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Perspective

Cyclic dipeptides and the human microbiome: Opportunities and challenges

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ABSTRACT

Research into the human microbiome has implicated its constituents in a variety of non-communicable diseases, with certain microbes found to promote health and others leading to dysbiosis and pathogenesis. Microbes communicate and coordinate their behaviour through the secretion of small molecules, such as cyclic dipeptides (CDPs), into their surrounding environment. CDPs are ubiquitous signalling molecules that exhibit a wide range of biological activities, with particular relevance to human health due to their potential to act as microbiome modulators.

1. Introduction

Our bodies possess an abundance of microbial symbionts which inhabit our skin, mucosal linings, gastrointestinal tract and various biofluids, with our resident commensals providing essential nutrients and metabolites in exchange for a propagation niche.[1–4] Given that the number of cells comprising our microbiome is thought to at least equal that of our own cells,[5] and with each strain known to possess a variety of unique genes,[6] the collective genome provided by our microbiome contributes greater genetic diversity than that of the human genome itself. Unsurprisingly, the human microbiome has become the focus of much research in recent years, the results of which have implicated its constituents in a variety of non-communicable diseases.[3] Given that microbes communicate and coordinate their behaviour at intra- and interspecies level through the secretion of specific chemical signals into their surrounding environment,[7] this perspective will focus on a class of small molecules, known as cyclic dipeptides (CDPs), as potential modulators of microbiome composition (see Scheme 1).

2. Characterising our ancillary genome

Despite rapid appreciation of their importance, identification of constituent microbial populations has historically proved difficult due to inherent issues with culturing microbes in a lab setting. In recent years, culturomics[8] and culture-independent omics technologies have played an instrumental role in furthering our understanding of the human microbiome, with shotgun metagenomics in combination with targeted sequencing methods identifying which prokaryote and fungal species comprise our microbial communities, as well as their relative abundance.[2,3,6,9] In fact, genomic investigations into the microbiome and mammalian phylogeny have revealed that although a core assemblage exists within conspecifics, microbial composition is more dependent on dietary habits than host lineage; in these studies, the most marked differences were observed between the herbivore and carnivore microbiomes, with their functional configurations distinguished mainly by enzymes involved in amino acid anabolism and catabolism respectively.[9] Furthermore, metanalyses of metagenomic research into the oral and gut microbiomes have shown this core assemblage to be enriched for primary metabolic processes, such as the citric acid cycle and amino acid biosynthesis; however, around 50% of all microbial genes present were specific to the individual and were enriched for a wide range of diverse biosynthesis and degradation pathways, thus providing each host with a unique functional repertoire.[6]

3. Biosynthesis of cyclic dipeptides

Owing to their relative stability and reactivity, cyclic nucleotides[10] and cyclic peptides,[11] which are ubiquitous signalling molecules, represent two classes of biologically significant compounds with particular relevance to the human microbiome. Of the natural peptide products, CDPs, or 2,5-diketopiperazines (DKP), are a diverse family of highly stable small molecules that exhibit a wide range of biological and pharmacological activities.[12] The DKP scaffold, an intrinsic feature of all CDPs, can be obtained via organic synthesis or enzymatic catalysis

Abbreviations: CDPS, cyclodipeptide synthase; CDP, cyclic dipeptide or cyclodipeptide; DKP, 2,5-diketopiperazine; aa-tRNA, aminoacyl-tRNA; IGF-1, insulin-like growth factor-1.

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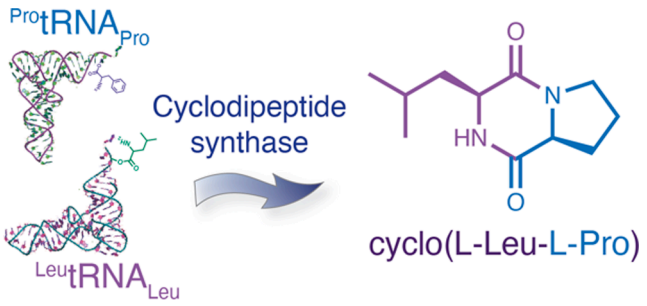

Scheme 1. Reaction catalysed by CDPS enzymes to form a cyclic dipeptide. Cyclo(L-Leu-L-Pro) is shown as an example.

