritional intervention decreased *Bacteroidales;other* compared to the EW group (*p* value = 0.028), while it increased *Lactobacillus* (*p* value = 0.045), restoring its levels to those of the NW group. Finally, appreciable levels of *Roseburia*, a beneficial gut microorganism, were only present when the mice underwent a normal weaning protocol (NW vs EW + B, *p* value = 0.007; NW vs EW, *p* value = 0.001).

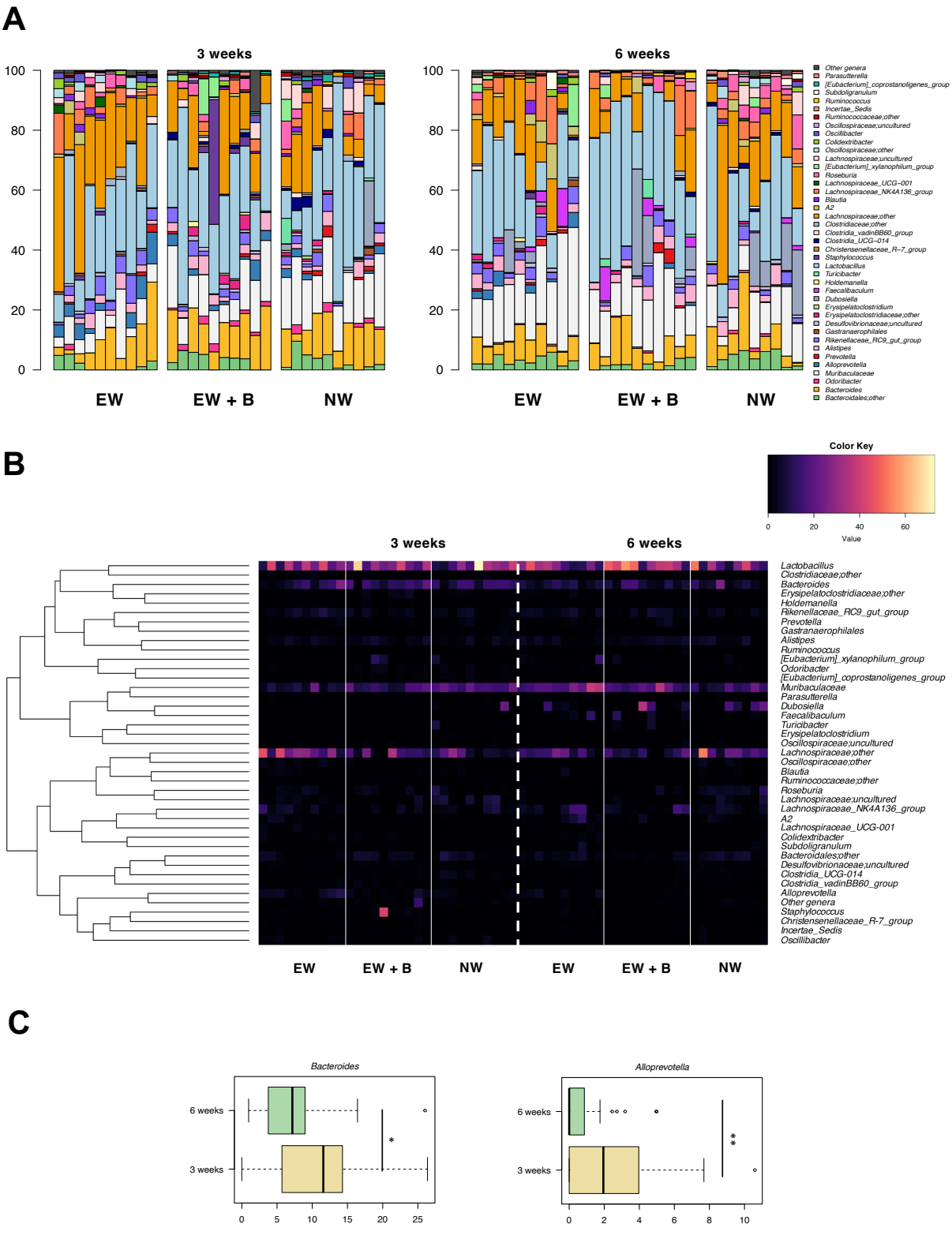

Figure 35. Genus-level compositional structure of 3- and 6-week-old mice. **A**, barplots showing the relative abundance profiles of the mice's colon content, at 3 and 6 weeks, divided by the three groups of study. **B**, heatmap showing the relative abundance profiles of the mice's colon content, at 3 and 6 weeks, divided by the three groups of study. **C**, boxplots showing the relative abundance distribution of genera between 3- and 6-week-old mice. Wilcoxon test, * p value ≤ 0.05; ** p value ≤ 0.01.

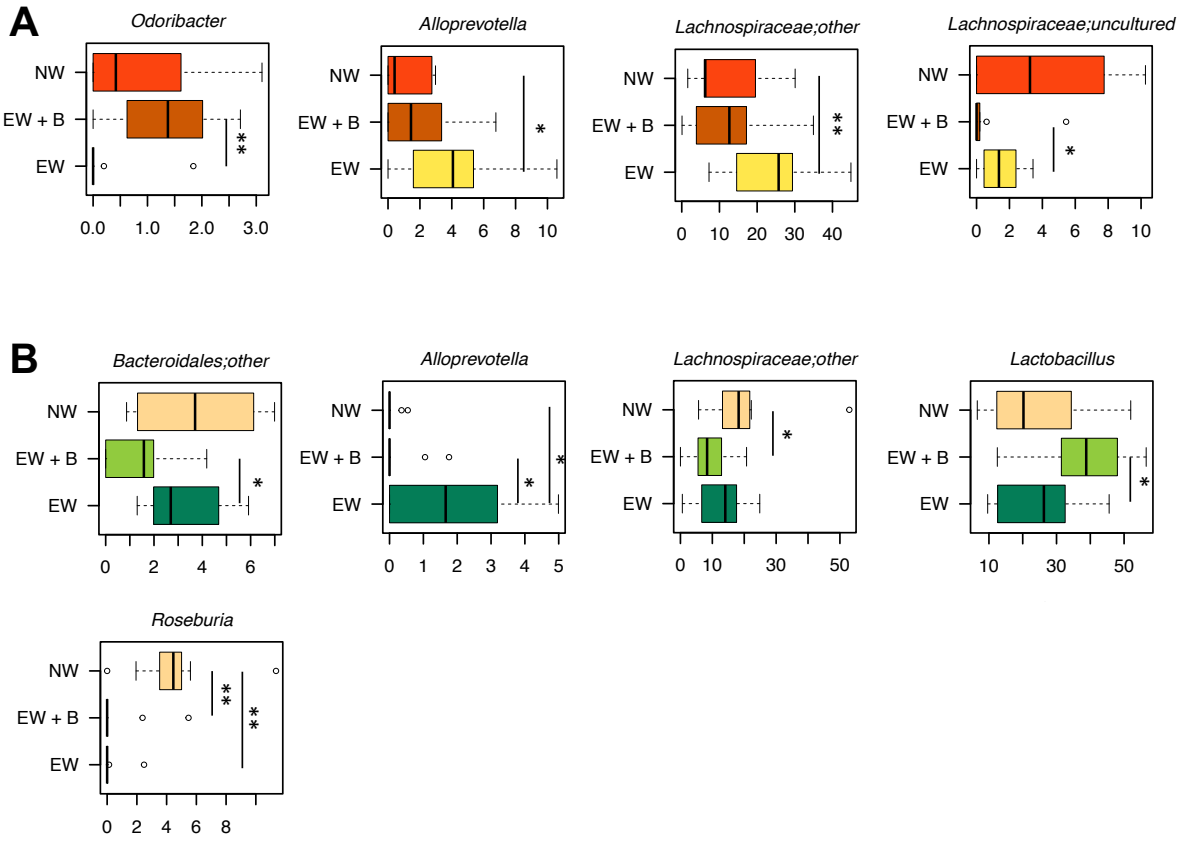

Figure 36. Genus-level relative abundance profiles differentially represented in 3- and 6-week-old mice. **A**, boxplots showing the relative abundance distribution of genera in the three groups at 3 weeks. **B**, boxplots showing the relative abundance distribution of genera in the three groups at 6 weeks. Wilcoxon test, * *p* value ≤ 0.05; ** *p* value ≤ 0.01.

