An update on the role of gut microbiota in chronic inflammatory diseases, and potential therapeutic targets.

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ABSTRACT

Introduction: The human microbiome plays a critical role in human health, having metabolic, protective and trophic functions, depending upon its exact composition. This composition is affected by a number of factors, including the genetic background of the individual, early life factors (including method of birth, length of breast feeding), nature of the diet and other environmental exposures (including cigarette smoking) and general life habits. It plays a key role in the control of inflammation, and in turn, its composition is significantly influenced by inflammation.

Areas covered: We consider metabolic, protective and trophic functions of the microbiome and influences through the lifespan from post-partum effects, to diet later in life in healthy older adults, the effects of aging on its composition, and its influence on health and potential therapeutic targets that may have anti-inflammatory effects.

Expert commentary: The future will see the growth of more effective therapies targeting the microbiome particularly with respect to the use of specific nutrients and diets personalised to the individual.

Keywords

Gut microbiota, chronic inflammatory diseases, therapeutic targets

1. Introduction

Chronic inflammatory diseases encompass a range of conditions, including osteoarthritis, autoimmune diseases such as rheumatoid arthritis (RA), various allergies, respiratory conditions such as asthma, and gut disorders such as inflammatory bowel disease (IBD). This latter group of diseases describes disorders that involve chronic inflammation of the digestive tract.

There is increasing evidence that microbiota are involved in chronic conditions as has been shown with IBD, and that the microbiota also play a role in determining the age at which symptoms become apparent [1,2]. The microbiome could also be an important target for therapy [1,3]. Indeed, faecal transplants have been suggested for IBD and other diseases [4,5], although selective use of probiotics, prebiotic foods and/or supplements that less invasively modulate the gut microbiome may be more palatable [5].

2. Establishment of the human microbiome

The microbial community within the microbiome shows high variability among healthy people. Evidence suggests there is no one core microbiome at species level but there may be at phylum level, or the core may be at the level of function rather than population. Each adult has a distinct microbial community in which one bacterial species may vary by between 12 and 2,187-fold [11,12]. It has been shown that in people with disorders like IBD and obesity, the composition of the microbiota is different to those found in healthy people [8,11,13]. Unlike the human genome which is inherited, the microbiome is acquired anew with each generation. The microbiome is also genetically alterable which enables microbiota to change with changing environments, for example in response to variable food intakes [10] . The microbiome also changes over the lifespan [8,13-15].

Until recently it was thought that the infant was born with a sterile gut and the first microbiota was conferred and influenced by the method of birth and initial feeding style. New evidence suggests that a foetal microbiome exists, being acquired in the last trimester from the microbiome in the mother's amniotic fluid (Figure 1). It has also been observed that microbiota diversity is lower among immature babies [16]. It is also established that the future composition of the infant's gut bacteria is affected by the delivery method i.e. whether Caesarean born (C-section) or vaginally born, and also whether breast fed or given infant formula. [17,18].

Insert here Figure 1: The balance of factors involved in gut dysbiosis in early infancy here

The type of first foods also contributes to the composition of the microbiota. Breast feeding promotes immune development and protects against many diseases. In a small study on 3-

month-old babies 1,214 probe sets for epithelial cells were significantly differently expressed in exclusively breast fed (n=12) and formula fed babies (n=10) [19]. This may explain some of the differences that have been noted in clinical and epidemiological observations between these two groups. The two groups have different gut development with breast-fed babies being described as 'less leaky'. *Bacteroidetes* species, which are important players in the commensal bacteria, were not found in the stools of the formula fed infants [19].

Work done by Donavan et al. on pig milk which is physiologically and compositionally similar to human milk analysed the microbiome gene expression at phylum level in the stools of piglets [20]. All formula fed pigs shared the same distinct signature but those that were suckled had highly variable microbiomes [19]. The microbiome of those that were suckled also had a higher number of virulence genes, thought to contribute to the host immune defence response. This could help explain why babies who are breast fed have a gut that is less leaky. Human breast milk also contains milk oligosaccharides which stimulate the growth of the intestinal flora acting as prebiotics for beneficial bacteria [21], thus contributing to the enhancement of gut barrier function.

Towards three years of age the microbiome of a child is thought to resemble the microbiota of an adult [22]. However, the ELDERMET study on those aged 65 years and older (n=126) found that it is quite different from middle aged adults, with a greater presence of Bacteroides species, increased taxonomic diversity and more individual variation [23]. Studies based on elderly people in other countries report different findings, reflecting the participant's country of residence [24]. This change in the microbiome over time and country of residence is in part possible because gut microbes regularly swap genes with environmental microbes via horizontal gene transfer. An example of this is with Japanese individuals who possess marine bacteria (associated with seaweed ingestion) that metagenomics does not show in North American individuals. This is probably because Japanese people consume the seaweed Nori used with sushi, and assimilate the bacterial genes associated with this seaweed [25].

3. Microbiota Composition and complexity

There are 50 bacterial phyla described to date, but these are dominated by two – the *Bacteroidetes* and *Firmicutes* – with *Proteobacteria, Verrucomicrobia, Actinobacteria,*

Fusobacteria, and *Cyanobacteria* being present in minor proportions [26,27]. Estimates of the number of bacterial species present in the human gut range up to 1,000 species [28].

