Probing and manipulating the gut microbiome with chemistry and chemical tools

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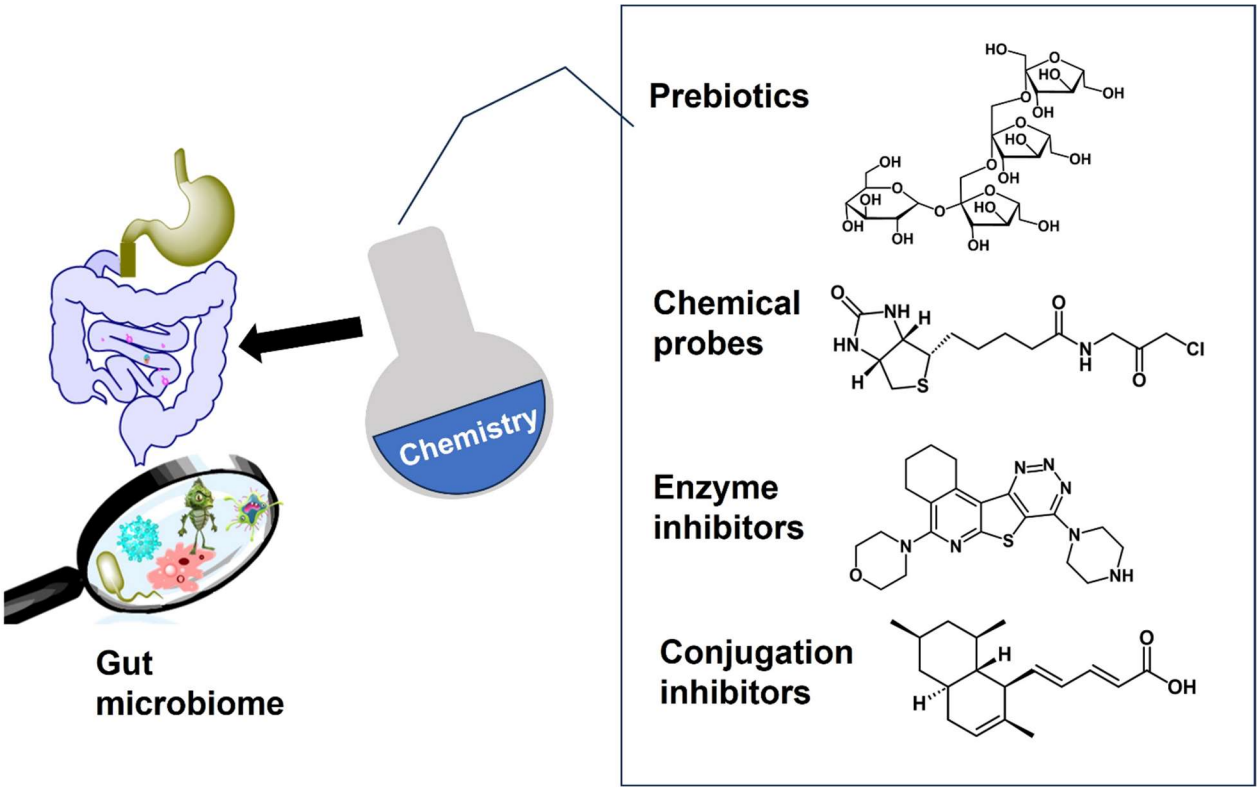
Graphical abstract



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Abstract

The human gut microbiome represents an extended "second genome" harbouring about 10^{15} microbes containing >100 times the number of genes as the host. States of health and disease are largely mediated by host-microbial metabolic interplay, and the microbiome composition also underlies the differential responses to chemotherapeutic agents between people. Chemical information will be the key in order to tackle this complexity and discover specific gut microbiome metabolism for creating more personalised interventions. Additionally, rising antibiotic resistance and growing awareness of gut microbiome effects is creating a need for non-microbicidal therapeutic interventions. We classify chemical interventions for the gut microbiome into categories like molecular decoys, bacterial conjugation inhibitors, colonization resistance-stimulating molecules, "prebiotics" to promote the growth of beneficial microbes and inhibitors of specific gut microbial enzymes. Moreover, small molecule probes including click chemistry probes, artificial substrates for assaying gut bacterial enzymes and receptor agonists/antagonists which engage host receptors interacting with the microbiome, are some other promising developments in the expanding chemical toolkit for probing and modulating the gut microbiome. This review explicitly excludes 'biologics' such as probiotics, bacteriophages, and CRISPR to concentrate on chemistry and chemical tools like chemoproteomics in the gut-microbiome context.

Keywords: Gut microbiome, chemistry, prebiotics, conjugation inhibitors, chemical probes

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1. Introduction

There are about 10^{13}-10^{15} symbiotic microbes residing inside of and on the surface of a human being which collectively constitute the human microbiome[1]. The microbiome plays a significant role in lifelong host health[2] and underlies a considerable proportion of the individual differences in drug metabolism[3]. Therefore, modulating the human microbiomes has triggered the interest of both academia and industry, and several interventions have been designed to either preserve or rebuild the function of the microbiome. In the period 2015-18, over 80 microbiome modulators entered the preclinical phase, while 15 were in phase I trials, 5 in phase II and 6 in phase III, according to the Pharmaprojects 2018 Microbiome Whitepaper[4]. The same report details that as of 2018, 10 modulators were in the pipeline for metabolic disorders, 21 for gastrointestinal disorders and 24 for infectious diseases.

The gut (gastrointestinal system) harbours the most extensive human microbiome, which is critical for host metabolic and immune functions[5]. Further, a healthy microbiome also prevents pathogens from colonizing the gut, a phenomenon known as colonization resistance (CR)[6]. The gut also contains the largest surface where immune system activity occurs inside the human body[7] and the development of the immune system itself is a delicate dance of balancing the host versus the gut microbes[8]. The gut connects to various distal organs via two-way signalling and therefore, the gut microbiome (GM) maintains far more than just gut health[9]. GM dysfunction is implicated in the development of infections, gastrointestinal cancers as well as liver, respiratory, neurological, cardiac, metabolic, and autoimmune diseases[10].

Antibiotics in particular cause deleterious changes to the function of the GM[11] and therefore preserving/restoring those functions is important. The antimicrobial resistance (AMR) crisis has also led to a search for less indiscriminate therapeutics which are GM-friendly[12]. Kang et al showed that gut bacteria such as *Clostridium scindens* and *Clostridium sordellii* which perform 7α-dehydroxylation of bile salts, also produced endogenous narrow-spectrum antibiotics derived from tryptophan, such as turbomycin A and 1-

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acetyl-β-carboline which inhibit *Clostridioides difficile*[13]. Indole-3-propionic acid (IPA), another tryptophan metabolite which is produced by *Clostridium sporogenes*, inhibits a variety of mycobacteria, including drug-resistant *Mycobacterium tuberculosis*[14]. IPA inhibited *M. tuberculosis* both *in vitro* and when administered in mice models via oral and intravenous routes (where it showed a seven-fold bacterial load reduction in the spleen[14, 15]). GM-derived IPA can bind and powerfully induce the aryl hydrocarbon receptor or AhR (a major regulator of both innate and adaptive immunity) and therefore modulate the susceptibility to *M. tuberculosis*[14]. The recovery of IPA in the serum[14] and the existence of the gut-lung[9] and gut-spleen[16] axes explains how the GM can influence both lung and immune function remotely.