involving a condensation reaction between two α-amino acids, followed by intramolecular cyclisation of the linear dipeptide. Methods for chemical synthesis of CDPs were thoroughly reviewed elsewhere, and beyond the scope of this article.[13]

The enzymes that catalyse CDP formation belong to two unrelated families, non-ribosomal peptide synthetases (NRPSs) and cyclodipeptide synthases (CDPSs), with the vast majority of CDPS genes and biosynthetic gene clusters found thus far in bacteria.[14–16] Since their discovery in 2002,[17] a combination of structure determination,[18–20] kinetics measurements,[21–24] computational methods[25] and enzyme engineering[26,27] has revealed the catalytic mechanism of CDPS enzymes and explored their potential as biocatalysts in the production of novel CDPs.[26,28,29]

CDPSs are aminoacyl-tRNA-dependent (aa-tRNA) enzymes that divert two aa-tRNAs from their canonical role in ribosomal protein synthesis,[30] and therefore represent a direct link between primary and secondary metabolism.[31] Biosynthetic gene clusters containing CDPS enzymes are typically associated with tailoring enzymes that can modify CDPs by inserting key functional groups that add substantial chemical complexity to the DKP scaffold.[14] Examples of common tailoring enzymes include methyltransferases, oxidoreductases, ligases, glycosyltransferases and P450 enzymes, with these catalysts facilitating complex condensation reactions such as nucleobase transfer and carbon–carbon bond formation.[16,32]

To date over 1,200 proteins are annotated as members of the cyclodipeptide synthase protein family (PF16715 on https://www.uniprot.org/), recently further enriched by the identification of fungal CDPS with a unique architecture.[33]

4. Microbial cyclic dipeptides

Given their role in bacterial communication, where they often serve as switches between symbiosis and virulence,[34–36] CDP-based therapeutics could act to antagonise or enhance the action of their native counterparts and may represent a novel means of modulating our microbial composition to maintain or achieve homeostasis. For example, a variety of proline-based CDPs from Korean fermented vegetable kimchi were found to display potent antimicrobial properties, with those from filtrates containing *Lactobacillus plantarum* LBP-K10 demonstrating antiviral[37] and antifungal[38] activities, and those from *Leuconostoc mesenteroides* LBP-K06 exhibiting activity against multidrug-resistant bacteria *Staphylococcus aureus* 11471 and *Salmonella typhimurium* 12219.[39] Interestingly, of a variety of foodstuffs investigated in the latter study, these CDPs were only detected when *L. mesenteroides* starter cultures were used to ferment Chinese cabbage, suggesting a possible link between dietary nutrient profile and microbial metabolic output. Additionally, proline-containing CDPs have also proved effective in disrupting biofilm formation by a variety of organisms, specifically, in a non-bactericidal manner, cyclo(L-Leu-L-Pro) was active against the foodborne pathogen *Listeria monocytogenes,*[40] as well as the opportunistic pathogen *Staphylococcus epidermis*[41] and toxic shock-inducing *S. aureus*.[42] These findings suggest proline-based compounds may present a promising means of treating infections caused by key human pathogens, with their non-bactericidal mode of action an attractive alternative to traditional antibiotics; however, the complex microbial networks and metabolites associated with homeostasis versus dysbiosis must be better understood, so that effects on microbes associated with health or disease can be better predicted.