Bacteroides thetaiotaomicron, a prominent foundation species for starch metabolism in the human gut is an anaerobic gram-negative saccharolytic bacterium with a highly developed genome encoding 226 glycoside hydrolases and 15 polysaccharide lyases. These include 64 arabinosidases, xylanases, and pectate lyases, whereas the human gut has none [29]. Their genome also enables two cell surface proteins to bind starch i.e. amylose, amylopectin, pullulan and maltooligosaccharides which allow the digestion of plants in the human gut [30]. *B. thetaiotaomicron* also contains an expanded set of environmental sensory and regulatory proteins to detect polysaccharides and coordinate and regulate genes involved in their metabolism [31].

Methanobrevibacter smithii, another important archaeal species, regulates the specificity of polysaccharide fermentation and influences the amount of calories deposited in fat stores [32]. It does this by improving fermentation efficiency through preventing hydrogen (H_2) (by combining H_2 with carbon dioxide to produce methane) and formate buildup. Accumulation of H_2 inhibits bacterial NADH dehydrogenases, thereby reducing the yield of ATP [33].

4. Functions of the microbiome

The microbiome has three important roles. It has a metabolic role by being able to increase the proliferation and differentiation of colonic cells. It has a protective role by inducing apoptosis in cancer cells and a trophic function by inducing anti-inflammatory responses. Its main metabolic function is to ferment unused energy substrates (Figure 2) [34].

Insert here Figure 2: Methods microbiome use to ferment unused energy substrates

Saccharolytic fermentation results in the production of short chain fatty acids (SCFA), the chief one being butyrate. Butyrate is an energy source for epithelial cells, prevents/inhibits colon carcinogenesis, has immuno-modulatory effects through suppression of the nuclear factor (NF-kB) pathway, modulates oxidative stress, enhances colonic defence barrier and increases satiety [35]. As part of its metabolic function the microbiota also produces vitamins (biotin, vitamin K) and regulates fat storage. This has been illustrated with germ free (GF) mice [36], in which the microbiota was shown to be responsible for rapid microbial induction of angiogenesis in small intestinal villi. GF mice had slower epithelial renewal rates and slower gut motility, were vulnerable to vitamin deficiencies, did not extract as much energy from their diet and had a lower metabolism [36,37]. Studies with obese people also illustrate the influence of microbiota, as illustrated in Figure 3 [38].

Insert Figure 3: Influence of microbiota in obese people and metabolic diseases [38]

Legend: Reprinted from Abstract figure legend 'Growing evidence suggests that the bacteria present in our gut may play a role in mediating the effect of genetics and lifestyle on obesity and metabolic diseases'. J. Physiol. (Lond.) 2017*, 595*, 477-487., DOI: 10.1113/JP272476 Copyright (2018), under Creative Commons licence

4.1 Protective functions

Because of constant threats from microbes and toxins, the human intestine has developed a number of protective mechanisms. In healthy individuals, the innermost layer of the intestine – a single layer of highly specialized epithelial cells – ensures that toxins and pathogenic microbiota are efficiently separated from the immune system of the gut. These cells are equipped with innate immune functions to prevent or control access of bacterial antigens to the mucosal immune cells and produce and maintain a double-layered mucous boundary [39]. Mucus has two main functions. One function is to act as a lubricant to reduce the stress on the epithelial cell layer with its constant exposure to food, antigens and pathogens. The other function is to act as a defence barrier through, for example, the use of antimicrobial peptides to prevent contact between bacteria and the epithelial cell layer by trapping them in the mucus and then eliminating them via peristalsis [39,40].

To maintain the integrity of the epithelium and its barrier function the microbiota have evolved a number of strategies. One such strategy is through cross-talk with cells in the epithelium. For example, *Bacteroides thetaiotaomicron* induces the epithelial Paneth cells to discharge angiogenin 4 (Ang4) into the lumen. This protein is abundant in the Paneth cell secretory

granules, and has potent antibacterial activity which targets gram positive bacteria. This may help maintain a gram negative population which is more prevalent in a healthy adult gut [41]. Mouse studies have established the link between Ang4 expression and the presence of commensal bacteria. Germ-free mice achieve high expression of Ang4 in their Paneth cells only when they are exposed to commensal bacteria [42]. This example illustrates how microbial composition can be regulated by one population impeding another via Paneth cell secretions. It also illustrates how the microbiota can accommodate a variety of microbes without mounting an immune response by using Ang4 to limit microbial access to the gut epithelium, so contributing to the maintenance of barrier function [42].

The microbiota also actively trigger mucosal repair through Toll-Like Receptor (TLR) signalling mechanisms. Signals from gut bacteria activate the Paneth cell MyD88-dependent TLR signalling pathway. This response limits the ability of the bacteria to penetrate the host's tissues, thus helping to maintain gut homeostasis. Rakoff-Nahoum *et al*. showed that the elimination of the commensal bacteria and TLR signalling by antibiotic treatment *in vivo* increased the vulnerability of mice to DSS-induced disease. Their work indicated that TLR signalling also induced protective and repair responses, and limited pathogenic bacterial growth. Their analysis suggests that this may occur through two possible processes. The first is the maintenance of protective factors, as the commensal bacteria constitutively bound TLRs expressed lumenally by the colonic epithelium. The second mechanism operates when epithelial damage leads to the activation of TLRs by the commensal microbiota and the induction of protective factors [43].