Endogenous narrow-spectrum peptide antibiotics with more complicated structures like bacteriocins also exist[17] and could become available for research via solid phase peptide synthesis since synthetic methods for cyclic peptides are rapidly improving[18]. Drug delivery targeted to different gut compartments[19] is already a burgeoning field. Therefore, chemically synthesised narrow-spectrum antibiotics could in the near future be delivered to specific gut compartments for directly or indirectly influencing the susceptibility and host-colonisation ability of major pathogens such as *M. tuberculosis*[14] and *C. difficile*[13] as well as modulating host immunity, to prevent infections or aid recovery from infections.

Direct chemical manipulation of the GM has been the most challenging to perform in the absence of prior knowledge of the targets. However, in a pioneering study, Chen et al. devised an *in vitro* screening protocol and were able to use the cyclic D,L-α-peptides they identified via screening, to change a GM induced by a Western diet into one reflecting a low-fat diet[20]. This not only ameliorated atherosclerosis in mice, but adjusted the levels of pro-inflammatory cytokines, short-chain fatty acids (SCFA) and bile acids to healthy levels, while improving gut barrier integrity and T-cell function. They described their approach as "directed remodelling", implying a deliberate manipulation of the GM in a predetermined manner from one state to another.

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Research is moving away from largely cataloguing microbial strains to examining and understanding the molecular basis of the GM's influence on human health[2]. Therefore, we argue that chemistry and chemical information will play an important part in unravelling GM interactions and manipulating the GM to promote health. With this in mind, we focus on the roles of chemistry and chemoproteomics, while excluding 'biologics' strategies such as probiotics, bacteriophages, and CRISPR. Narrow spectrum antibiotics and directed chemical remodelling are only two recent examples of the potential of chemistry in the GM story. Whether preparing prebiotics, inhibiting bacterial conjugation in the gut, stimulating colonization resistance, probing GM-host interactions, or altering the GM composition to promote host health, the versatile toolkit of chemistry offers abundant opportunities to explore and modulate the GM.

2. Molecules which preserve/restore the gut microbiome

These are classified based on their mode of action as shown in **Fig 1A** and some example chemical structures are shown in **Fig 1B**.

2.1 Prebiotics: Prebiotics are selectively fermented ingredients that trigger specific changes in the microbiome composition and activity to promote host health[21]. Safely administering live microbes and establishing their colonization in the gut is difficult and faces regulatory hurdles, making small molecule interventions more attractive[22]. Small molecules, especially endogenous metabolites can accumulate to high concentrations with negligible toxicity, remain stable in the systemic circulation and obey the principles of pharmacokinetics. The major prebiotics are human milk oligosaccharides (HMOs), inulins (**1** in **Fig 1B**), fructose oligosaccharides (FOS), xylooligosaccharides (XOS), mannan oligosaccharides (MOS) and galactooligosaccharides (GOS), which are polymers/oligomers of glucose, fructose, mannose, fucose, galactose, sialic acid, xylose, uronic acid, and arabinofuranose units linked together with β2, β3 and β4 linkages[23].

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Developments in chemical synthesis are bringing the goal of complex carbohydrate assembly closer. Difficulties arise mainly from 1) the need to selectively protect and deprotect monosaccharides, and 2) regio- and stereoselectivity. Improved glycosylation strategies have been reported, which enables glycosyl donors to react in a specific order, yielding a single oligosaccharide product[24]. Automated glycan assembly (AGA) currently enables access to a maximum length of 100, while convergent block coupling of 30- and 31-mer oligosaccharide fragments made by AGA was used to make a multiple-branched 151-mer polymannoside[25].

Enzymatic and chemoenzymatic processes offer better region- and stereoselectivity, along with fewer steps in the synthesis which makes them faster and more cost effective[26]. For example, the HMO 2'-fucosyllactose (2'FL) has been synthesized in engineered *Escherichia coli* strains[27]. One-pot multi-enzyme (OPME) synthesis has been reported which employs glycosyltransferases to synthesize sialyl- and fucosyl-derivatives[28]. Sialylated HMOs with high region- and stereoselectivity have been synthesized using a chemoenzymatic strategy, whereby automated solid phase synthesis of the glycan backbone was followed by α-(2,3)-sialyltransferase treatment[29]. Interest in sustainable chemical feedstocks has led to method development for the conversion of lignocellulose biomass into valuable prebiotics such as XOS[30].

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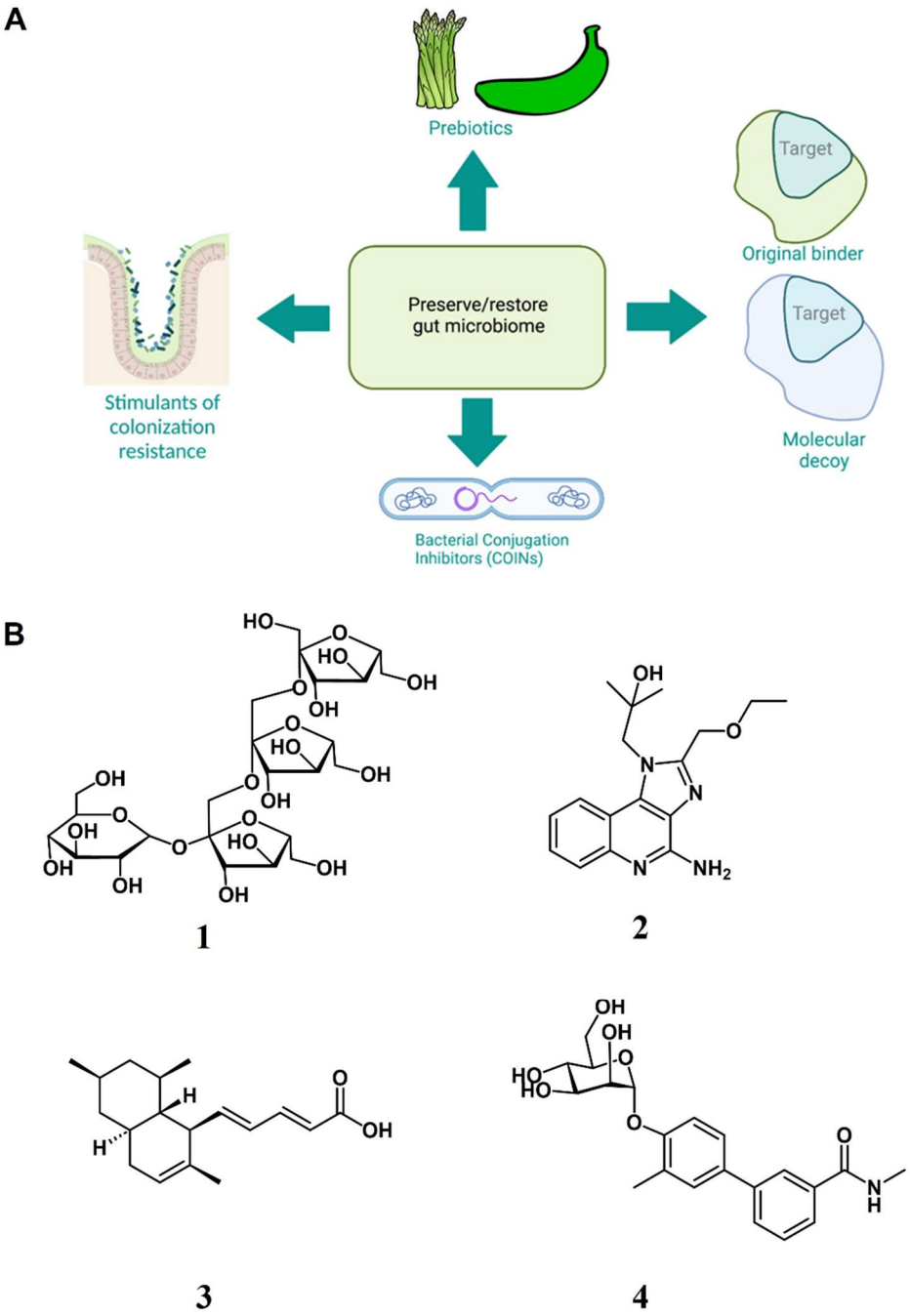