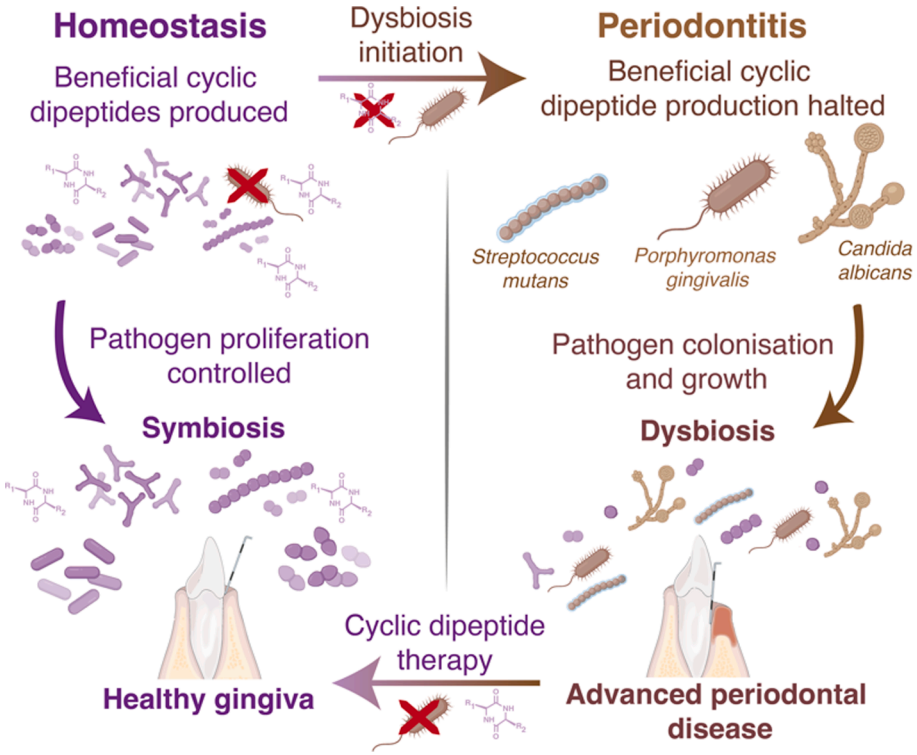

Fig. 1. Homeostasis and dysbiosis in the oral microbiome. During homeostasis, commensal organisms coexist and help keep pathogen numbers under control via processes mediated by small molecules and other factors, including CDPs. When pathogens prevail and proliferate, they dominate their surrounding environment and subsequently increase competition for key resources, ultimately resulting in dysbiosis by decreasing the abundance and diversity of symbiont populations. This process could potentially be reversed by administration of small molecules, including CDPs, that either disrupt pathogen growth or promote colonisation by beneficial commensals.