4.4 Discussion

Early weaning represents a concern due to its short- and long-term effects on the physiology and physiopathology of a plethora of NCDs. In all cases where prevention is not possible, an alternative to drugs (that can have a strong impact on the GM) could be the use of probiotics, especially *ad hoc* consortia. In this study, a model of EW was established, firstly to explore the effects of EW on the GM of mice, for which data are still lacking in the literature, and secondly to test innovative probiotic formulations specifically targeting SCFA production, with the aim of ameliorating the gut ecosystem.

Early weaning effect on mice

EW showed its effects already from the results of weight, which was higher at both 3 and 6 weeks, compared with normal weaning. These findings seem to be inconsistent with what has been found in the literature, where mouse weight did not change or was even lower between groups (when compared to normally weaned mice)[577, 578, 579]. This could be due to the fact that in both EW groups, mice were supplemented with formula feeding (since previous experiments showed higher mortality rate in EW compared to NW), resulting in a higher weight. Systemically, EW led to an inflammatory pattern, decreasing at 3 weeks TAC levels and increasing a biomarker of intestinal and metabolic inflammation, Lipocalin-2, whose higher levels are observed in obesity and related disorders[580]. Furthermore, serum IgA levels were found to be slightly higher in the EW group compared to the NW group, but not significantly. Moreover, the results of TAC, weight and Lipocalin-2 analysis at 6 weeks on serum samples were comparable to those of the previous experiment. Considering the effect of EW without the nutritional intervention compared to NW at 3 weeks, it was found that the biodiversity of colon content was decreased by EW, along with differences in β-diversity. However, these differences flattened out at 6 weeks. Taxonomically, the colon microbiota of early weaned mice was characterized by a decrease in the *Muribaculaceae* family at 3 weeks, while an increase in this family occurred at 6 weeks. This is in line with the literature because this taxon is generally recognized as an health indicator and is often

[577] Bailoo, J.D. et al.; "Effects of Weaning Age and Housing Conditions on Phenotypic Differences in Mice" in *Scientific Reports* 10, no. 1 (**2020**): 11684.

[578] George, E.D. et al.; "Maternal Separation with Early Weaning: A Novel Mouse Model of Early Life Neglect" in *BMC Neuroscience* 11, no. 1 (**2010**): 123.

[579] Kikusui, T., S. Ichikawa, & Y. Mori; "Maternal Deprivation by Early Weaning Increases Corticosterone and Decreases Hippocampal BDNF and Neurogenesis in Mice" in *Psychoneuroendocrinology* 34, no. 5 (**2009**): 762–772.

[580] Moschen, A.R. et al.; "Lipocalin-2: A Master Mediator of Intestinal and Metabolic Inflammation" in *Trends in Endocrinology & Metabolism* 28, no. 5 (**2017**): 388–397.

negatively correlated with inflammation-associated parameters and diseases[581, 582, 583]. Additionally, EW led to a total depletion of *Roseburia*, an important recognized marker of gut health. Alterations in *Roseburia* spp. levels may influence diverse metabolic pathways and have been linked to a range of diseases, including irritable bowel syndrome, obesity, T2D, allergies and neurological disorders[584, 585, 586]. EW also led to an increase in some important core-gut taxa such as *Alloprevotella* (also confirmed at family level), at both 3 and 6 weeks, and *Lachnospiraceae;other* at 3 weeks.

Nutritional intervention mitigates some of the early weaning effects

The probiotic consortium administered to the mice appeared to mitigate some EW effects. For instance, there was a trend towards increased TAC levels at 3 weeks (while at 6 weeks levels decreased as in the EW group). Interestingly, when focusing on the IgA levels in serum and colon samples, the nutritional intervention decreased their levels and restored them to levels more closely resembling those observed under NW.

Another effect associated with the probiotic consortium was the marked increase of serum IgM levels, especially in 3-week-old mice. This could probably be related to the nature of the intervention; the administration of probiotics in murine models has found to raise the levels

[581] Berlin, P. et al.; "Dysbiosis and Reduced Small Intestinal Function Are Required to Induce Intestinal Insufficiency in Mice" in *American Journal of Physiology-Gastrointestinal and Liver Physiology* 324, no. 1 (**2023**): G10–G23.

[582] Shang, L. et al.; "Core Altered Microorganisms in Colitis Mouse Model: A Comprehensive Time-Point and Fecal Microbiota Transplantation Analysis" in *Antibiotics* 10, no. 6 (**2021**): 643.

[583] Sibai, M. et al.; "Microbiome and Longevity: High Abundance of Longevity-Linked Muribaculaceae in the Gut of the Long-Living Rodent Spalax Leucodon" in *OMICS: A Journal of Integrative Biology* 24, no. 10 (**2020**): 592–601.

[584] Kasahara, K. et al.; "Interactions between Roseburia Intestinalis and Diet Modulate Atherogenesis in a Murine Model" in *Nature Microbiology* 3, no. 12 (**2018**): 1461–1471.

[585] Luo, W. et al.; "Roseburia Intestinalis Supernatant Ameliorates Colitis Induced in Mice by Regulating the Immune Response" in *Molecular Medicine Reports* 20, no. 2 (**2019**): 1007–1016.

[586] Tamanai-Shacoori, Z. et al.; "Roseburia Spp.: A Marker of Health?" in *Future Microbiology* 12, no. 2 (**2017**): 157–170.

of IgM in the literature, even if these formulations included just *Lactobacillus* spp.[587] and *Bifidobacterium* spp.[588]. Also, even if the nutritional intervention effects were not visible at 3 weeks on serum Lipocalin-2 levels, they became clearly evident at 6 weeks locally, with markedly reduced Lipocalin-2 levels. Surprisingly, at 6 weeks, the probiotic intervention caused a reduction in α-diversity and a difference in β-diversity between the EW group and the EW + B group, for both weighted and unweighted UniFrac (for this latter metric, differences were also observed at 3 weeks), suggesting profound differences in composition. These results highlighted the role of the intervention, since in both groups EW was performed. Compared with the EW group, at 3 weeks the intervention restored the abundances of *Marinifilaceae*, *Muribaculaceae* as well as the butyrate producer *Odoribacter*, a beneficial microorganism whose decrease has been reported to be associated with non-alcoholic fatty liver disease, cystic fibrosis and inflammatory bowel disease[589]. A significant decrease was also found in *Lachnospiraceae* and *Oscillospiraceae*, two butyrate-producing families[590]. A decrease in *Lachnospiraceae* could be positive since this family has been correlated with some diseases, even if it is overall considered beneficial[591]. At 6 weeks, the nutritional intervention led to an increase in *Lactobacillaceae* (and its genus *Lactobacillus*), while a decrease in *Lachnospiraceae* compared to the NW

[587] Kadafi, K.T. & S. Wibowo; "Differences in Systemic Humoral Immune Response among Balb/c Mice Administered with Probiotic, LPS Escherichia Coli, and Probiotic-LPS E. Coli" in *Iranian Journal of Microbiology* 11, no. 4 (**2019**): 294–299.