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140 **Fig 1. A)** Functional classification of molecules to preserve/restore the gut microbiome; **B)**
141 Chemical diversity of molecules with microbiome preserving/restoring functions; **1** = General
142 structure of inulins (endogenous prebiotic), **2** = resiquimod or R848 (synthetic stimulant of
143 colonization resistance); **3** = tanzawaic acid B or TZA-B (natural product colonization inhibitor); **4**

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= a mannoside (mannose-containing decoy for urinary pathogens which preserves the gut microbiota).

Prebiotics can have synergistic interactions with approved drugs. Konjac MOS from the plant *Amorphophallus konjac* are prebiotics containing β-D-mannose and β-D-glucose residues linked by 1-4 linkages[31]. The combined administration of the drug metformin and konjac MOS mitigates insulin resistance and glucose tolerance, while also improving islet and hepatic tissue function[32]. The beneficial effects were correlated with the reduced abundance of the Rikenellaceae family and the Clostridiales order, with an increased relative abundance of *Bifidobacterium pseudolongum*, *Akkermansia muciniphila* and OTU05945 of family S24−7[32]. Further studies focussing on prebiotic-drug interactions could lead to more targeted application of prebiotics in combination with approved drugs to mitigate the impact of specific diseases.

2.2 Stimulants of colonization resistance (CR): CR is a mechanism by which the gut microbiota protects itself against the incursion and establishment of largely harmful microorganisms. This protection can be accomplished by several routes, such as antimicrobial secretion, nutrient limitation, stimulation of gut barrier integrity and the action of bacteriophages[6]. Disturbances to the gut resulting from the use of antibiotics, other drugs or inflammation can reduce CR, allowing enteric pathogens such as *C. difficile*, *Salmonella enterica* serovar Typhimurium, *E. coli*, *Shigella flexneri*, *Campylobacter jejuni*, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Listeria monocytogenes*, to colonize the niches vacated by microbiome disruption[33]. Both endogenous molecules such as SCFA and tryptophan metabolites produced by the gut microbiome and exogenous synthetic small molecules can restore CR function. Synthetic molecules are beginning to be used in efforts to stimulate CR following disturbances to the GM, for example, after antibiotic administration. For example, vancomycin-resistant enterococci (VRE) flourishes when CR is compromised following antibiotic treatment. A synthetic molecule, resiquimod or R848 (**2** in **Fig 1B**), binds to a Toll-like receptor 7 (TLR-7) that stimulates innate immune defences, leading to the restoration

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of CR against VRE by triggering the expression of the antimicrobial peptide Reg3γ[34]. R848 can be taken orally and induces the secretion of the interleukins IL-23 and IL-22.

2.3 Bacterial conjugation inhibitors (COINs): Antibiotic resistance is spread by several mechanisms including horizontal gene transfer mediated by plasmids. Analysis of *Bacteroidetes* strains sharing the intestinal niches of specific individual humans, demonstrated the extensive occurrence of horizontal gene transfer among those strains. In this case, the genetic elements exchanged coded for orphan DNA methylases, fimbriae synthesis proteins, novel metabolic enzymes, antibiotics, and proposed type VI secretion systems (T6SS)[35]. More recent studies have recorded extensive plasmid exchange in the gut environment using CRISPR-Cas spacer acquisition analysis in an *E. coli* strain[36]. Unlike earlier studies which relied on phenotypic markers or post-transfer replication to detect mobile genetic elements, the spacer acquisition analysis reveals plasmid transfer in real time, and the results showed that the IncX plasmid type was most frequently transferred[36]. Therefore, inhibiting bacterial conjugation in a bacteria-dense environment could enable the host to mitigate antibiotic resistant infections. In general, resident bacteria in the healthy GM may be able to suppress the evolution of antibiotic resistance *in vivo*. However, the wide distribution of plasmid-borne resistance in the environment is well-known and exposure to them might be common. Moreover, gut inflammation boosts plasmid transfer between pathogenic and commensal Enterobacteriaceae[37]. Therefore, inhibiting plasmid transfer in the gut is expected to promote host health and COINs are unlikely to disturb the GM composition unlike conventional antibiotics. We describe a few known COINS, but some need to be further specifically tested in the gut environment.

Early studies to identify COINs unearthed many unspecific molecules which affected DNA replication or growth[38]. Plant phenolics seems to be a good source of COINS and have yielded two molecules which specifically inhibited bacterial conjugation, namely rottlerin and 8-cinnamoyl-5,7-dihydroxy-2,2,6-trimethylchromene[39]. Screening of a library of over 12,000 NPs (NatChem library) based on high throughput whole cell-based assays enabled the discrimination between true COINS and false “hits” which merely

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affected cell growth, leading to the discovery of the COIN dehydrocrepnynic acid (DHCA)[40]. DHCA belongs to the chemical family of unsaturated fatty acids (UFAs), which is generally a good source of COINS. DHCA is derived from a tropical seed and its supply is limited. However, it was used as the starting point for the synthesis of other COINS, particularly 2-hexadecynoic acid (2-HDA) and other 2-alkynoic fatty acids (2-AFAs) which specifically inhibited the transfer of a range of plasmids, including the common and highly infective IncF, in various bacteria[41]. 2-HAD was later reported to prevent bacterial conjugation in the mouse gut[42]. A series of UFA NPs called tanzawaic acids were discovered (tanzawaic acid B or TZA-B is depicted as (**3** in **Fig 1B**); they mainly inhibited conjugation by the IncW and IncFII-based plasmids. Other plasmids classified under the IncFI, IncI, IncL/M, IncX and IncH incompatibility groups were less affected, while IncN and IncP plasmids were unaffected[43].

Conjugation is driven by the type 4 secretion system (T4SS) whose architecture is conserved in most bacteria, and contains the pilus, the core channel complex, the inner membrane platform and the ATPases that provide energy for substrate transport and pilus biogenesis[44]. Nicking the DNA to relax the plasmid, DNA transfer to the secretion channel, the transfer of pilin molecules during pilus biogenesis, and pilus biogenesis are performed by four distinct ATP-ase enzymes, among which carboxylic acid COINS were shown to target the last step (TrwD protein). Based on structural and computational data, the UFAs and AFAs were suggested to bind at the end of the N-terminal domain as well as the beginning of the linker region that connects the N-terminal and C-terminal domains, likely hindering the swapping movements of the domains needed for the catalytic cycle[45].