The microbiome also regulates neutrophil trafficking to maintain host resistance. Neutrophils are key cells of the innate immune response for defence against pathogenic bacteria. Gut epithelial cells recruit neutrophils from the vasculature through chemokine signalling. Neutrophils are necessary in controlling infections such as those of *E. coli* serotype K1 [44]. Deshmukh *et al*. in their work with neonatal mice exposed to a three or five-antibiotic protocol followed by inoculation with *E. coli* have shown how microbiota can influence neutrophil activity. Antibiotic exposure reduces the composition and total numbers of the intestinal microbiota in the neonatal mice. For example, *Gammaproteobacteria* were not present on day three, and *Bacilli* on day 14 failed to colonise as normal. When normal intestinal microbiota

was transferred into these antibiotic exposed mice, bone marrow and circulating neutrophils and plasma G-CSF levels increased, and resistance to *E. coli* K1 was partially restored [45].

In studies with humans it has been shown that although the microbiota exhibit resilience[1] to antibiotics, repeated exposure can induce an importunate regime shift which can lower resistance. [14,48]. Repeated courses of antibiotics can also lead to the development of what is termed 'the resistome' – a collection of genes in bacteria that confers resistance to antibiotics [49]. This is possible because of lateral gene transfer between microbes which can be a common phenomenon among some microbial communities. In an investigation by Rasko *et al.* [50], where 17 genomes of both commensal and pathogenic strains of *E.coli* were compared, approximately half the genes were conserved in the group with the rest being components of a large reservoir of genes, known as the pangenome. This ability by microbiota to transfer genes not only contributes to antibiotic resistance but also to pathogenicity and virulence [51]. Lateral gene transfer would also appear to be accelerated by inflammation. In the study by Stecher *et al.* [52] on the transfer between pathogenic and commensal *Enterobacteriaceae* in a colitis mouse model it was noted that gene transfer can occur at unprecedented rates in an infected gut. This microbiota behaviour would contribute to breaches of the barrier function of the gut which is a particular feature of Crohn's disease (CD). The genes involved in this inflammatory process will be discussed later in this review.

The ability of the microbiota to function in this way is possible because of microbe 'mutualism'; as the microbiota sense the fluctuations in their environment, they work co-operatively to promote their survival using a signalling pattern known as quorum sensing. This is where small signalling molecules are secreted and the microbiota are able to sense the molecules' concentrations [53]. When a certain level is reached the whole population responds, co-ordinated by the expression of particular target genes. One of these signalling molecules is acyl-homoserine lactone (acy-HSL), which has been identified in more than 200 species of Proteobacteria. This group includes a large number of pathogenic bacteria. They can use quorum sensing to increase the production of virulence factors i.e. activate a disproportionate number of genes which encode harmful products for the host [54], and overrun host defences

[1] The capacity of a system to absorb disturbance and reorganise while undergoing change so as to retain essentially the same function, structure, identity and feedbacks [46,47].

which can include the gut barrier. This use of quorum sensing has given these pathogenic bacteria the title of ‘cheaters’ as they use the co-operative behaviour without paying for their metabolic production ‘costs’ [55]. Fortunately other mechanisms like kin discrimination, high relatedness and pleiotropy can limit cheaters’ impact [56].

Another facet of the microbiota is biofilms which is where most of the microbiota lives. Biofilms are complex groups of various species that thrive on multiple surfaces [57,58]. One example of a biofilm that impinges on the barrier function of the oral cavity is dental plaque which is associated with tooth decay. Dental plaque contains multiple species and strains of bacteria (as do all biofilms). It is surrounded by a matrix polymer which protects the microbe residents from environmental pressures and host immune defence systems [57]. This makes biofilms very resistant to antimicrobial agents. They have been shown to resist concentrations of these agents which are 1000x higher than what would kill genetically equivalent bacteria which do not reside in biofilms. This makes chronic wounds, where they can also be found, very resistant to treatment [59].

4.2 Trophic Function

As part of its trophic function, the microbiota can control the proliferation and differentiation of epithelial cells and have a role in the development of the immune system. For example, in the gut mucosa, T cells and B cells of the immune system have various functions that are influenced by the composition of the microbiota. These cells play vital roles in the maintenance of immune homeostasis by suppressing responses to harmless antigens and by enforcing the integrity of the barrier functions of the gut mucosa [60]. This enables continuous sampling of the luminal bacteria. Imbalances in the gut microbiota, known as dysbiosis, can trigger several immune disorders through the activity of various T-cells [61].

Commensal bacteria are essential to break food down into nutrients, ensuring constant communication between the luminal bacteria and the intestinal immune cells in homeostasis. If this homeostasis is disrupted for any reason, it will lead to chronic inflammation. Clarification of the mechanisms that distinguish between homeostatic and pathogenic microbiota-host interactions could lead to optimisation of currently available therapies and identify new therapeutic targets for preventing or modulating inflammatory diseases.