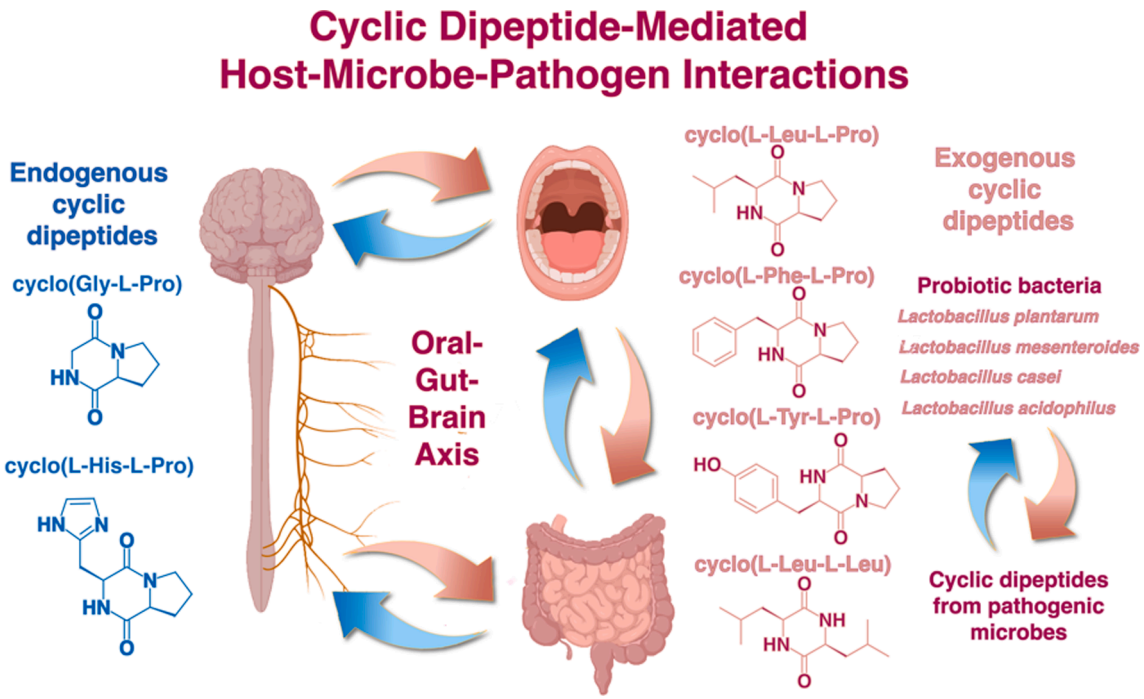

Fig. 2. Interactions between host and microbial cyclic dipeptides (CDPs). Humans produce two cyclic dipeptides, cyclo(Gly-L-Pro) and cyclo(L-His-L-Pro) (depicted in blue). These molecules can exert effects on microbial populations in our gut and oral microbiomes. Alternatively, several microbes that form part of our microbiota can either act as probiotics or agents for dysbiosis via secretion of CDPs – for example, cyclo(L-Leu-L-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Tyr-L-Pro), and cyclo (L-Leu-L-Leu) (depicted in salmon) – which can affect other microbes and their hosts.

5. Cyclic dipeptides and the oral microbiome

The Global Burden of Disease Study (2016) estimated that oral diseases affect around half of the world's population, with severe periodontal disease identified as the 11th most prevalent disease globally.[43] A mounting body of evidence has implicated keystone-pathogens in the development of several human diseases via perturbation of the microbiome, such as *Porphyromonas gingivalis* which induces dysbiosis and inflammatory periodontitis.[44] Importantly, multiple studies have demonstrated the efficacy of CDPs against pathogens commonly linked to dysbiosis. For example, out of a library of 75 novel CDPs, five were found to significantly inhibit biofilm formation by *Candida albicans* and *Streptococcus mutans* – the primary causative agent of dental caries.[45] Considering the association between periodontal and systemic disease,[3,4,43,44] as well as its relative ease of access, the oral cavity and its associated microbiome appears to be a promising starting point to test strategies for CDP-based therapeutic interventions. Progression from homeostasis to dysbiosis and its possible reversal using CDP-based therapies is depicted in Fig. 1.

6. Endogenous cyclic dipeptides

In addition to their impact on microbial composition and pathogen colonisation, CDPs can also affect mammalian cellular processes. Specifically, endogenously generated cyclo(L-His-L-Pro) and cyclo(Gly-L-Pro) discovered in the central nervous system of various mammals, including humans,[46–48] suggests CDPs may be common signalling molecules utilised by prokaryotes to higher eukaryotes. The subsequent identification of the cyclo(L-His-L-Pro) cognate receptor, the sodium-independent organic cation transporter (OCT) 2, showed the receptor to be preferentially expressed in dopaminergic brain regions, with the highest density found in the substantia nigra pars compacta.[49] Additionally, cyclo(L-His-L-Pro) has been shown to reduce glial cell-mediated inflammation[50] and exert cytoprotective effects on neurons expressing the OCT2 transporter.[49] In these investigations, when an OCT2 gene variant - with an ethnic-specific allelic frequency of 1.5% - encoding amino acid substitution R400C was expressed, cells displayed reduced transport efficiency for cytoprotective cyclo(L-His-L-Pro) and a subsequent increase in susceptibility to salsolinol-mediated cell death. These findings highlight a crucial role for cyclo(L-His-L-Pro) in maintaining neuronal integrity within the nigral dopaminergic system - a finding with clear implications for neurological disorders such as Parkinson's disease.[51,52] Other research has also shown cyclo(Gly-L-Pro), a metabolite derived from insulin-like growth factor-1 (IGF-1), to play a neuroprotective role, with a single dose treatment of this CDP conferring protection against vascular damage when administered to rat models for hypoxic-ischemic brain injury.[46] Interestingly, in the same studies, cyclo (Gly-L-Pro) was also found to significantly inhibit IGF-1-dependent tumour growth, implying a key regulatory role for CDPs in homeostasis and pathogenesis. Additional investigations have also found endogenous cyclo(Gly-L-Pro) to be present in the milk of lactating rats and demonstrated oral bioavailability and effective maternal-infantile transfer of exogenous cyclo(Gly-L-Pro) through milk, with elevated serum levels of this CDP during offspring development associated with enhanced recognition memory during behavioural tests.[48] Furthermore, consumption of blackcurrant anthocyanins, which were found to contain cyclo(Gly-L-Pro), resulted in increased levels within the cerebrospinal fluid of Parkinson's disease patients,[47] providing evidence for the oral bioavailability of cyclo(Gly-L-Pro) in humans as well as demonstrating its effective uptake into the central nervous system. Taken together, these results suggest that CDPs may have therapeutic utility in a wide variety of neurological and systemic inflammatory disorders and that dietary supplementation of cyclo(Gly-L-Pro) could prove a viable means of administration. Cyclo(-L-Phe-L-Pro) produced by *Vibrio vulnificus* can affect bacterial and viral infections. It inhibits production of interferon-β, enhancing host susceptibility to several viruses such as hepatitis C and influenza,[53] in addition to affecting gut microbe survival in mice.[54] Therefore, given that several microbes have been shown to produce proline-containing CDPs, these molecules may represent a mode of communication between mammalians and microbes