2.4 Molecular decoys: These are molecules which bind enteric pathogens and stimulate their elimination from their gastrointestinal tract. This binding is thought to “fool” pathogens by mimicking receptors used by them to attach to the gut epithelia in the lower gastrointestinal tract. The global burden of disease caused by enteric pathogens is substantial and cases may number in the hundreds of millions annually. HMOs act as soluble decoys for receptors and block the binding of enteric pathogens. Rotavirus infection is prevented

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most effectively by the HMO 2'FL, although several other HMOs also have similar inhibitory effects[46]. *Campylobacter jejuni* infects the mammalian gut and causes diarrhoea and sometimes also motor neuron paralysis. The infection is initiated by the bacterium binding to the fucosylated intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc). However, FOS in human milk can act as decoys, binding to the pathogen instead and preventing infection[47].

Uropathogenic *E. coli* (UPEC) uses the extracellular appendages called Type 1 pili to colonize the intestine by binding a mannosylated host receptor; the Type 1 pili are also essential for colonization and infection in the bladder. Mannosides (**4** in **Fig 1B**) are small-molecule drugs bearing mannose group(s) which act as decoys by mimicking the mannosylated receptor and can clear both bladder and intestinal UPEC upon oral administration in mouse models, leaving the GM largely intact[48]. The decoy approach has been further extended to combat cholera, and in this case also employs nanotechnology. The *V. cholerae* toxin binds to the host receptor monosialotetrahexosylganglioside (GM1), and coating GM1 on the surface of polymeric nanoparticles was enough to reduce cyclic-AMP production in epithelia and fluid responses to live *V. cholerae* in both cell cultures and a mouse infection model[49]. The modulation of disease via molecular mimicry extends to non-sugar molecules, such as metalloenzymes allows for the manipulation of the gut chemical environment using synthetic catalysts. A metalloporphyrin mimic of the enzyme superoxide dismutase could reduce lipid peroxidation levels and thereby shielded epithelial cells from damage in rats injected with the common antigen bacterial lipopolysaccharide (LPS)[49].

3. Chemical probes of the gut microbiome

The majority of recent chemistry-oriented studies did not deal with direct chemical manipulation of the GM but focussed on probing the GM using bio-orthogonal strategies such as alkyne-cycloazide addition, Staudinger ligation and tetrazine ligation to create "chemical reporters"[50]. Bacterial surface glycans, peptidoglycans, lipopolysaccharides, capsular polysaccharides, glycoproteins, lipids, and other molecules

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246 such as bile acids have been labelled[50]. In addition to such surface targeting, protein function may be probed
247 by ABPP (activity-based protein profiling), which involves small molecules reacting with mechanistically
248 related enzymes[51]. In ABPP, the probe usually contains a reactive group and a tag. Microbiota-metabolite
249 interactions as well microbiome composition and dynamics can be interrogated via ABPP, while
250 chemoproteomics advances have made the detection of covalent probe-tagged proteins following ABPP
251 routine[50].
252
253 **3.1 Fluorophores:** The most common tools for probing the GM are fluorophores, which may be attached
254 to different types of other chemical entities. Commensal anaerobic bacteria including *B. fragilis* when fed
255 azide-labelled sugars, which subsequently conjugated with alkyne-fluorophores via click chemistry,
256 facilitate the imaging of bacteria in live mice[52]. Three different bacterial surface molecules from the GM,
257 which interact with the host immune system, namely LPS, capsular polysaccharide (CPS) and peptidoglycan
258 (PGN) can be tracked[53], helping to dissect host-microbe interactions. Azide-bearing amino acids when fed
259 to complex gut microbial communities showed that newly synthesized proteins could be visualized *in situ*[53].
260 Two D-Amino acid based fluorescent probes, TADA and Cy5ADA (**5,6** in **Fig 2**), which get incorporated
261 into bacterial peptidoglycan have been instrumental in enabling live monitoring of GM growth and division
262 patterns in mice[54]. Probes based on D-amino acids are also being used to track the viabilities of bacteria in
263 faecal transplants by using sequential tagging[55]. In this approach, the bacteria are treated with a probe before
264 the transplantation and then the recipient mice are fed a second probe following the transplantation.
265 Therefore, the bacteria surviving the process show the emission for both probes, enabling the identification
266 of viable bacteria in the transplant[55].
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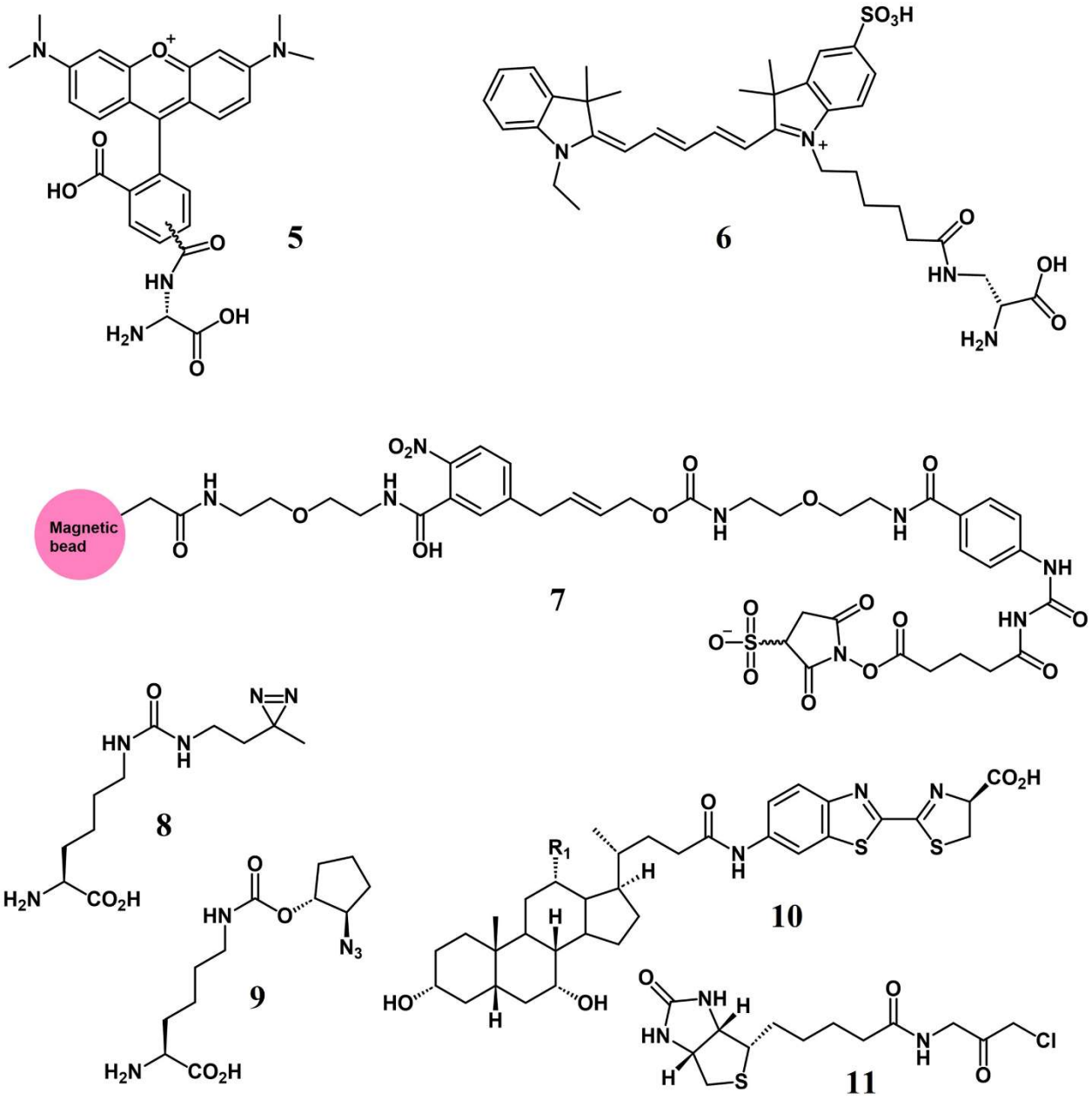