[588] Vieira, A.T., M.M. Teixeira, & F. dos S. Martins; "The Role of Probiotics and Prebiotics in Inducing Gut Immunity" in *Frontiers in Immunology* 4 (**2013**); Martins, A.K.S. et al.; "Evaluation of in Vitro Antagonism and of in Vivo Immune Modulation and Protection against Pathogenic Experimental Challenge of Two Probiotic Strains of Bifidobacterium Animalis Var. Lactis" in *Archives of Microbiology* 192, no. 12 (**2010**): 995–1003.

[589] Hiippala, K. et al.; "Novel Odoribacter Splanchnicus Strain and Its Outer Membrane Vesicles Exert Immunoregulatory Effects in Vitro" in *Frontiers in Microbiology* 11 (**2020**).

[590] Plomp, N. et al.; "A Convenient and Versatile Culturomics Platform to Expand the Human Gut Culturome of Lachnospiraceae and Oscillospiraceae" (**2024**).

[591] Vacca, M. et al.; "The Controversial Role of Human Gut Lachnospiraceae" in *Microorganisms* 8, no. 4 (**2020**): 573.

group as at 3 weeks. Also, *Roseburia*, a *Lachnospiraceae* genus, was completely absent at 6 weeks in the EW + B group. This pattern was also seen for the EW group, suggesting that the effect of early weaning on this genus cannot be reversed by the probiotic intervention. Finally, no significant differences were found in the relative abundances of the taxa belonging to the bacterial consortium.

Chapter 5: CONCLUDING REMARKS

The two works here presented explored the potential of two different interventions (drug- and probiotics-based) in *in vitro* and *in vivo* models.

In the first study, the Batch Gut Model Cultures assay provided the opportunity to evaluate a plethora of different compounds, without the immediate need for an *in vivo* model. This approach significantly reduced the time and resources, required while adhering to the principles of the 3Rs (Replacement, Reduction, and Refinement)[592]. By pre-screening various compounds, this method minimized unnecessary animal use and allowed for more focused and precise analysis. The study demonstrated the efficacy of the four novel compounds against AR *C. difficile*, even at low doses, and also highlighted their impact on the GM. However, there are certain limitations, such as the absence of an immune component within the multi-well system, restricting immediate exploration of immunological responses.

In the second study, the effects of an *ad hoc* probiotic consortium were assessed to mitigate some signatures associated with early weaning practice. The model employed in this study has the potential to elucidate intestinal microbial invasion and its resilience during healthy adulthood. This study underscored the importance of designing customized probiotic formulations, and suggested that the GM may play a critical role in modulating the effects of early weaning in mice. Nonetheless, the project will proceed with an investigation into the effects of nutritional intervention in the F_4 generation (refer to **Figure 22**, Chapter 4.2.1), aiming to elucidate the long-term impact of vertical microbiota transmission. Moreover,

[592] MacArthur Clark, J.; "The 3Rs in Research: A Contemporary Approach to Replacement, Reduction and Refinement" in *The British Journal of Nutrition* 120, no. s1 (**2018**): S1–S7.

metabolomic analyses to evaluate the restoration of SCFAs levels are essential and pivotal for determining whether the selected microbial consortium is effective in reestablishing GM eubiosis. Furthermore, considering the limited research addressing the interplay between early weaning and the microbiota, this novel model provided a valuable framework for advancing future studies exploring GM dynamics in greater detail, as well as other health outcomes.

Chapter 6: LIST OF PUBLICATIONS FROM THE AUTHOR

Mengoli, M., Conti, G., Fabbrini, M., Candela, M., Brigidi, P., Turroni, S., & Barone, M. (2023). Microbiota-gut-brain axis and ketogenic diet: how close are we to tackling epilepsy?. *Microbiome Research Reports*, 2(4).

Mengoli, M., Barone, M., Fabbrini, M., D'Amico, F., Brigidi, P., & Turroni, S. (2022). Make it less difficile: Understanding genetic evolution and global spread of *Clostridioides difficile*. *Genes*, 13(12), 2200.

Roggiani, S., **Mengoli, M.**, Conti, G., Fabbrini, M., Brigidi, P., Barone, M., D'Amico, F., & Turroni, S. (2023). Gut microbiota resilience and recovery after anticancer chemotherapy. *Microbiome Research Reports*, 2(3).

Fabbrini, M., D'Amico, F., Barone, M., Conti, G., **Mengoli, M.**, Brigidi, P., & Turroni, S. (2022). Polyphenol and tannin nutraceuticals and their metabolites: How the human gut microbiota influences their properties. *Biomolecules*, 12(7), 875.

genes

MDPI

Review

Make It Less *difficile*: Understanding Genetic Evolution and Global Spread of *Clostridioides difficile*

Mariachiara Mengoli [1], Monica Barone [1,2], Marco Fabbrini [1,2], Federica D'Amico [1,2], Patrizia Brigidi [1] and Silvia Turroni [2,*]

[1] Microbiomics Unit, Department of Medical and Surgical Sciences, University of Bologna, 40138 Bologna, Italy
[2] Unit of Microbiome Science and Biotechnology, Department of Pharmacy and Biotechnology, University of Bologna, 40126 Bologna, Italy
* Correspondence: silvia.turroni@unibo.it

Abstract: *Clostridioides difficile* is an obligate anaerobic pathogen among the most common causes of healthcare-associated infections. It poses a global threat due to the clinical outcomes of infection and resistance to antibiotics recommended by international guidelines for its eradication. In particular, *C. difficile* infection can lead to fulminant colitis associated with shock, hypotension, megacolon, and, in severe cases, death. It is therefore of the utmost urgency to fully characterize this pathogen and better understand its spread, in order to reduce infection rates and improve therapy success. This review aims to provide a state-of-the-art overview of the genetic variation of *C. difficile*, with particular regard to pathogenic genes and the correlation with clinical issues of its infection. We also summarize the current typing techniques and, based on them, the global distribution of the most common ribotypes. Finally, we discuss genomic surveillance actions and new genetic engineering strategies as future perspectives to make it less *difficile*.

Keywords: *Clostridioides difficile*; PaLoc; antibiotic resistance; worldwide spread; ribotypes; genomic surveillance; genetic engineering

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1. Introduction

Clostridioides difficile, formerly known as *Clostridium difficile* [1], is an obligate anaerobic pathogen among the most common causes of healthcare-associated infections [2]. It was first described in 1935 by Hall and O'Toole, following isolation from a newborn's stool, and initially called *Bacillus difficilis* due to the difficulty of cultivating it [3].

C. difficile is a Gram-positive, spore-forming, rod-shaped bacterium. Its spores are able to resist oxygen, heat, and common disinfectants (e.g., ethanol) thanks to their low water content and other properties, such as high levels of dipicolinic acid and saturation of DNA with soluble proteins [4–7].

This pathogen is transmitted via the fecal–oral route: if ingested, *C. difficile* spores withstand the acidic conditions of the stomach and then germinate in the intestine. This happens in the lower gastrointestinal tract where the oxygen concentration is lower [8]. Spore germination is triggered by certain compounds such as the primary bile acid, cholic acid, and cholesterol derivatives, including taurocholate. On the other hand, chenodeoxycholate is able to inhibit the germination of *C. difficile* spores [9–11]. Once germination has begun, the vegetative cells are able to colonize the colon and start producing toxins [12].