5. Effects of genetic background on the microbiome

In 2012, [62] Jostins *et al*. performed a meta-analysis of Genome-Wide Association Studies (GWAS) previously reported on IBD, followed by extensive validation of significant findings, leading to a combined total of more than 75,000 cases and controls. This led to many new loci being identified and many of these have been implicated in immune-mediated disorders. Many of the key loci found by this (and other studies cited therein) shows that they play an essential role in defence of the host against infection.

Two important groups of genes confirmed by this study and implicated in triggering commensal bacteria to shift from a symbiotic to pathogenic relationship with their host were the nucleotide-binding oligomerization domain (*NOD*) genes, *NOD1* and *NOD2* [63]. The proteins produced by these genes, through peptidoglycan recognition, enable detection of intracellular bacteria and promote their clearance through initiation of a pro-inflammatory transcriptional programme and other host defence pathways, including autophagy.

Autophagy has emerged as a crucial defence mechanism against bacteria, but the host intracellular sensors responsible for inducing autophagy in response to bacterial infection were until recently largely unknown. An important gene that associates with microbiota is Autophagy Related 16-Like 1 (*ATG16L1*). In 2010, Travassos *et al*. [64] demonstrated that the intracellular sensors NOD1 and NOD2 act through recruiting the ATG16L1 protein to the plasma membrane at the bacterial entry site. In cells homozygous for the CD-associated NOD2 frameshift mutation, mutant NOD2 failed to recruit ATG16L1 to the plasma membrane and wrapping of invading bacteria by autophagosomes was impaired. These results linked bacterial sensing by NOD proteins to the induction of autophagy, providing a functional link between *NOD2* and *ATG16L1*, which are considered two of the most important genes associated with CD.

Chu and co-workers considered the question of how the human commensal *Bacteroides fragilis* delivers immunomodulatory molecules to immune cells via secretion of outer membrane vesicles (OMVs) [65]. They demonstrated that this requires both *ATG16L1* and *NOD2*, to activate an autophagy pathway during protection from colitis. ATG16L1-deficient dendritic cells do not induce regulatory T cells (Tregs) to suppress mucosal inflammation. Immune cells from human subjects with a major risk variant in *ATG16L1* are defective in Treg responses to

OMVs. They suggested that polymorphisms in susceptibility genes promote immune-related diseases such as IBD through defects in detecting protective signals from the microbiome.

Another of the important groups of genes confirmed by the Jostins study are the TLRs innate immune sensors [62] described earlier. One of the TLRs that has received significant attention is TLR4; gene knockout studies have confirmed that it plays an important role in both intestinal inflammation and microbiota recognition [68]. Increased epithelial TLR4 expression is observed in patients with IBD, for example. To understand the effect of increased TLR4 signalling on intestinal homeostasis, transgenic villin-TLR4 mice that overexpress TLR4 in the intestinal epithelium were used. It was shown that such mice are characterized by increases in the density of mucosa-associated bacteria and bacterial translocation. Furthermore, increased epithelial TLR4 signalling led to an impaired epithelial barrier, altered expression of antimicrobial peptide genes, and altered epithelial cell differentiation. The changes in the microbiota induced by increased epithelial TLR4 signalling are transmissible, and exacerbate dextran sodium sulphate-induced colitis.

6. Gut microbiota mediated inflammation

The mechanism by which inflammation is activated by the gut microbiota is unclear. In this section we highlight some of the latest studies looking at the various processes identified in gut microbiota-mediated inflammation. These include changes in Toll-Like Receptor (TLR) expression and Interleukin (IL) 1β, conflict between host and gut microbiota interests and the metabolizing of tryptophan by the gut microbiota.

The interaction between the gut microbiota and the host is known to be mediated by pattern recognition receptors (PRRs) [69]. Mice treated with antibiotics as a model of intestinal dysbiosis showed increased damage to, and infiltration of inflammatory cells in, the colon and ileum compared to control mice [69]. In addition, antibiotic treatment increased the expression of TLR4, TLR5, and TLR9 in the ileum and TLR3, TLR4, TLR6, TLR7, and TLR8 in the colon, while reducing the expression of TLR2, TLR3, and TLR6 in the ileum and TLR2 and TLR9 in the colon [69]. Recently, TLR5 was studied in relation to gut microbiota-induced inflammation in obesity, a chronic inflammatory disease [70]. The authors observed that women with high expression levels of TLR5 signalling genes in their adipose tissue also had higher TLR5 inflammatory activating, *flagellated Clostridium cluster XIV* abundance when compared to women who had low expression of TLR5 signalling genes in their adipose tissue [70]. These studies suggest the inflammatory response mediated by the gut microbiota is

associated with abnormal expression of PRRs.

IL1β is another well-studied inflammatory signalling molecule in intestinal inflammation [71] and studies have shown that the production of this molecule is triggered by the gut microbiota. A recent study in mice has shown that particular commensal bacteria, specifically *Proteus mirabilis* induce IL1β from monocytes during intestinal injury leading to inflammation [72]. The distinct role of commensal and pathogen bacteria in the gut microbiota has been further complicated by another group recently identified as the pathobiont [73]. These bacteria function symbiotically in the normal gut but negatively affect the host during disease, instigating a pro-inflammatory response [74]. Another recent study has shown that the pathobiont *Helicobacter bilis* can result in mild inflammation in germ free mice that have intestinal injury [75]. However, the mice exhibited severe disease when a microbiota was present during intestinal injury demonstrating synergy between the pathobiont and microbiota in exacerbating pathology [75].