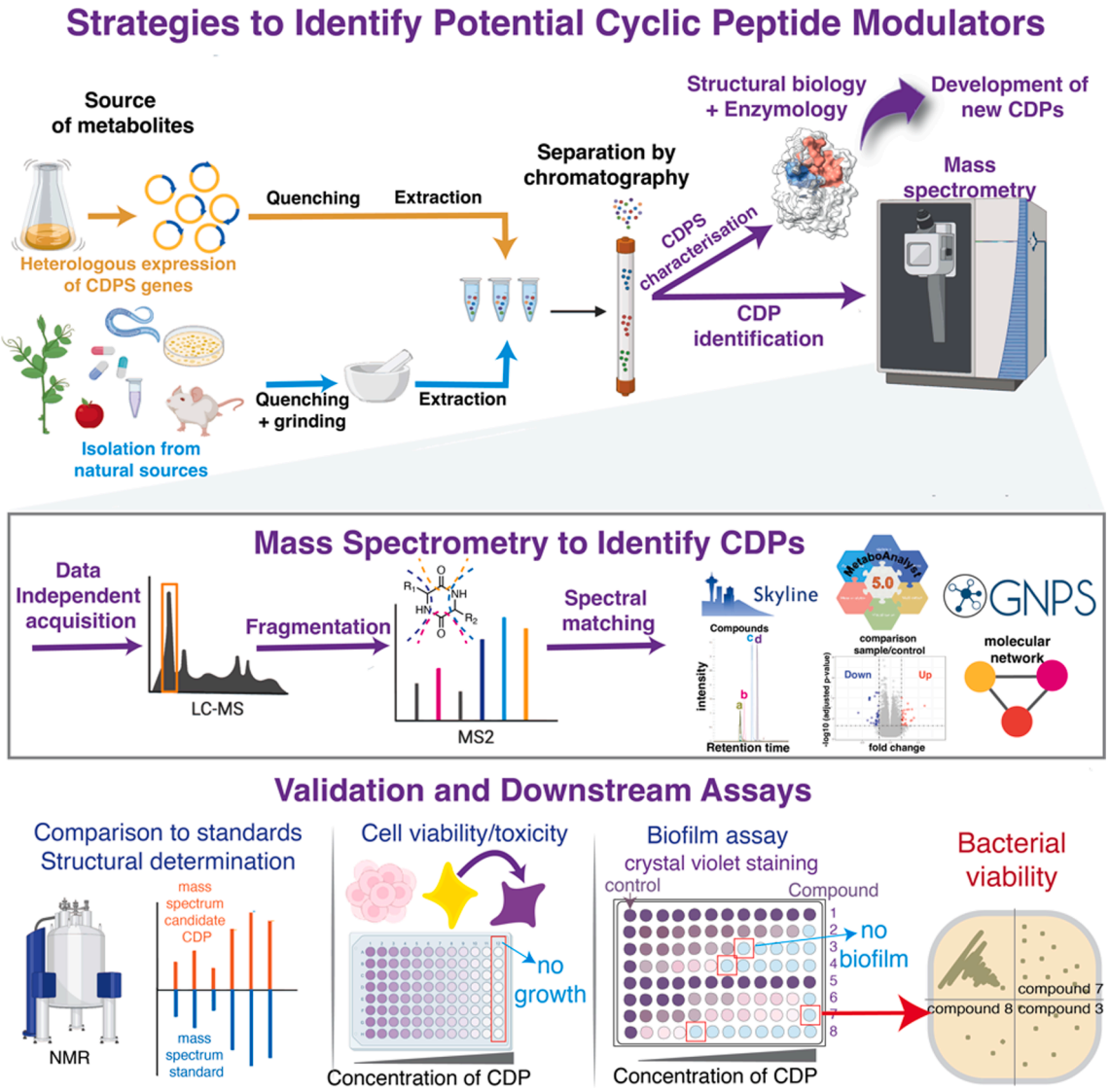

Fig. 3. Strategies to discover and develop novel CDPs that can modulate microbial communities. CDPs can be isolated from natural sources or produced by heterologous expression of CDPS enzymes. Following CDP extraction and separation via chromatography, mass spectrometry is used along with a variety of computational tools to enable CDP identification (depicted here Skyline,[63] MetaboAnalyst[64] and GNPS.[65] This can be followed by a series of downstream assays which, for example, determine CDP toxicity and biological activity against biofilms.

(Fig. 2).

7. Host-microbiome crosstalk via cyclic dipeptides

A wide variety of hormone-like molecules have been implicated in interkingdom signalling, with particular eukaryotic hormones thought to mimic quorum sensing signals and certain bacterial products known to modulate signal transduction pathways in their eukaryotic hosts.[7] Specifically, it is thought that microbe-derived tryptophan metabolites are crucial regulators of human metabolism and immune function.[1–3]

Many instances of interkingdom crosstalk occur between plants and prokaryotes. For example, *Pseudomonas aeruginosa*, a plant and human pathogen, was found to synthesise three proline-containing CDPs which replicate the effects of the phytohormone auxin (indole-3-acetic acid) on *Arabidopsis thaliana* seedlings.[55] Interestingly, four CDPs harvested from human probiotic bacteria, *Lactobacillus casei* CRL 431 and *Lactobacillus acidophilus* CRL 730, demonstrated anti-biofilm and anti-virulence activity against three strains of *P. aeruginosa*[56] - further suggesting our commensal microbes confer protection against key pathogens via CDP-mediated mechanisms. Other investigations found a variety of tryptophan-containing CDPs to significantly disrupt virulence factor production, swimming motility, biofilm formation and adhesion by *P. aeruginosa*.[57] Given the fundamental importance of indole derivatives in both plant and human metabolic processes, coupled with the fact that many pathogenic bacterial species, including *E. coli*, *P. gingivalis* and *Vibrio cholerae*, are known to produce large quantities of indole to modulate various aspects of bacterial physiology, such as drug resistance, virulence, spore and biofilm formation, the possible significance of indole-mimetic compounds, including CDPs, to human health should not be overlooked.[58]

8. Conclusions