C. difficile generally produces two exotoxins, namely toxin A (TcdA) and toxin B (TcdB), which are both enterotoxic and cytotoxic. In addition, some *C. difficile* strains are capable of producing *C. difficile* transferase (CDT or binary toxin). These toxins damage the cytoskeleton of epithelial cells, causing breakdown of tight junctions, fluid secretion, and adhesion of neutrophils, thus leading to disruption of the intestinal barrier integrity, loss of function and local inflammation. In addition to toxin production, *C. difficile* virulence is

also attributable to enzymes, such as collagenase, chondroitin-sulfatase, and hyaluronidase, which are capable of disrupting tight junctions as well, leading to fluid secretion and promoting inflammation [2,13,14].

With these prerequisites, the clinical picture of *C. difficile* infection (CDI) ranges from mild asymptomatic carrier status and diarrhea to fulminant colitis associated with shock, hypotension, or megacolon [15]. In the most severe cases of CDI, symptoms are critical (e.g., colon perforation, intestinal paralysis, septicemia) and can lead to death [15]. The mortality rate due to CDI is estimated at 5%, but the mortality associated with CDI complications ranges from 15% to 25% (up to 34% in intensive care units) [16–18].

Among all the potential risk factors for CDI, we can list increasing age (>65 years) and disorders such as inflammatory bowel disease, malignant tumors, diabetes mellitus and suppression of gastric acidity. However, the strongest risk factor for CDI is antibiotic therapy because it allows for the disruption of the gut barrier integrity [19]. In the study by Webb et al. [20], it is suggested that cumulative exposure to antibiotics, prior to admission for hospitalized patients, is the main contributor to CDI risk. Another risk concerns antibiotic classes: late-generation cephalosporins and carbapenems present a higher risk [21], but even relatively narrow-spectrum antibiotics, such as ampicillin, are associated with CDI. A recent work by Anjewierden et al. [22] has identified risks for asymptomatic CDI, such as hospitalization within six months prior to infection, nasogastric tube feeding, use of gastric acid suppression therapies, as well as use of corticosteroids in the previous eight weeks.

The gut microbiota is also known to play an important role in the pathogenesis of CDI. For example, the presence of bacterial consortia consisting of *Porphyromonadaceae*, *Lachnospiraceae*, *Lactobacillus*, and *Alistipes* might limit the growth of *C. difficile* [23]. On the other hand, in the case of dysbiosis exacerbated especially by the loss of primary fermenters, such as *Lachnospiridae* and *Lactobacillus*, a niche is established in which alternative primary fermenters such as *Bacteroides thetaiotaomicron* can proliferate. These microorganisms can produce various metabolic end-products that have been shown to be used by *C. difficile* as a carbon source [23–25]. Furthermore, some members of the gut microbiota are responsible for the generation of secondary bile acids, which, as anticipated above, can have an impact on the germination of *C. difficile* spore [23]. In particular, bacterial 7alpha-dehydroxylation of primary bile acids appears to protect against CDI [26], potentially representing a biomarker for a *C. difficile*-resistant environment. However, cefoperazone and other broad-spectrum antimicrobials have been shown to disrupt the bile acid-modifying activity of the gut microbiota, thus facilitating the colonization and outgrowth of *C. difficile* [27]. As described by Spigaglia et al. [28], the administration of certain antimicrobials (e.g., cephalosporins and carbapenems) is in fact more commonly associated with the induction of CDI than other pharmacological treatments.

One of the most common problems with CDI is recurrent CDI, which is generally associated with poor clinical outcomes. Some of the risk factors for recurrent CDI, recently described by Alrahmany et al. [29], are over 76 years of age, the total length of hospital stay (>7 days), previous exposure to clindamycin, and concomitant use of aztreonam.

Constituting one of the most important nosocomial infections, several guidelines have been developed to counter the spread of *C. difficile* [15,30]. However, ironically, the use of antibiotics is still considered first-line therapy. Of course, prolonged administration of antibiotics leads to the onset of antimicrobial resistance. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has established epidemiological cut-off values (ECOFFs), i.e., values that distinguish wildtype microorganisms from those with phenotypically detectable acquired resistance mechanisms (non-wildtype) to a given pharmacological agent [31]. According to EUCAST data, in some cases, *C. difficile* shows minimum inhibitory concentration (MIC) values above the ECOFF limit, especially against antimicrobials recommended by European and American guidelines, such as vancomycin, fidaxomicin and metronidazole [31]. However, fortunately, the resistance to these antimicrobials remains quite rare (see paragraph on "Antimicrobial resistance genes") [32,33]. It should also be noted that a correlation between vancomycin treatment to counteract intestinal colonization

of *C. difficile* and the acquisition of vancomycin-resistant *Enterococcus* (VRE) species has been demonstrated [34–36]. In particular, Fujitani et al. [35] found that the rate of VRE exceeded 50% in CDI patients, and patients with VRE had a higher rate of co-infection with multi-drug resistant pathogens. In an epidemiological study of hospital-acquired CDI, involving intensive care units, nearly one-third of patients with CDI were found to be colonized with VRE [37]. The use of intravenous vancomycin or metronidazole could be the main contributing factor to the risk of VRE colonization or infection [35].

Several studies focusing on both human and animal isolates have suggested a possible role for animals as sources of *C. difficile* [38–43]. A small Italian study, conducted on 39 samples, identified 14 different *C. difficile* ribotypes (RTs, see paragraph "Molecular typing techniques for *C. difficile* strains"), of which four (i.e., RT020, RT078, RT106, RT126) were detected in both animal and human samples. It should be noted that RT018 and RT078 were the two most frequently identified RTs, making up nearly 50% of all animal and human strains [44]. However, transmission of *C. difficile* can occur not only in a hospital setting or via animals. There is indeed evidence that *C. difficile* spores can also persist in the food chain, from farm to fork [45].

In light of the serious health implications, it is of the utmost urgency to better understand how to treat and contain the spread of this pathogen. The present review aims to investigate the genetic variation of *C. difficile* pathogenic genes and their relationship with the clinical issues of CDI. Then, we discuss the current typing techniques and the global distribution of the most common RTs, putting forward some hypotheses on the absence of hypervirulent RTs in some countries. Finally, this review summarizes actual genomic surveillance with a focus on novel genetic engineering strategies to make it less *difficile*.

2. Genetic Evolution of *C. difficile* Virulence

The *C. difficile* genome is characterized by a high level of plasticity. In particular, the evolution of its virulence concerns a specific genomic region, namely the Pathogenicity Locus (PaLoc). PaLoc consists of 19.6 kb of a chromosomally located element [46], which includes *tcdA* and *tcdB*, the genes encoding the two main toxins (TcdA and TcdB, respectively). PaLoc also comprises three accessory genes, namely *tcdR*, *tcdC* and *tcdE*. Specifically, *tcdR* encodes a positive regulator of toxin expression, while *tcdC* a negative regulator. *tcdE* encodes a putative holin-like protein, responsible for the secretion of microbial toxins [46,47]. Another toxin produced by *C. difficile* is the binary toxin (CDT), encoded by the Ctd Locus (CdtLoc), a distinct chromosomal region that carries *cdtA* and *cdtB*, the genes for catalytic and binding/translocation proteins, as well as *cdtR*, coding for a regulatory protein. The CdtLoc can be found in two versions, whole or truncated. In strains lacking CdtLoc, a unique 68-bp sequence is found inserted in the same genomic location [48,49]. In non-toxigenic strains, such as the recently discovered NTCD-035 by Maslanka et al. [42], PaLoc is replaced by a conserved non-coding sequence of 115 bp [46,50–52]. Ongoing works on the comparative genomics of *C. difficile* are highlighting some important features on the evolution of these two pathogenic regions. The entire PaLoc region appears to have a modular structure and its variability may be due to the substitution of single nucleotides and to recombination events that played a pivotal role in the evolution of PaLoc variants. This structure also affects the *tcdA* and *tcdB* genes. Interestingly, the CdtLoc region appears to be more conserved than the PaLoc region, but it is mainly observed that full-length CdtLoc is associated with *C. difficile* strains exhibiting significantly altered PaLoc [53]. Mansfield et al. [54] highlighted a difference between *tcdA* and *tcdB*: while the evolutionary history of *tcdB* may depend on extensive homologous recombination, *tcdA* shows a greater degree of sequence variation and a greater number of subtypes [54]. Therefore, the authors suggest that the extreme recombination events observed in *tcdB*, but not in *tcdA*, could lead to increased selective pressure for *tcdB* diversification, highlighting the potential role of *tcdB* in the pathogenesis of *C. difficile*. This observation seems to be confirmed also by studies focused on the use of monoclonal antibodies (e.g., bezlototumab and actoxumab) both in animal models (i.e., gnotobiotic piglets) [55] and in humans [54,56,57].