The immune system is capable of tolerating a high microbial density in the colon, but a similar density in the distal gut (defined as small intestine bacterial overgrowth) is associated with illness including diarrhoea and abdominal pain [76]. It is suggested that the severe reaction to the high microbial density in the distal gut is a result of a strong host immune reaction due to direct competition for fat and carbohydrate used up by some bacteria and absorbed mostly by the host in the distal gut but not the colon [77].

Microbiota metabolites are known to regulate immunity and inflammation [78]. Recently, the essential amino acid tryptophan was shown to regulate inflammation via the gut microbiota [79]. One study showed that disease-causing microbiota can be transferred from a knockout mouse to wild type mice through faecal transfer. When the microbiota was analysed, it was shown to lack bacteria capable of catabolizing tryptophan into aryl hydrocarbon receptor (AHR) ligands [80]. In another study, it was shown that a tryptophan-depleted diet resulted in inflammation in the central nervous system which was associated with AHR [81].

The role of the gut microbiota in activating inflammation is complex. In this section we have highlighted some of the ways in which inflammation may be activated by the gut microbiota in different environments.

7. Environmental changes that disturb the human microbiome

The nature of the microbiome community changes through the lifespan (Figure 4) [82].

Insert here Figure 4: Microbiome changes through the Lifespan [82].

Legend: Reprinted from Gastroenterology **2011**, *140*, 1713-1719 Dominguez-Bello, M.G.; Blaser, M.J.; Ley, R.E.; Knight, R. Development of the Human Gastrointestinal Microbiota and Insights from High-Throughput Sequencing., Copyright (2018), with permission from Elsevier

Pregnancy leads to changes in the maternal microbiome just before childbirth (Figure 1). Factors such as peripartum antibiotics and C-sections may disrupt microbial balance, with unintended and adverse effects on new born babies [1]. Miyoshi and co-workers used a rodent model deficient in Interleukin-10 (IL-10), with a study design where confounding factors were stringently controlled to show the effect of peripartum antibiotics. [83]. The commonly used antibiotic, cefoperazone, was administered to both control and genetically susceptible mice during the peripartum period. Their results showed that offspring from dams who had peripartum exposure to this antibiotic developed dysbiosis that continued into adult life.

Microbial colonization of the infant as well as being affected by dietary exposures, exposure to antibiotics and environmental toxicants [84] is also thought to be governed by early immune programming, subsequent infections and risks of developing allergies. As mentioned earlier, feeding practices, especially in relation to breastfeeding duration, appear critical [12]. Studies have established that the delivery method (natural vs. C-section) strongly affect the predominant bacterial species. Vaginally born infants have a higher proportion of beneficial intestinal microbes such as *Bacteroides* and *Bifidobacterium*, while those delivered via C-section had more of the mother's skin and mouth microbes present. The study by Madan *et al.* showed the role of gut microbiota in the production of essential amino acids and vitamins for the growing infant [84]. Duration of breastfeeding also influences the composition of the infant microbiome, with up to one year of breastfeeding appearing to have desirable effects. Glycans of the intestinal mucosa and oligosaccharides of human milk influence the early colonization of the infant gut, and establishment of mucosal homeostasis [85]. The period in which the infant gut needs maximum protection from hypersensitive inflammation overlaps with the recommended period of exclusive breastfeeding. Especially for premature infants, using an

artificial formula that lacks human milk protective glycans may lead to inflammation. The balance of all these factors is shown in Figure One.

7.1 Postpartum effects

Necrotizing enterocolitis (NEC) afflicts approximately 10% of extremely preterm infants leading to a high rate of fatality. Inappropriate bacterial colonization with Enterobacteriaceae has been suggested as an important cause, but no specific pathogen has been identified. Ward and co-workers identified uropathogenic *Escherichia coli* (UPEC) colonization as a significant risk factor for the development of NEC, with subsequently high mortality [86]. They used a large-scale deep shotgun metagenomic sequence analysis to compare the early intestinal microbiome of 144 preterm and 22 term infants. This method enabled them to identify genes associated with NEC and mortality that indicated colonization by UPEC. In addition, they were able to define NEC-associated strains as sequence types often associated with urinary tract infections, including ST69, ST73, ST95, ST127, ST131, and ST144 [86].

7.2 Diet later in life

Once a solid diet is established, the nature of this diet strongly influences the composition of the microbiome. Dietary patterns, such as a Western diet as compared with a more traditional diet, have strong effects on this composition. Graf and co-workers reviewed the accumulating evidence on dietary patterns, whole foods and food constituents as well as food-associated microbes, which all impact on the nature and composition of the gut microbiota [87]. A Mediterranean-style diet has been clearly associated with the acquisition of a beneficial microbiome [88].