Fig 2. Examples of chemical probes used to interrogate the GM - D-amino acid based fluorescent probes = TADA (**5**) and Cy5ADA (**6**); a multifunctional probe showing different parts shaded in distinct colours = amine directed probe based on sulpho-N-hydroxysuccinimide (**7**); photoactive unnatural amino acid probes = DiZPK (**8**) and ACPK (**9**); a cysteine-targeted probe = Biotin-Gly-CMK (**10**); bioluminescent bile acid-luciferin conjugates for Bile Salt Hydrolase (BSH) activity = series of compounds with H or OH at the positions R1 and R2 (**11**).

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3.2 Multifunctional selective probes: Direct extraction from human faecal samples and release under mild conditions is possible using multifunctional chemo selective probes[56], allowing for the analysis of femtomole levels of metabolites with enhanced sensitivity. Probe **7** in **Fig 2** is anchored at one end to magnetic beads, linked by a spacer to a novel *p*-nitrocinnamyloxycarbonyl biorthogonal cleavage site, while the reactive site features an amine-selective sulpho-N-hydroxysuccinimide (sulpho-NHS) "warhead", which reacts with metabolic amines[56]. Since 2011, it has been possible to monitor enteric pathogens via the incorporation of the photoactive unnatural amino acids DiZPK and ACPK (**8,9** in **Fig 2**) into specific pathogen proteins, which react to form cross links revealing the interactions between the modified protein and its client proteins[57]. This approach is enabling the direct identification of proteins involved in pathogenesis and acid-stress defence mechanisms, which is quite challenging to perform with conventional methods.

3.3 Simple reactive probes: Sphinganines are bioactive components of foods, but the GM also modifies them. The use of alkyne-tagged sphinganines allows for the identification sphinganine-utilising GM strains based on labelling followed by a cell sorting workflow[58]. The subsequent sequencing of the sorted bacteria revealed that this metabolism is nearly exclusively performed by members of the *Bacteroides*[58]. An activity-based probe, Biotin-Gly-CMK (**10** in **Fig 2**), has been used to differentiate between mice models harbouring "normal" human GM and "Inflammatory Bowel Disease" (IBD) affected human GM, whereby a novel cysteine-reactive probe tagged several proteases and hydrolases in the IBD model, but not in the healthy controls[59].

An elegant recent study by Nie et al. using a click chemistry strategy isolated and identified a previously unknown bile acid 3-succinylated Cholic Acid (3-sucCA) correlated with reduced progression of metabolic dysfunction associated steatohepatitis (MASH) in humans[60]. Using this discovery, the authors were able to

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characterise an annotated β-lactamase in the GM member *Bacteroides uniformis* as the enzyme catalysing the 3-succinylation of CA[59].

3.4 Bioluminescent probes: Luciferin-based bioluminescent probes (**11** in Fig **2**) have been employed to detect Bile Salt Hydrolase (BSH) activity in a wide variety of sample environments including purified enzymes, bacterial cells, faecal slurries as well as non-invasive imaging in mice and humans[61]. BSH activity releases luciferin from the conjugated bile acid and can be further assayed using luciferase. These bile acid-luciferin probes were useful in demonstrating the stimulatory effect of prebiotics on BSH activity and as diagnostic tests which non-invasively detect the clinical IBD status in human patients[61].

4. Modulating specific enzyme functions in the gut microbiome

Targeting specific enzymes among the thousands of proteins actively produced by the gut microbes is a viable strategy for microbiome modulation.

4.1 Choline metabolism: A 'chemically guided functional profiling' could be a strategy to uncover the presence of novel enzymes in the GM and subsequently, to modulate their function to achieve therapeutic effects. The conversion of choline into trimethylamine (TMA) by anaerobic gut bacteria is correlated with disease conditions in humans, and more specifically, the production of TMA in both isolated bacteria and complex communities can be inhibited by betaine aldehyde (**12** in **Fig 3**)[62]. The identified target is GM choline TMA-lyase (CutC) and this opens up the scope for the development of other inhibitors.

4.2 Bile salt metabolism: Bile salts have major effects on the physiology and virulence of *C. difficile*. When patients are restored to a *C. difficile*-resistant state, it is observed that the production of deoxycholate from cholate by 7α-dehydroxylating gut bacteria occurs[63]. Broad spectrum antibiotics block the production of secondary bile acids and kill the 7α-dehydroxylating bacteria, thereby enabling *C. difficile* to colonize the

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gut[63]. BSH enzymes expressed by the GM and bile salt metabolism affects the immune and metabolic processes via engaging host receptors. Therefore, inhibiting BSH enzymes would enable the dissection of the role of bile salts in host-microbe interactions. Screening a library of compounds, Adhikari et al, zeroed in on a covalent suicide inhibitor containing an α-fluoromethyl ketone moiety (**13** in **Fig 3**) which reacts with the active site cysteine of BSH enzymes, as way to globally modulate BSH and understand their physiological roles[64].

4.3 Glucuronidase inhibitors: β-Glucuronidase (GUS) enzymes harboured by gut microbes can cause severe toxicity reactions to certain pharmaceuticals including cancer drugs, and therefore, GUs inhibitors have been developed (**14,15** in **Fig 3**) to ameliorate these toxic side effects. Pellock et al. reported the discovery of piperazine-based GUS inhibitors by combining chemical biology, protein structural data and mass spectrometry with cell-based assays[65]. Their GUS inhibitors interrupt the catalytic cycle of the enzyme and are substrate-dependent, binding to the catalytic intermediate by means of a piperazine-linked glucuronide. The inhibitor-glucuronide conjugates were detected by LC-MS[66].