One of the few works so far focused on the evolutionary history of *C. difficile* is a study by Dingle et al. [52], which paves the way for understanding the genomic background of this bacillus. According to the authors, PaLoc acquisition occurred at separate times between the *C. difficile* clades. Based on multi-locus sequence typing (MLST), *C. difficile* strains can be stratified into at least eight phylogenetic clades: clade 1–5 and clades C-I, C-II and C-III [58]. Clade 1 includes over 200 toxigenic and non-toxigenic sequence type (STs). Clade 2 also contains several highly virulent strains (e.g., ST1). Little is known about clade 3, although it includes ST5, a toxigenic CDT-producing strain [58]. Clade 4 contains ST37, which is responsible for much of the endemic CDI burden in Asia [59] despite the absence of the *tcdA* gene. Clade 5 contains several CDT-producing strains (e.g., ST11), which are highly prevalent in production animals worldwide [60]. Clades C-I, C-II, and C-III, known as "cryptic clades", were first described in 2012 [52,61] and contain more than 50 STs [52,61–64]; however, their evolution remains poorly understood [58,63]. In the study by Dingle et al. [52], the authors suggest that each lineage acquired its current PaLoc variant after the divergence and that the common ancestor of all modern *C. difficile* strains may have been non-toxigenic. In particular, clade 1, which includes the greatest diversity of toxigenic genotypes, may exemplify the most ancient acquisition; this fact would also explain the emergence of non-toxigenic strains within this clade, as sufficient time has elapsed for occasional PaLoc losses to occur. Moreover, the most recent PaLoc loss event occurred about 30 years ago within a genotype belonging to clade 1. In contrast, clades 4 and 5 exemplify the most recent PaLoc acquisition (about 500 years ago), because of their narrow genotypic diversity [52].

3. Antimicrobial Resistance Genes

One of the major issues related to CDI (and recurrent CDI) is antimicrobial resistance, which plays a crucial role in the pathogenesis and spread of *C. difficile* [33]. Resistance to specific antimicrobials may actually support the spread of *C. difficile*, as in the case of tetracycline resistance in RT078 [65] and clindamycin resistance in RT017 [66]. Although resistance to antimicrobials recommended by guidelines for the treatment of CDI remains quite rare [32,33], these guidelines have been modified and reshaped over the years, also to address this issue. In particular, the first guidelines (1995–1997) were focused on the administration of vancomycin and metronidazole, while from 2014 to date they also include treatment with fidaxomicin [67]. Fortunately, most countries in a pan-European study [32] reported metronidazole, vancomycin and fidaxomicin resistance below 10% (most of them reported 0%), and a species-wide genomic study [33] could identify pCD-METRO plasmid (conferring metronidazole resistance) in only 15 of > 10,000 strains tested. Below, we discuss the antimicrobial resistance mechanisms for medications commonly recommended for the treatment of CDI. Regarding the mechanisms of resistance, *C. difficile* strains can encode a vanG-type gene cluster (*vanGCd*), conferring resistance to vancomycin, an antimicrobial glycopeptide that inhibits bacterial cell wall biosynthesis [67]. Specifically, resistance is conferred by the production of an alternative lipid II carrying a D-alanine-D-serine terminus that is seven times less bound by vancomycin than the D-alanine-D-alanine terminus [68]. Constitutive expression of *vanGCd* has been shown in vancomycin-resistant strains isolated in the clinical setting as well as in laboratory-generated mutants, which carry mutations in the two-component VanSR system that regulates *vanGCd* [68].

Metronidazole-resistant *C. difficile* strains are occasionally isolated in clinical practice, but some strains appear to need the heme cofactor to be detectable [69,70]. The authors suggest that *C. difficile* may use heme in oxidative stress responses to metronidazole, as a source of iron and cofactor for redox-associated proteins, as shown in other bacteria [70,71]. Metronidazole belongs to the nitroimidazole class of antimicrobials that can block the helical structure of DNA, leading to strand breakage, inhibition of protein synthesis and cell death [67]. As anticipated above, resistance to metronidazole appears to be mediated by a high-copy-number plasmid, pCD-METRO [72]. However, so far, there are no data available as to which plasmid gene may actually be responsible for the resistance. Another genetic

mechanism involved in metronidazole resistance in clinical isolates is the modification of the catalytic domains of pyruvate-ferredoxin/flavodoxin oxidoreductase (PFOR), a protein encoded by the *nifJ* gene [73]. This modification is mediated by a synonymous codon change in putative xanthine dehydrogenase, which is likely to affect mRNA stability, and frameshift and point mutations that inactivate the iron-sulfur cluster regulator [73].

Another drug commonly used to treat CDI is fidaxomicin, a narrow-spectrum macrocyclic antimicrobial that inhibits RNA synthesis. This drug acts through lipiarmycin A3 (Lpm), its active component, which inhibits bacterial RNA polymerase [74]. Hence, resistance to fidaxomicin is conferred by mutations affecting RNA polymerase, especially the β-subunit of the RNA polymerase [75,76]. Alternative therapies for the treatment of CDI recommended by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the Infectious Diseases Society of America (IDSA), and the Society for Healthcare Epidemiology of America (SHEA) [30,77] include rifaximin and tigecycline. Like fidaxomicin, rifaximin, which belongs to the rifamycin family, inhibits bacterial RNA polymerase, especially the β subunit [28,78]. The β subunit appears to be the primary site of mutations responsible for modifying the rifamycin binding pocket, thus limiting the interaction between the target and the antimicrobial [79]. Tigecycline, a glycylcycline chemically and structurally similar to tetracyclines, has been proposed for the treatment of CDI due to its ability to inhibit spore formation and reduce the toxin production by the pathogen [80]. It should be noted that some *tet* genes (i.e., *tet*(X3) and *tet*(X4), encoding a flavin-dependent monooxygenase), which [81–87] typically confer tetracycline resistance, also appear to mediate tigecycline resistance in various microorganisms of clinical relevance [81,82], thus challenging the clinical efficacy of the entire family of tetracycline antibiotics, including any derivatives.