There is good evidence that the gut microbiome in humans 65 years and older is quite different from that seen in middle-aged adults. There is also a phenomenon of immune-senescence [89], in which immune function becomes compromised with age; this includes a reduced ability to respond to infections [90]. Ageing is also associated with chronic low-grade inflammation, referred to as inflammaging [89]. Because of the close links between the microbiome and immune function, there is general agreement that there is also an association between age-related microbial and immune changes, however the nature of this relationship is still not defined. It is unclear if the relationship is cause-and-effect (and if so which component is causal) or whether both result from other factor(s). A recent animal study [89] showed increased intestinal inflammation and leakiness when an “aged microbiota” was transferred

into germ-free mice, which the authors conclude indicates that the aged microbiota is a contributory factor to inflammaging. However, in the same study the authors observed that the aged microbiota changed after transfer into the young recipient mice, being more similar to a "young" microbiota after four weeks. This latter observation shows the microbiota adapting to the host, potentially via immune signalling, and reflects the complex, two-way system of communication between the host's immune system and the microbiota. In another animal study, a relationship between microbial dysbiosis, loss of barrier function, and inflammation was reported [91], but again a clear cause-and-effect was not demonstrated; while the microbiome from older rats led to a loss of barrier integrity when transferred into germ-free mice, the microbiome also altered in response to TNF, again indicative of a complex two-way relationship. Similarly, in a recent human study in which corn fibre and a probiotic were consumed (either alone, or in combination), changes were observed in both the microbiota and a range of immune markers [92]. This study suggested that the intervention could be a useful intervention for hypercholesterolaemia, but there was no clear indication of whether changes in microbiota or immunity were primarily responsible.

7.3 Exercise

Although there has been less research into the effects of exercise on the gut microbiota compared with those of diet, a clear picture is emerging of a relationship between the two. Studies in rats and mice have shown that exercise can influence the composition of the microbiome, having a particular effect on the levels of *Bacteroidetes* and *Firmicutes* and the ratio between them [93-95]. Many of these effects appear to occur regardless of diet (for example, occurring in the context of either a normal diet or a high-fat diet), suggesting that the effects of diet and exercise on the microbiota may occur independently [96]. Recent human studies are consistent with this idea. A six week period of endurance exercise in previously sedentary subjects led to changes in the gut microbiota, and in faecal short chain fatty acid levels, with these changes reversing when exercise ceased [97]. In this study the changes were independent of diet, but were dependent on obesity status, with the effects only occurring in lean individuals. In a comparison of professional athletes with size, age, and gender-matched controls, there were significant differences in the gut microbiome, with the athletes having a higher microbial diversity [98]. Studies such as these highlight the complexity of the host-microbiome relationship, with factors such as diet, exercise, and the inherent physiology all potentially influencing the composition and functional characteristics of the microbiome, with varying levels of interdependence.

7.4 Smoking

Several studies have confirmed that cigarette smoking is a risk factor for some chronic diseases e.g. for CD but not Ulcerative Colitis [99-102]. Some studies also show smoking affects the course of CD with an increased involvement of the ileal site, fistulas, stenosis and recurrence, if smoking continues after resection. Immunosuppressive treatments are also more likely to be used as medical treatment with smokers [103-106]. A murine study on the gut showed not only microbial, but also inflammatory and mucin changes, with exposure to smoke [107]. The bacterial community structure and activity were significantly altered in the colon of smoke-exposed mice (n=12) compared to air-exposed mice (n=12) after 24 weeks' exposure. Lachnospiraceae species increased in smoke-exposed mice, as did the mRNA expression of Muc2 and Muc3, along with increased Cxcl2 and decreased Ifn-γ in the ileum, and increased Muc4 in the distal colon. Increased Il-6 and decreased Tgf-β were also observed in the proximal colon.

There have been a number of recent studies investigating the effects of cigarette smoking and the microbiota in different sites on the human body. Wu and associates looked at the oral microbiome in an American adult group (n= 1204) [108]. They reported an overall significant difference in the oral microbiome composition between current and non-smokers. 16S rRNA gene sequencing of microbes collected in oral mouth wash samples showed that Proteobacteria were depleted with lower relative abundance at class, genus and OTU levels. Three genera (*Capnocytophaga*, *Peptostreptococcus* and *Leptotrichia*) were depleted, and two genera (*Atopobium* and *Streptococcus*) were enriched in current smokers compared to non-current smokers. They concluded that alteration of the oral microbiome with smoking could lead to alterations in functional pathways which would affect outcomes in diseases consequent to smoking. Another smaller adult study, assessing oral sites and the nose, showed that cigarette smoking had a significant effect with lower alpha (mean) diversity of the microbiota on the buccal mucosa site [109].

Charlson *et al.* in their study of adult cigarette smokers (29 smokers and 33 non-smoking) on the effect on the microbiome in the upper respiratory tract describe the microbial communities there as 'disordered' [110]. They observed distinct differences in samples from the right and left nasopharynx and oropharynx of healthy asymptomatic adults. 16S rRNA analysis of these samples showed that smokers' microbiota compared to non-smokers were significantly

different in diversity and several genera were altered (in individual components and global structure) by smoking in both areas of the respiratory tract. This was thought to lead respiratory tract complications in the smoking population [110].

These changes in microbiome composition, epithelial mucus profiles and immune factors as a result of chronic cigarette exposure were thought to be the drivers behind this exposure being a risk factor in chronic conditions as IBD [107]. A French review on intestinal microbiota and smoking observed that microbiota changes through a result of smoking were similar to those in people who were obese, with more efficient extraction of calories. This may explain the weight gain observed in people who have given up smoking, suggesting that regulation of microbiota could be a new source of diagnostic and therapeutic approaches [111].