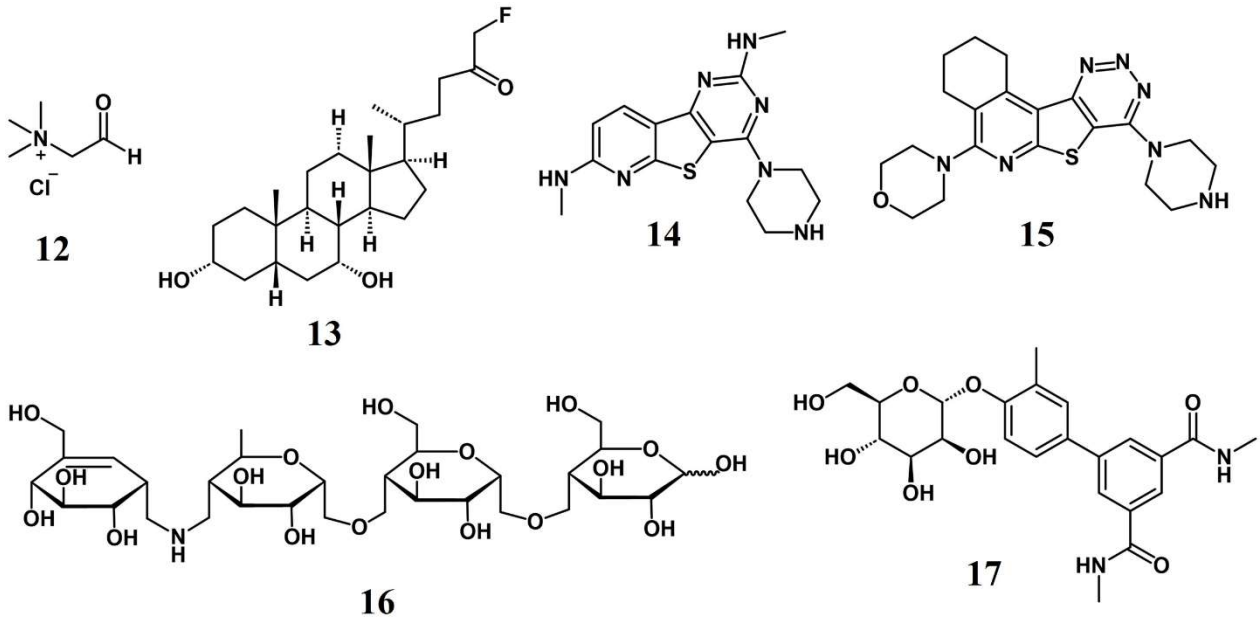

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Fig 3. Specific enzyme inhibition can be a strategy to selectively manipulate the gut microbiome, and some inhibitors of gut bacterial enzymes are shown. **12** = betaine aldehyde, inhibits choline TMA-lyase (CutC); **13** = fluoromethyl ketone suicide inhibitor of Bile Salt Hydrolase (BSH); **14, 15** = piperazine-containing β-glucuronidase inhibitors; **16** = acarbose, inhibits starch and pullulan utilization; **17** = M4284 mannoside, inhibits FimH in uropathogenic *E. coli*.

4.4 Carbohydrate metabolism: The prospects for chemical precision editing of the GM are improving due to an expansion in the knowledge of its metabolism. GM diversity is promoted by the metabolism of complex plant polysaccharides. Selective manipulation of polysaccharide metabolism without microbicidal effects has been achieved using a small molecule inhibitor, acarbose (**16** in **Fig 3**), which abolished the ability of *B. thetaiotaomicron* and *B. fragilis* to utilize potato starch and pullulan by interfering with the Starch Utilization System[67]. Shifting the GM metabolic activity selectively in this non-lethal fashion alleviated colitis. Until recently, it was not known if single bacterial species or a small community is needed to drive the degradation of any highly complex polysaccharide. The most complex polysaccharide characterized in the gut environment is rhamnogalacturonan-II, which is depolymerized by *Bacteroides thetaiotaomicron* with the cleavage of 20 out of its 21 distinct glycosidic bonds[68]. Further analysis revealed several previously unknown bacterial enzymes were responsible for the degradation of rhamnogalacturonan-II.

4.5 Miscellaneous inhibitors: Zhu et al., showed that dysbiosis-linked gut inflammation caused by the expansion of facultative anaerobic Proteobacteria could be blocked via tungstate administration, which inhibits molybdenum-cofactor respiratory chain enzymes[69]. GM composition was undisturbed when tungstate was administered under homeostatic conditions. Recurrent infections of the urinary tract caused by UPEC occur in 30-50% of patients even after antibiotic treatment. This persistence is linked to the type 1 pilus adhesin, FimH, which binds mannose and aids the colonization of the bladder surface. Type 1 pili were also shown to aid UPEC colonization in the gut and the administration the high affinity FimH inhibitor

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mannoside M4284 (**17** in **Fig 3**) reduced gut colonization and urinary tract infection caused by genetically distinct UPEC isolates, without disrupting the GM composition[70].

5. Chemoproteomics tools for GM studies

Over 1900 uncultured gut microbes were discovered in 2019[71], showing enormous potential for finding metabolic diversity in the GM. Metagenomics projects including the Human Microbiome Project show that identification of the biochemical functions of genes encoding metabolic enzymes in the human gut microbiome accurately is fraught with difficulty. In a survey of 139 stool metagenomes, only around 30% of them could be assigned a GO (Gene Ontology) or EC (Enzyme Commission) annotation; of these annotations, 50% have previously unknown functions[72]. Even in the case of enzymes/pathways that could be annotated, the gut microbiota contains many uncharacterized gene products detected in genomics/ metagenomics analysis. Therefore, chemical information-based analyses (including analysis of chemical structure, chemical reactivity, and potential biological interaction partners) which predict potential GM metabolism, and chemoproteomics methods are better placed to elucidate those "unknown" metabolic functions rather than purely metagenomics. Examples of the chemical information-based include the design of gut-targeted drugs[73] and predictions of potential drug/xenobiotic metabolism in the GM[74]. Herein, however we focus on some chemoproteomics/metabolomic tools developed for specific metabolite groups.

5.1 Enzyme-based sulphated metabolome analysis: Sulphated compounds are derived from gut microbial transformation of dietary material and relate to disease states. Using an arylsulfatase enzyme to hydrolyse sulphated compounds and mass spectrometry-based metabolite analysis, Correia et al have characterized and validated 235 sulphated metabolites in a single study, which were the products of gut microbiota and subsequent host transformations and discovered eleven previously unknown sulphated metabolites[75]. The metabolites reported in this study could form the basis of classification of human subjects as harbouring

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high or low sulphate metabolizing microbiota for future cohort studies. Further, the arylsulfatase-based method may be useful for discovering novel sulphated metabolites.

5.2 Bile salt hydrolase and bile acid-based chemoproteomics: As mentioned before, bile acids are secreted by the liver and further converted into secondary bile acids by the action of the GM. The latter participate in several processes including the metabolism of glucose and lipids, and immune homeostasis. The key reaction of secondary bile acid biosynthesis is catalysed by bile salt hydrolases (BSH). BSH are bacterial cysteine hydrolases whose activity precedes other kinds of bile acid transformations[76]. Parasar et al., developed a strategy based on the covalent labelling of the active site cysteine using a substrate analogue[77]. When the substrate analogue is covalently bound, biorthogonal click chemistry could be applied to attach either a fluorescent contrast agent or a biotin affinity tag to the enzyme-bound analogue. In the first case, in situ imaging could be performed following gel electrophoresis, and in the second, affinity purification using streptavidin (the samples were subsequently analysed using proteomics).