4. Molecular Typing Techniques for *C. difficile* Strains

Several molecular methods are available for typing *C. difficile*, and routine typing is not performed with the same techniques across countries. The most widely used *C. difficile* typing technique is PCR ribotyping. Specifically, this technique consists in the amplification of the intergenic spacer region (ISR) of the 16S-23S rRNA gene using primers targeting the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene [83]. Due to its high discrimination capacity, PCR ribotyping is currently recommended by the European Centre for Disease Prevention and Control (ECDC) for surveillance of the *C. difficile* spread [84]. However, this molecular technique has a delivery time of up to one week and often requires in-house protocol optimization, thus not being fully transferable between laboratories [85]. Another technique in use is pulsed-field gel electrophoresis (PFGE). This technique is based on DNA digestion, through enzymatic restriction, and separation of DNA fragments on gel. This method therefore provides for the clonal assignment of the bacterial strain based on PFGE banding patterns [86]. The criticality of this method can be identified in the tendency of *C. difficile* DNA to rapid degradation, resulting in non-typable isolates. To overcome this problem, a modified PGFE method has been designed, with different plate cultures compared to the standard protocol, but its application is rare in light of the other methods adopted for molecular typing [87]. Among these, restriction endonuclease analysis (REA) is a technique based on the use of restriction enzymes (e.g., HindIII) but, unlike PFGE, the digestion fragments obtained are separated by classical electrophoresis on agarose or polyacrylamide gels [83]. Other noteworthy typing methods are Multilocus VNTR Analysis (MLVA) and MLST. Specifically, the target of MLVA are variable number tandem repeats (VNTR), disseminated throughout the genome, while MLST uses PCR amplification of housekeeping genes to generate a complete allelic profile [88].

Since there are a multitude of different typing techniques, a web-accessible database [89] (always updated) was set up in 2011 by Griffiths et al. [90]: they typed a total of 49 isolates by MLST and classified them into 40 STs. Since MLST and PCR ribotyping are very similar in discriminatory abilities, they found a correspondence between RTs and STs: multiple RTs for the same ST and multiple STs for the same RT usually had very similar profiles.

Some STs correspond to a single RT (e.g., ST54/RT012), while others to multiple (e.g., ST02/RT014, RT020, RT076 and RT220). However, it should be noted that RTs were not always predictive of STs [90].

In recent years, with the advancement of whole genome sequencing (WGS) techniques, the scientific community is moving towards these methods instead of standard PCR ribotyping and is trying to develop new methods for the characterization of *C. difficile* strains [85,91]. The two main approaches to discover genomic variations are single nucleotide variant (SNV) analysis and core genome or whole genome MLST (cgMLST, or wgMLST). The first technique is based on comparing the differences in single nucleotide polymorphisms (SNPs) while the second is based on the analysis of multiple genes across the whole genome. Cg- or wgMLST typing works according to the same principles as the classic MLST [91]. Three publicly available schemes for *C. difficile* are available for cg- and/or wgMLST typing, and analysis can be performed using commercial software (e.g., BioNumerics, Ridom) or freely accessible online resources (e.g., EnteroBase). Eyre et al. [92] were the first to use WGS of *C. difficile* genomes on benchtop sequencing platforms to investigate its transmission, demonstrating that the use of these technologies could improve infection control and patient outcomes in routine clinical practice. Since then, WGS typing has been widely adopted for CDI surveillance and has revealed some novel insights concerning the spread of *C. difficile* [91].

5. Worldwide Distribution of *C. difficile* Ribotypes

In the following chapters, we will offer a glimpse into the global distribution of the *C. difficile* RTs, divided by country. These data are summarized in Figure 1, while the toxin gene profile of the mentioned RTs is shown in Table S1.

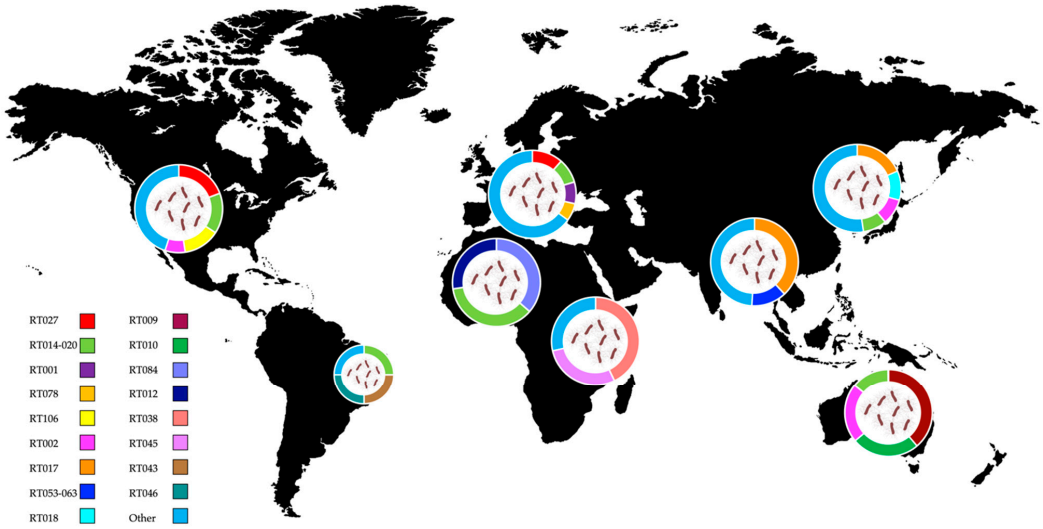

Figure 1. Distribution of *C. difficile* ribotypes in the United States, South America, Europe, North and Central Africa, Asia and Australia. Data are shown in pie charts as percentage, from studies covering different countries [93–95] or single country [96–100]. The smaller pie chart is representative of a single hospital study. The world map was obtained from Freepik.com. See also Table S1 for the toxin gene profile of listed ribotypes.

5.1. Europe

In Europe, several studies have highlighted a common genetic profile of *C. difficile* [93,101–103]. In particular, in 2021, a global study by Zhao et al. [101], based on data obtained from the MODIFY I (NCT01241552) and MODIFY II (NCT01513239) clinical

studies using a WGS approach [104], found that in Europe there is a predominance of clade 1, with the exception of Poland where clade 2 predominates. Clade 2 was found to be one of the most virulent, along with clade 5. Interestingly, these two hypervirulent clades show the lowest recombination rates, while clade 3 and clade 4 show similar recombination rates [101]. Regarding the classification into RTs, clade 1 includes the non-toxigenic RT009, RT010, and RT039 [105], while the hypervirulent RT027 belongs to clade 2 [101]. The study conducted by Zhao et al. offers a broad view of the *C. difficile* genotype distribution at the global level but lacks comprehensive data. In fact, the study took into consideration only 1501 clinical isolates distributed globally. Another less recent but more accurate study analyzed 3499 isolates from 40 sites across Europe [93]. This study describes the 2011–2016 epidemiological framework. In particular, there was a well-defined predominance of RT027 during the five years, followed by RT001 in 2011 and RT014 in 2012, 2013 and 2014 (both RTs are toxigenic). In 2015, a predominance of RT014 was observed, followed by RT106 and RT002 [93]. These results appear to be in contrast with the observation by Zhao et al. [101], but they are probably more accurate due to the greater number of isolates analyzed. It is also worth mentioning the two works by Abdrabou et al., who considered Germany in the periods 2014–2019 and 2019–2021 [102,103]. The authors noted that in the first period there was a prevalence of RT027, with a decrease in the following years [102,103]. As discussed by the European Medicines Agency (EMA), the Centers for Disease Control and Prevention (CDC) and the U.S. Food and Drug Administration (FDA), such a decrease could be due to a potential reduction in fluoroquinolone administration [106–108]. Fluoroquinolones have in fact been associated with CDI outbreaks with RT027, which is highly resistant to them [109,110].

Similarly, comparing these results with other studies, a decrease in the prevalence of RT001 in Germany over time was observed [102,111,112]. It is also interesting to note that certain foods, such as potatoes, could be a vector for the introduction of *C. difficile* spores into the food chain or household environment. Indeed, potatoes have the highest rates of *C. difficile* contamination tested to date [113,114]. A recent study analyzed the positivity rate and distribution of RTs on potatoes in 12 European countries in the first half of 2018. Thirty-three of the 147 samples tested were positive for *C. difficile*, and the most common RTs were RT126, RT023, RT010, and RT014, in part overlapping what was discovered for human samples. The multiplicity of RTs was found to be substantial and the overlap between countries moderate.