Smoking has also been observed to have an effect on the vaginal microbiome. Women who lacked significant numbers of *Lactobacillus* species (which are thought to provide broad-spectrum protection from infections) in their vaginal microbiota had a 25-fold higher reporting of current smoking when compared with women with microbiota dominated by *L. crispatus* [112]. Further research by this group also showed that smokers had significant differences in 12 metabolites in the vaginal metabolome, particularly the biogenic amines, especially in women with a low-*Lactobacillus* CST-IV. The cadaverine and putrescine biogenic amines are associated with the fishy odours and the bacterial contamination linked with bacterial vaginitis [113].

8. Potential anti-inflammatory therapies mediated through gut microbiota

Recent analysis on the natural disease course of CD during the first 5 years after diagnosis found that the progression of the disease remained unchanged with the use of advanced therapies – hence other therapies need investigating [114].

In this section, we highlight some of the latest gut microbiota-mediated treatments currently being evaluated or considered as potential therapies for diseases with an underlying inflammatory component.

Preterm Neonates are at risk of infectious inflammatory disease such as late onset sepsis (LOS) and NEC which can result in adverse neurodevelopmental issues later in life [115]. Dysregulated immune system in the context of gut dysbiosis is thought to be a major contributing factor in NEC and LOS [115-118]. Currently probiotics have been shown to decrease the risk of LOS and NEC by lowering the risk of inflammation and dysbiosis in

preterm neonates [119]. They are now commonly used in neonatal intensive care units with preterm neonates [120]. More recently, studies suggest lactoferrin (found naturally in breast milk) is a promising treatment for NEC and LOS due to its antimicrobial and anti-inflammatory effects [121]. There are ongoing feeding trials such as the lactoferrin Infant feeding trial (trial registration ID ACTRN12611000247976) and the enteral lactoferrin in Neonates that are evaluating the benefits of bovine lactoferrin [115].

Faecal Microbiota Transplantation (FMT) is an effective treatment of *Clostridium difficile* infection and is acknowledged as a promising treatment of other intestinal disease such as IBD [122]. More recently, there have been trials looking at the impact of FMT on extra-intestinal disorders. In 2012, a double-blind randomized control trial was conducted on patients with metabolic syndrome [123]. The results showed that six weeks of FMT increased insulin sensitivity compared to the control group [123]. Case studies have shown reduction of symptoms or complete treatment for patients suffering from Parkinson's disease [124], multiple sclerosis [125] and myoclonic dystonia [126]. Studies show that there is an association between the gut microbiota and cancer, tumour development [122] and in particular colorectal cancer (CRC) (reviewed in [127]) suggesting that FMT should be considered as a potential therapeutic.

Probiotic formulations have already been shown to have a beneficial effect in relation to CRC [127]. In CRC patients, bile acids and cholesterol are converted into microbial products too quickly in the colon which leads to generation of harmful enzymes [128]. These harmful products can be reduced by probiotic formulations that contain *Bifidobacterium* or *Lactobacillus* through decreasing the dehydroxylation of bile acids and reducing faecal deoxycholic acid concentrations [129]. One study assessed the link between CRC and probiotics in humans [130]. The study correlated yoghurt consumption and CRC risk in over 4,000 healthy people, with yoghurt intake being directly related to lower CRC risk [130]. This suggests the use of probiotics as an effective potential therapy for CRC in combination with more traditional treatment such as surgery and chemotherapy.

Another study The "RISTOMED project" using a randomized controlled trial tested the impact of a personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota on community dwelling healthy adults (n=26, 65-85years) [131]. Blood and stool samples were collected on days 1 and 56. The biomarker of inflammation used was high-sensitivity C-reactive protein (hsCRP). The adults were given an eight week web-

based dietary advice ('RISTOMED' platform, optimized to reduce inflammation and oxidative stress) alone, or with probiotic supplementation (VSL#3, 2 capsules per day). Diet alone significantly reduced Erythrocyte Sedimentation Rate (ESR) as well as plasma levels of cholesterol and glucose. Addition of the probiotic further reduced ESR and improved folate, vitamin B12 and homocysteine plasma levels. Neither intervention demonstrated any further effects on inflammation. However, this study points to the health inducing role probiotics can play in conjunction with an optimised diet.

The gut microbiota has also been shown to play a role in successful bone barrow transplantation (BMT) [132]. The success of BMT is limited by graft-versus-host disease (GVHD) that results in activation of host immune and inflammatory responses against the donor cells [133]. In a study using a mouse model of BMT, mice that underwent BMT but exhibited no GVHD were characterised by a lower loss of overall diversity of the gut microbiota following antibiotic treatment, compared to mice that exhibited GVHD [132]. Additionally, eliminating *Lactobacillus* from the gut microbiota of mice before BMT aggravated GVHD, whereas reintroducing the predominant *Lactobacillus* species mediated significant protection against GVHD. The authors found similar patterns when they characterized the gut microbiota of patients during onset of intestinal inflammation caused by GVHD [132]. The authors suggested that manipulation of the gut microbiota can improve success of BMT.