While the expression of metagenomic fragments in well-studied model microbes showed that at least three distinct phyla possess BSH activities in the GM[78], genome-based strategies suffer from the issues of potential toxicity, incomplete coverage, incomplete BGC expression, unintended changes in enzyme levels and tissue localization, all of which led to deviations from the physiologically relevant states of the BSH enzymes. By comparison, the covalent modification of the active sites of BSH enzymes coupled with proteomics has avoided many of the pitfalls of the genome-based methods and enabled the direct identification of these enzymes.

While bile acids (BA) promote CR, little was known about the target proteins affected in the gut pathogens inhibited by BA action. Photoaffinity probes based on chenodeoxycholic acid (CDCA) were able to crosslink many host and pathogen proteins in Salmonella enterica serovar Typhimurium infection models, of which direct protein inhibition by CDCA probes was reported for HilD, a key regulator of Salmonella

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pathogenesis and virulence[79]. Chemical proteomics and photoaffinity labelling based on lithocholic acid (LCA) were also used to identify a previously unknown BA-binding transcriptional factor called BapR in *C. difficile*[80].

5.3 Direct lysine-acylation chemoproteomics: In a 2022 report, abundant post-translational lysine-acylation by RACS (reactive acyl-CoA species) was discovered, whereby the acyl motifs found on several differentially expressed proteins corresponded to the metabolism of specific carboxylic acids in syntrophic bacteria[81]. The importance of cross-feeding in the gut environment, the abundance of SCFA and the ability to analyse the proteome for post-translational modifications without highly biased pre-enrichment, direct analysis of lysine acylation in the GM has good potential to shed light on metabolomic aspects.

5.4 Vitamin affinity probe chemoproteomics: *Bacteroidetes* are one of the four major GM phyla; their genomes usually encode several B_{12}-dependent enzymes, although they lack the ability of *de novo* cobamide synthesis[82]. It is therefore likely that they could harbour B_{12} transport proteins different at the sequence level from canonical *E. coli* counterparts. The use of B_{12}-based affinity probes and subsequent application of chemoproteomics in *Bacteroides thetaiotaomicron* samples revealed the presence of proteins without previously unknown functions; one of these, BtuH2 was shown to capture and transport B_{12} directly *in vitro* and responsible for gut fitness of these bacteria in gnotobiotic mice[83].

6. Modulating host receptors

The intestinal surface senses bacterial surface molecules and GM metabolites through several types of cell-surface receptors and further effects are exerted by receptor protein complexes inside various types of gut cells. Here, we will briefly consider only selected agonists/antagonists linked to GM activity of a few cell-surface, nuclear and peroxisome-linked receptors.

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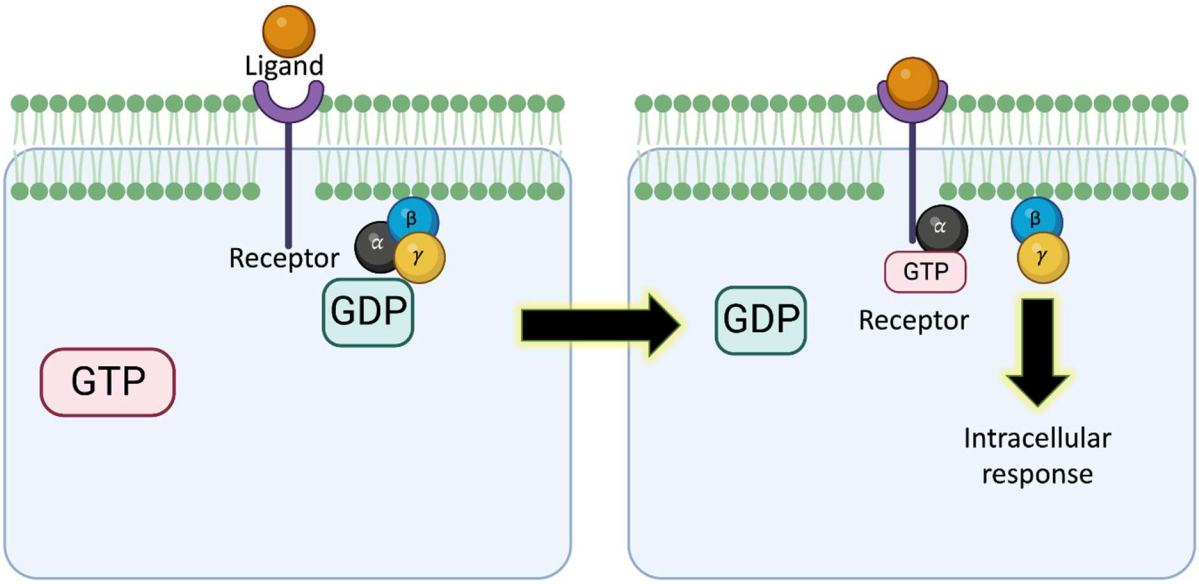

Fig 4. Molecular mechanism of G-protein coupled receptors on the cell surface. The ligand binds to the receptor protein causing the G-protein subunits to disassemble and exchange bound GDP with GTP. The G-protein α-subunit is bound to the receptor, while the other subunits signal to other proteins involved in intracellular responses. GTP hydrolysis drives the dissociation of the α-subunit from the receptor and a return to the GDP-bound multi-subunit G-protein complex.

6.1 Cell-surface receptors

G-protein coupled receptors (GPCRs) are the largest membrane protein family in humans and sense their ligands through a mechanism outlined in **Fig 4.** GPCR complexes contain a transmembrane subunit (green in **Fig 4**) which binds a small molecule (ligand) at the cell surface, while a linked trimeric G-protein bound to GDP is located inside the cell. Once the ligand has been captured by the receptor subunit, then a conformational change occurs in the complex, allowing GTP to bind the trimeric G-protein, which usually dissociates, triggering an intracellular response via further downstream events. There are a variety of GPCRs in the gut for various microbial metabolites such as SCFA[84], bile acids[85] and several other types of effectors[86]. Gut bacteria synthesise molecules such as commendamide, which mimic the human (endogenous) ligands of GPCRs[87].

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A forward genetics screen (i.e., trying to identify genes leading to a phenotype) based on the Tango β-arrestin recruitment assay (PRESTO-Tango), was able to measure the activation processes of almost all the non-olfactory human GPCRs[88] and revealed several novel GPCR ligands such as L-phenylalanine secreted in the GM[89]. Several other ligands which bind GPCRs (including in immune and nerve cells) such as phenylpropanoic acid, cadaverine, 9-10-methylenehexadecanoic acid, and 12-methyltetradecanoic acid were identified in a high throughput screening of 241 GPCRs[90], using seven gut microbes to represent a simplified human microbiome (SIHUMI) consortium[90,91].

6.1.1 Free fatty acid receptors (FFAR): SCFA are sensed by specialized GPCRs called the FFAR, a family of cell surface receptors[91-93]. FFAR2 and FFAR3 signalling links the GM and the β-cells in the pancreas and therefore are important targets in type-1 and type-2 diabetes[93,95]. In pigs, the use of trans-glycosylated starches (TGS) led to downregulated FFAR2 via GM modulation, which decreased obesity[95]. GM-derived SCFA and LPS also participate in the gut-lung immune axis since these molecules can travel to the lungs and modulate FFAR2/3 activity there[84].