5.2. America

In the USA, clinical specimens acquired from 2011 to 2015 showed a prevalence of RT027, belonging to the hypervirulent clade 2, as well as a prevalence of clade 1 [101]. Interestingly, the prevalence of clade 2 was higher on the East Coast and West Coast than inland. This observation was reversed for clade 1, which was more prevalent inland [101]. Another recent study [94] focusing on stool specimens recovered from 2011 to 2016 in the states of Illinois, Minnesota, New York, Massachusetts, California, and Virginia showed a marked decrease in the prevalence of RT027 over these six years in agreement with what was seen in Europe and Canada [94,112,115–118]. This decrease was also found in a 2020 study [119], covering the 2011–2018 period in Texas, where the most common RT was RT027, followed by RT014-020, RT106, and RT002. Curiously, the authors found a novel emerging RT, RT255 [119]. The complete RT255 genome has recently been made publicly available [120] and has occasionally been isolated [94]. However, its attributes and associated clinical outcomes are not yet well described [119].

Considering the transmission of *C. difficile* between humans and animals, an interesting study of samples collected from 13 Ohio swine farms (from farrowing rooms, nursery rooms and workers' breakrooms) showed high contamination with toxigenic *C. difficile* [121]. The same *C. difficile* RTs recovered from most of the farm breakrooms were also recovered from at least one swine environment in those same farms. Furthermore, three RTs (i.e., RT078, RT005 and RT412) identified in the environment had previously been found in association

with CDI in humans [122,123] and with animal-to-human transmission in Europe (i.e., Italy) [44]. Water and soil are also an important reservoir for this pathogen. At the Flagstaff site (Arizona), researchers found potential novel strains belongings to clades C-I, C-II and C-III and a hypothetical additional clade (C-V) [124].

In contrast to the literature describing the situation in North America, few recent articles are available on what concerns South America, mainly from Brazil. Regarding the latter, a study using MLST found that patient stool samples from different hospitals were positive for *C. difficile* distributed in 14 STs [125]. In particular, it was the first description of the STs 15 and 54 in the Brazilian country. ST15 has already been described in the UK [90], but as it is a non-toxigenic ST, there are not many data in the literature, while ST54 is already widespread in South America [125]. Unlike Europe and North America, the RT027 epidemic has never been reported in Brazil and the epidemiology of CDI is still underexplored. This is partly due to the lack of specialized technologies and facilities for the detection of obligate anaerobic bacteria, which is not a routine procedure in clinical laboratories [126]. However, numerous *C. difficile* RTs involved in CDI cases have been detected in Brazilian hospitals (e.g., RT014, RT043, RT046, RT106, RT132-135, RT142, and RT143) [96,126–128].

5.3. Asia and Middle East

While for Europe and North America there are numerous studies on the genetics and epidemiology of *C. difficile* strains, Asia appears to be split in half. For example, there is a lack of scientific reports on the prevalence of *C. difficile* in South Asian countries such as India, while these works appear to be abundant in East and Southeast Asian countries such as China and Japan. In India, a recent study using MLST on samples from a tertiary care center found that the most common STs were ST17, ST54, and ST63, with ST17 being the most prominent [129]. In Bangladesh, the first report on the prevalence of CDI in the hospital setting found that the most common RTs in stools (i.e., present in $\geq$10% of isolates) were RT017, RT053–163 (the same RTs found in environmental isolates), and a new RT (i.e., FP435) [130].

Furthermore, another study conducted in 2020 on the antimicrobial susceptibility of *C. difficile* in the 2014–2015 period in Asia-Pacific countries highlighted the prevalence of RT017 [95], confirming what was observed in a previous study [130]. Interestingly, the hypervirulent RT027 and RT078 have only rarely been isolated in the Asia-Pacific region [131–134]. In Japan, evidence suggests that the most common RT is RT018 [133–136]. In China, one of the dominant circulating RTs is RT017 [59,137,138]. However, another study published in 2021 on samples from different sources (e.g., soil, animals) found that the predominant RTs in China are RT001, RT046, and RT596 [139]. In 2021, the study by Zhao et al. found that the most common clade in the Asian country is clade 4 [101]. Furthermore, another report focusing on antibiotic resistance and molecular features of economic animals in China showed that RT126 is the most prevalent in Shandong province [140]. As for the Middle East region, RT001 is the most prevalent in Iran, followed by RT126, while RT258 is widespread in Qatar, RT139 in Kuwait and RT014 in Lebanon [141–144].

5.4. Oceania

Most of the data on the incidence of CDI come from Australia, the largest country in Oceania, particularly Western Australia. However, probably due to the variety of patient characteristics such as age, there is a discrepancy between the studies available to date.

For example, a study of *C. difficile* isolated from pediatric patients hospitalized in Perth, capital of Western Australia, in the period 2019–2020, reported a prevalence of RT002 (toxigenic) and RT009 (non-toxigenic) (on a total of 427 stool samples) [98]. On the other hand, a study conducted in the earlier period 2013–2018 in a geographically larger area covering 10 diagnostic laboratories from five states in Australia (Western Australia, New South Wales, Victoria, South Australia, and Queensland), reported 203 different RTs in predominantly elderly subjects, with RT014/020 being the most common, while RT027 and

RT078 only rarely found [145]. Additionally, toxigenic RT014/020 was found to be more common among clinical cases in a tertiary hospital in Perth, while non-toxigenic RT010 was prevalent among floor samples and shoe soles of hospital staff, visitors and patients [146]. Outside of Australia, few research works are available. In particular, for New Zealand, a 2021 study reported that the two most common RTs were the same found in Australia (i.e., RT014, RT020) [147]. Interestingly, the dominance of RT014 has also been reported in Europe, particularly in 2015 [93]. The dominance of RT014 was also found in Auckland in 2014 [148]. In the study by Zhao et al., clade 1 was found to be the predominant one in Oceania [101].

5.5. Africa

In Africa, *C. difficile* is generally considered a minor pathogen, while the most common causative agents of diarrhea are *Escherichia coli*, *Cryptosporidium*, *Shigella,* and rotavirus [149]. No less important, there is a lack of recent literature on ribotyping and molecular characterization of *C. difficile*. For example, for Northern Africa, the latest PCR ribotyping report was released in 2018 in two Algerian hospitals, revealing the predominance of RT014, RT020 and non-toxigenic RT084, but only in 11 out of 159 stool samples collected between 2013 and 2015 [99]. In sub-Saharan Africa (i.e., Tanzania), a 2015 study identified RT038 among non-toxigenic strains, RT045 among toxigenic strains, and an unknown RT for two strains resulted positive for both *tcdA* and *tcdB*. Further analyses conducted on one of the two unknown strains highlighted a similarity with RT228 and RT043 [100]. Another study conducted in rural Ghana showed a high rate of non-toxigenic strains (e.g., RT084) isolated from patients with diarrhea and no hypervirulent strains [150]. A 2018 multi-centric cross-sectional study conducted between Germany, Ghana, Tanzania, and Indonesia showed that non-toxigenic strains were more abundant in Africa, with a prevalence of RT084 in the Ghanaian site and RT038 and RT045 in the Tanzanian site [151]. In light of this evidence, there appears to be a shortage of hypervirulent RTs in Africa (as opposed to Europe and the USA), while most of the CDIs found are attributable to non-toxigenic strains. Finally, it should be noted that in the Zimbabwe region, RT084 was the most common in human samples, while RT103, RT025, and RT070 were prevalent in chicken isolates, and RT025 and RT070 in soil samples [152]. Based on the study by Zhao et al., in South Africa, there is a prevalence of clade 1 [101].