Considering the complex metabolic interplay between host and commensal organisms, as well as that of competing and cooperating microbes, in conjunction with diet and lifestyle, which can yield microbiota with distinct taxonomic compositions,[1,4,9] both human and microbial metabolomes should be fully interrogated if we are to gain a comprehensive understanding of this multifaceted relationship. To enable this, determination of microbial metabolic output and metabolite

biosynthesis is essential and can be achieved through a combination of techniques (Fig. 3). This presents challenges, given that the metabolome is highly chemically diverse, further complicated by the presence of novel molecules as their unequivocal structural assignment can be arduous. Significant progress has, however, been enabled by rapidly improving bioinformatic tools allowing prediction of biosynthetic gene clusters and their products as well as aiding metabolite assignment, in comparison to databases and using artificial intelligence, somewhat alleviating these challenges. Strategies for these workflows have been reviewed and described elsewhere.[59–62]

Although characterising human and microbial metabolomes is an important endeavour, given that a single nucleotide polymorphism can result in structural abnormalities, impede receptor function, disrupt key metabolic pathways or lead to aberrant immune responses, more work remains to be done on identifying pathogenic gene variants within the human population.[66] Initiatives such as Our Future Health,[67] the UK's largest ever research program, which aim to genotype a diverse sample of the human population, are therefore an essential step towards tackling the growing burden of disease; only by having a clear understanding of host genetics can we fully comprehend the driving forces behind dysbiosis, as well as its association with various disease states, and begin to infer causality. Lastly, given the uniqueness of each person's endogenous and ancillary genomes, attempts to improve the composition of our microbiota should move towards more tailored interventions that consider human-microbiome individuality. This would involve understanding key metabolite differences between healthy and diseased states, prior to introducing the appropriate microbial consortia or metabolites which reinstate homeostasis or prevent dysbiosis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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