6.1.2 Hydroxy carboxylic acid receptor (HCAR): This is yet another class of GPCRs which regulate immunity and energy homeostasis and sense hydroxycarboxylic acids. Most mammals have HCA1 which senses lactic acid, and HCA2 which senses 3-hydroxybutanoate and butyrate[96]. Recently, a third HCAR called HCA3 was detected in hominin genomes and described in humans; it senses and is potently activated by D-phenyllactic acid (D-PLA)[97], which is produced as an antimicrobial by GM Lactobacilli. HCA2 is expressed in not only the intestinal epithelial cells, but also adipocytes, immune cells, hepatocytes, retinal epithelium, and Langerhans cells[98], suggesting involvement in communication between the gut and the fatty tissues, liver, eyes, and skin. It is implicated in pathological states such as intestinal inflammation and cancers, making it a possible therapeutic target in several diseases[98].

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6.2 Nuclear receptors

The major nuclear receptors in the gut are the aryl hydrocarbon receptor, the farnesoid X receptor and the pregnane X receptor.

6.2.1 Aryl hydrocarbon receptor (AHR): This receptor is a transcription factor with a helix-lop-helix motif, and senses compounds bearing an aromatic ring such as indole/tryptophan compounds, polyphenols, flavonoids, and synthetic pollutants like dioxins and polycyclic aromatic hydrocarbons. It controls immunity at the gut barrier via the differentiation and inflammatory responses of innate and adaptive immune cells[99, 100]. GM tryptophan catabolism produces AHR ligands such as indole-3-aldehyde, which stimulate intestinal immunity against *C. albicans* colonization via IL-22[101]. Tryptophan metabolites also communicate bi-directionally between the GM and the brain (gut-brain axis) via the AHR[102]. The natural dye indigo binds the AHR and induces the production of the interleukins IL-10 and IL-22, which confers protection against high-fat diet (HFD)-induced insulin resistance and fatty liver disease in mice[103]. This was linked to specific increases in *Lactobacillus* cell counts and the elicitation of IL-22 secretion in the gut[104]. Intestinal inflammation can be modulated by AHR ligands such as oxazoles[105] and 6-formylindolo (3,2-b) carbazole (Ficz)[106].

6.2.2 Farnesoid X receptor (FXR): FXR is activated by bile acids and are involved in lipid and glucose metabolism as well as energy homeostasis through the enterohepatic route[107,108]. The antioxidant compound tempol leads to the accumulation of tauro-β-muricholic acid (T-β-MCA) in mice by blocking BSH enzymes in the Lactobacilli; T-β-MCA inhibits FXR signalling, consequently reducing obesity[109]. Glycine-β-muricholic acid (Gly-MCA) prevents obesity, insulin resistance, and fatty liver disease in mice by decreasing the Firmicutes to Bacteroidetes ratio, leading to reduced SCFA levels[110]. Bile acids conjugated to the amino acids phenylalanine, tyrosine and leucine are FXR agonists and are elevated in cystic fibrosis and inflammatory bowel disease (IBD)[111].

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The bile acid derivative obitecholic acid (OCA) can reshape the small intestine microbiome in humans and mice via the FXR receptor[112]. These studies demonstrated the links between the GM, FXR and metabolic disease and showed that FXR agonists could be promising anti-obesity leads via microbiome remodelling. In addition, OCA could also reduce the severity of *C. difficile* infection in mice fed a high-fat diet by an FXR-mediated drop in primary bile acid levels, which decreases *C. difficile* spore germination[113]. Owing to the communication between the GM and the brain (the gut-brain axis), OCA can influence the GM-triggered microglia accumulation in the brain and ameliorate the anxiety associated with metabolic disease of treated mice[114]. Small-molecule manipulation of the GM therefore enables the modulation of distant organs via the gut-brain, the gut-liver, the gut-heart, and the gut-lung axes.

6.2.3 Pregnane X receptor (PXR): PXR is implicated in the metabolism of xenobiotic compounds, expressed in the vascular endothelium lining the blood vessels and is in direct contact with the serum[115]. It is involved in innate immunity via the inflammasome and protection of the endothelia from oxidative damage[116]. The natural product tanshinone IIA protects the endothelial cells from ROS damage via PXR activation[117], while the GM metabolite indole-3-propionate (IPA) regulates PXR-dependent vasodilation[118]. Using IPA as a scaffold, Dvořák et al, synthesized a series of indole derivatives which were the first ever non-cytotoxic PXR agonists which reduced inflammation in mice[119], suggesting that GM metabolite mimicry might be a viable strategy to discover novel drugs with good efficacy and low toxicity.

6.4 Peroxisome proliferator-activated receptors (PPARs)

PPARs are found throughout the gut tissue and have roles fatty acid sensing, metabolism, and modulation of immunity; PPARα is crucial for fatty acid and branched chain amino acid catabolism in the mitochondria and peroxisomes[120], while PPARγ is important in innate immunity[121]. Double agonists of both these receptors have been successful in animal models of *Citrobacter rodentii* and DSS-induced colitis of reducing tissue damage and bacterial loads leading to infection clearance and resolved inflammation, compared to agonists of each receptor separately[122]. PPARα and γ activation has been reported for keto- and

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hydroxy-octadecanoic acid species, which were produced by *Lactiplantibacillus plantarum*[123]. Oleoylethanolamide (OEA), an endogenous PPAR ligand can be administered exogenously in mice to shift the microbiota in the colon to higher Bacteroidetes/Firmicutes ratio, with corresponding increases in *Bacteroides*, *Prevotella* and *Parabacteroides* and decreases in *Bacillus* and *Lactobacillus* strains[124]. The GM has also been modulated also by synthetic agonists, such as fenofibrate, which led to increased SCFA in serum and tissues in mice fed high-fat diets (HFD)[125]. Dysbiosis induced by either high-fructose diets or HFD in mice could be remediated by the PPAR agonist Wy-16434, whereby the Bacteroidetes/Firmicutes ratio increased (reduced Proteobacteria and increased Actinobacteria)[126, 127].

6. Future directions and conclusions

As outlined in this article, approaches such as the inhibition of specific GM metabolism, the use of COINS, prophylactic use of small-molecule determinants of CR, and GM metabolite mimicry could emerge as therapeutic avenues in GM modulation and precision medicine. Outside the coverage of this article, developments in canonical amino acid modification, biorthogonal chemistry, non-canonical amino acids, ribosome engineering, mass spectrometry, natural product databases and machine learning have increased the scope of chemical and chemical information-based tools to interrogate GM-related metabolism and discover GM-related natural products. The emergence of chemical and informatics technologies alongside advances in deep sequencing[128], improvement in technologies to cultivate "uncultivable microbes"[129] and isolate GM-specific microbes via "culturomics"[130] make it an exciting time to be a chemical biologist interested in GM research, with expanding opportunities for chemistry-based discovery and interventions to benefit human health.

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relationships that could be construed as a potential conflict of interest. The paper was initiated by Dr. Anutthaman Parthasarathy and Dr. Ganesh Pandian Namasivayam, while the former was on an academic visit to Japan.

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