6. Future Perspectives to Make It Less *difficile*

6.1. Genomic Surveillance

Recently, the COVID-19 pandemic has exposed the challenges for genomics in public health surveillance systems, given the rapid spread of new viral variants [153]. Genomic surveillance is transforming public health action by providing a deeper understanding of pathogens, their evolution and circulation. As mentioned earlier, new technologies in sequencing and bioinformatics have recently emerged and we have now reached a point where genomic surveillance has a clear role to play for public health. Specialized laboratory techniques such as WGS are increasingly used according to the WHO Global Surveillance 2022 report, in the investigation and acute management of diseases that could constitute public health emergencies, including cholera, influenza, Ebola virus disease, bacterial meningitis and poliomyelitis [154]. Public health responsiveness to pathogens could also benefit from larger-scale genomic surveillance, for example to monitor the occurrence and spread of *C. difficile* variants. However, for this to happen, several actions would be required. First, there is a strong need to improve access to tools for a better geographical representation of the pathogen spreading, sampling not only hospitalized patients with symptoms, but also environmental hotspots that can be enriched in human-derived pathogenic microorganisms, such as wastewater. Recently, sewage systems have gained increasing attention for health surveillance purposes due to their relevance in improving responsiveness to SARS-CoV-2 infection peaks, enabling early detection of viral loads and the emergence of new viral variants [155,156]. *C. difficile* is commonly found in raw sewage and survives the wastewater

treatment process, thus being disseminated into the environment via effluent and land application of biosolids [157]. A genomic investigation conducted by Moradigaravand et al. [158] in 65 patients showed that CDI was attributable not to hospital-derived spores, but rather to strains isolated from effluent water from nearby treatment facilities, bolstering the need for bacterial genomic control in the environment [158]. This first action implies the need to strengthen the workforce to support this monitoring burden between countries and improve data sharing in a local-to-global fashion, enabling shared decision-making. Furthermore, the production of big data on a global scale encompassing genomic data, geographic origin, and spread of the isolates in the population will serve as a starting set to train a genomic-informed, real-time, global pathogen predictive model that might allow for greater control of infection outbreaks and conscious management of wastewater and other pathogen reservoir hotspots. Surveillance, outbreak detection, and response fully adhere to the One Health concept, which encompasses the domains of human, animal, and environmental health [159]. A deep global genomic characterization of *C. difficile* isolates could definitely help improve not only the profiling, but also the treatment of infected patients in a more rational, knowledge-based way.

6.2. Genetic Engineering

In recent years, a second-line approach leveraging synthetic biology is receiving particular attention, as an innovative strategy to limit *C. difficile* outbreaks by reducing its pathogenicity. Indeed, a wide range of tools are now available to precisely delete genes, change single nucleotides, complement deletions, integrate novel DNA, or overexpress genes [160]. Among these, the CRISPR/Cas system is of great interest for its ability to produce targeted modifications with high precision and efficiency, and to act on multiple strains [161]. CRISPR-mediated genome editing has been successfully implemented to tackle *C. difficile* infectious process, by hijacking the native system to introduce selective knock-outs [154] of several virulence factors involved in its pathogenicity in humans, deepening our knowledge over certain pathogenic processes [162]. For example, the study of the flagellin FliW-CsrA-*fliC*/FliC regulation mechanism implementing a CRISPR-based deletion of *fliW*, *csrA* and *fliW-csrA* genes demonstrated that the RNA-binding protein CsrA negatively modulates *fliC* expression, whilst FliW indirectly affects *fliC* expression through inhibition of CsrA post-transcriptional regulation [163].

Understanding *C. difficile* virulence regulation and pathogenicity factors through gene editing techniques also paves the way for efficient attenuation and possible therapeutic strategies. For example, toxicity and early-stage adhesion can be counteracted by knocking-out the *tcdA* and *cpw84* genes, respectively [164]. While *tcdA*, as previously mentioned, encodes toxin A, *cpw84* encodes a cell wall protein, the main protease of *C. difficile*, important in the early stages of colonization [164]. Another example of effective attenuation of *C. difficile* virulence was proposed by McAllister et al. [165], who produced a CRISPR-Cas9 mutant of *C. difficile* lacking the selenophosphate synthetase gene *selD*, resulting in a growth-deficient mutant [165]. Similarly, Wang et al. [166] achieved the complete deletion of the sporulation protein spo0A in a wildtype strain of *C. difficile*, undermining its ability to survive environmental stresses. Finally, Selle et al. [167] proposed an interesting delivery system for in vivo targeting of *C. difficile*, by developing a phage-delivered CRISPR/Cas3-mediated antimicrobial [167]. Phage infection was selective and apparently safe, activating a repurposing response of the endogenous CRISPR/Cas system that induced self-targeting endonuclease activity.

Such synthetic biology studies pave the way for new treatments in the field of personalized medicine, with in vivo strain-specific targeted genomic modifications to make *C. difficile* more vulnerable to therapies, thus providing the patient with a rapid and relapse-free recovery. Furthermore, such strategies can be virtually translated to other bacterial infections and could provide powerful and reliable strategies for overcoming the challenges of infections with multidrug-resistant microorganisms directly in the context of complex microbial communities in vivo.

7. Conclusions

This review gives a glimpse on the worldwide spread of *C. difficile* from a genetic standpoint. *C. difficile* is nowadays a global threat due to the clinical outcomes of its infection and growing concern about resistance to antimicrobials. From a methodological point of view, it should be remembered that *C. difficile* is not easy to grow and, although the ECDC guidelines suggest using PCR ribotyping as the main typing technique, many studies from different countries have been conducted with other methods, obviously challenging the overlap of results. Additionally, typing data are absent in Northern and Central Africa or some Asian countries, making the view of what *C. difficile* burden really represents even more fragmented. However, to date, as far as we know, there is a majority of toxigenic RTs in Western countries (e.g., RT027 in Europe), as well as in Asian countries (e.g., RT017), while in Africa, the most abundant strains are non-toxigenic (e.g., RT084). Failure to detect hypervirulent RTs could be explained by multiple factors, related to populations and their lifestyle, as well as poor typing effort. For example, as regards the African continent, CDI patients are often younger than in European and North American contexts, but typing data are rarely reported [168]. Furthermore, co-infection with the causative agents of tuberculosis or malaria has been reported to affect the virulence of *C. difficile* RTs [169], although the underlying mechanisms are unknown. Finally, the low availability of antimicrobials in some Asian and African countries and the suggested reduction in their use by international agencies [170,171] could certainly be linked to lower selective pressure on circulating *C. difficile* strains, potentially helping to foster the emergence of non-hypervirulent RTs.

Another relevant issue in CDI management is the lack of data on non-clinical transmission, i.e., through the food chain or the environment. In addition to a homogenization of methodological approaches, future studies should therefore aim at a better sampling and geographic representation of the *C. difficile* spread, in order to better understand (and interfere with) its transmission dynamics. With specific regard to therapies, in recent years, genome editing technologies such as the CRISPR/Cas system are demonstrating their potential in improving the susceptibility of *C. difficile* to treatments, thus opening up unprecedented opportunities for personalized medicine. Coupled with the mapping effort, advances in genomics and bioinformatics could lead to a better understanding of what this pathogen is and, more importantly, how to make it less *difficile*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13122200/s1, Table S1: Toxin gene profile of the ribotypes mentioned in the main text.

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