Other possible therapeutic targets are those genes involved in vitamin D, selenium (Se) and long chain omega-3 polyunsaturated fatty acid (n3-PUFA) metabolism. It has been well established that these three nutrients have effects on inflammation through key metabolic pathways [134-139].

The vitamin D receptor (*VDR*) has a large impact on immune regulation. There have been over 2000 binding sites identified for *VDR*, which has also been shown to be involved in the transcription of more than 913 genes [140-142]. Through its metabolites, vitamin D also supports the anti-inflammatory pathways which enable the tight junctions between the intestinal epithelial cells [143,144].

Studies are also beginning to clarify the role of Vitamin D and *VDR* on the microbiota. In one murine study comparing caecal and faecal samples from VDR knockout and wildtype mice

there were differences in the microbiota composition as the gut contents moved through the intestine, potentially implicating the loss of VDR with dysbiosis [145]. A further study on VDR knock out mice by Chen *et al.* proposed that vitamin D supplementation could help deter dysbiosis through reducing Innate Lymphoid Cell (ILC3) numbers and thus regulating the pathogenic microbiota [146]. Pattern recognition receptors like *NOD2* which can promote antimicrobial production are also regulated by vitamin D [147-151]. Genes associated with the microbial pattern recognition receptors cluster of differentiation 14 (*CD14*) and *TLR2* are also stimulated by vitamin D in wound healing [149,152]. Macrophages and dendritic cells also use Vitamin D to stimulate antimicrobial peptides like B-defensin and cathelicidin [153,154]. These studies suggest vitamin D supplementation can be used as a therapy to target anti-inflammatory genes and metabolic pathways which impact on the microbiota. A recent exploratory study with Vitamin D supplementation in cystic fibrosis and its effects on airway microbiota is consistent with this idea [155].

Chronic inflammatory diseases such as cancer, IBD and heart disease have also been shown to be affected by modest Se deficiencies. The effect of this is to decrease the activity and expression of selenoproteins. Se is a trace element which is involved in epigenetic modulation, cell cycle arrest, and initiation of reactive oxygen species [156].

A study by Kasaikina *et al.* examined the intestinal microbiota composition in wild type and germ free mice on various diets (Se-deficient, -sufficient and -enriched) and found that sufficient Se increased the diversity of the microbiota and the colonisation of the gut. Se status of the mice was independent of other trace mineral levels [157]. In another study investigating the emerging approach of using nanoparticles to counteract pathogens, Se (0.9 mg/kg) was introduced into chicken feed in this manner and was found to increase beneficial bacteria such as *Lactobacillus* and *Faecalibacterium*, as well as SCFAs, chiefly butyric acid [158]. This latter study is in contrast to an earlier study which found no effect of Se delivered through yeast into chicken feed [159]. This implies that the method of delivery used is important and nanoparticles maybe a more effective delivery system. Low Se levels have been associated with CD in a New Zealand cohort [139]. This important trace element needs to be considered more when looking for anti-inflammatory vectors that increase the beneficial bacterial composition of the microbiota in the future.

Similar findings with respect to the impact of n3-PUFA supplementation on the microbiota are also beginning to emerge (as reviewed by Costantini and colleagues [160]). In one randomised

control study two forms (a capsule and a functional drink) of n3-PUFA supplementation were given to human adults. Both resulted in an increase in the abundance of butyrate-producing bacterial genera, with the functional drink having a greater effect [161]. This again illustrates that the mode of delivery can alter the microbiota response. In another cross-sectional study using a dual-omics approach to elucidate the gut microbiome-metabolome axis, people with IBD were compared to a control group. Those with IBD showed an increase in *Escherichia, Faecalibacterium*, and *Streptococcus* genera, and a decrease in *Bacteroides*, *Flavobacterium*, and *Oscillospira* genera [162]. It has been hypothesised that n3-PUFA supplementation for those in this study could be a means to normalise the microbiota in IBD people resulting in improved pathology. The n3-PUFAs may have their effect on the microbiota by increasing SCFA and thus inducing an anti-inflammatory response [160].

In future studies, the appropriate levels and method of delivery of vitamin D, Se, and n3-PUFAs will be assessed to maximise their beneficial effect on the microbiota, and consequently their anti-inflammatory effects in chronic inflammatory conditions.

9. Expert Commentary and Five-Year View

This review provides an overview of the increasing importance associated with the microbiome, not only in the gastro-intestinal tract, but in other body parts. It also identifies stages in the life cycle at which the human microbiome can be influenced, and the ways in which these happen.

More international collaborations such as the International IBD Genetics consortium will enhance our understandings of the importance, not only of human genes *per se*, but also their interplay with the microbiome in a range of inflammatory conditions.

The future will see the growth of more effective therapies targeting the microbiome, particularly with respect to the use of specific nutrients and diets personalised to the individual as described in this review. A recent review on CD showed the natural disease course remained unchanged with the use of current advanced therapies – hence other therapies need investigating. This means that personalised medicine and precision medicine will come of age.

Declarations of Interest:

Competing Interests

The authors declare they have no competing interests

Authors' contributions

BL, MB, GM, NAN and LF had roles in concept and design. All contributed to the manuscript. All authors read and approved the final manuscript.

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