



Troublesome friends within us: the role of gut microbiota on rheumatoid arthritis etiopathogenesis and its clinical and therapeutic relevance

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Abstract

The role of gut microbiota on immune regulation and the development of autoimmune diseases such as rheumatoid arthritis (RA) is an emerging research topic. Multiple studies have demonstrated alterations on gut microbiota composition and/or function (referred to as dysbiosis) both in early and established RA patients. Still, research delineating the molecular mechanisms by which gut microorganisms induce the loss of immune tolerance or contribute to disease progression is scarce. Available data indicate that gut microbiota alterations are involved in RA autoimmune response by several mechanisms including the post-translational modification of host proteins, molecular mimicry between bacterial and host epitopes, activation of immune system and polarization toward inflammatory phenotypes, as well as induction of intestinal permeability. Therefore, in this review we analyze recent clinical and molecular evidence linking gut microbiota with the etiopathogenesis of RA. The potential of the gut microbiota as a diagnostic or severity biomarker is discussed, as well as the opportunity areas for the development of complementary therapeutic strategies based on the modulation of gut microbiota in the rheumatic patient.

Keywords Rheumatoid arthritis · Autoimmunity · Microbiome · Gut dysbiosis · Probiotics · Fecal microbiota transplant

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic pain, joint inflammation, autoantibodies production and progressive disability [1]. This condition is associated with multiple comorbidities including cardiovascular disease (CVD), depression, infections, osteoporosis, cancer, among others [2]. The etiology of RA remains incompletely understood, but there is consensus that a complex interaction between genetic and environmental factors is involved on its development [3]. Genetic factors are estimated to contribute with up to 50% of RA risk.

Among these, the allelic variants in the *HLA-DRB1* gene that codes for the DRB1 chain of the human leukocyte antigen (HLA) molecule, with a common amino acid sequence known as the shared epitope (SE), are the strongest risk factor [1, 4], followed by multiple low-magnitude risk variants mostly on immunoregulatory genes located outside the HLA-region, including *PTPN22*, *PADI4*, *STAT4*, *TNFAIP3*, *CTLA4*, among others [4]. The remaining RA risk is attributed to environmental and lifestyle-related factors such as smoking, silica exposure, diet, infections and the oral, lung and intestinal mucosa-associated microbiota [3, 5].

Microbe infections have been associated with RA for more than a century, although the etiology of this condition could never be confirmed by the single presence of an infectious agent [6]. Among the microorganisms commonly associated with RA are bacteria such as *Porphyromonas gingivalis*, *Proteus mirabilis*, *Streptococcus pyogenes* and *Mycobacterium*, as well as Epstein-Barr virus and cytomegalovirus infection [7, 8]. In the last years, there has been a renewed interest in the study of microbes as implicated agents on the etiopathogenesis of autoimmune diseases, partly, due to the development and widespread use of techniques for massive DNA sequencing that allow the

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characterization of trillions of microorganisms, that is the microbiota, that resides within the human body in conditions of health and disease.

The mucosa-associated microbiota of the oral cavity, the lungs and the gastrointestinal tract is in close relationship with the immune system and mounting evidence suggests that the high load of microbial antigens to which these mucosal sites are constantly exposed to may lead to the loss of immune tolerance and the development of RA in genetically predisposed individuals [3, 5]. Intestinal dysbiosis, that is alterations on the composition and/or function of the gut microbiota, has been extensively demonstrated in early [9–11] and established RA patients [12, 13]. Still, data delineating the molecular mechanisms by which microorganisms can induce autoimmunity are scarce and constitute an emerging research topic. Therefore, in this article we provide an overview of the current clinical and molecular evidence linking the gut microbiota with RA etiopathogenesis. The potential of gut microbiota as a diagnostic or severity biomarker is presented, and finally, we discuss the opportunity areas for the development of complementary therapeutic approaches based on the modulation of gut microbiota in the rheumatic patient.

Search methodology

For this narrative review, original and review articles published until March 2020 were searched in the NCBI PubMed database using the terms rheumatoid arthritis OR arthritis AND one of the following terms: gut microbiota OR microbiome OR probiotics OR intestinal flora OR a specific bacterium. Studies of gut microbiota characterization by metagenomics in RA patients were systematically analyzed and are resumed in Table 1. The reference lists of some articles were considered for the identification of papers of interest. Additional information presented in the review is based on meta-analyses or systematic reviews; in the absence of such studies we searched for experimental studies, clinical trials or epidemiological studies.

Gut microbiota and immune response: ¿from the gut to the joints?

The gastrointestinal tract is an essential constituent of our immune system, as it hosts the greatest density and diversity of microorganisms of our body. Its major functions include the nutrient transport from the luminal space into the circulation and providing a selective barrier that prevents translocation of pathogenic bacteria toward internal tissues [14]. The intestinal barrier is composed by a monolayer of cells with predominantly absorptive functions, distributed

from the small intestine (enterocytes) to the large intestine (colonocytes), as well as specialized subsets of cells, including Goblet cells, M cells, zymogenic cells, enteroendocrine cells and Paneth cells [15]. At the submucosa of digestive tract, there is the gut-associated lymphoid tissue (GALT), which is formed by organized cell structures (such as the Peyer patches, intestinal lymphoid follicles and the mesenteric lymph nodes), as well as individual cells interspersed through the gut, including intestinal intraepithelial lymphocytes (IELs), macrophages, dendritic cells (DCs), natural killer (NK) cells, polymorphonuclear (PMN) leukocytes and innate lymphoid cells (ILCs) [15, 16]. The GALT is a key modulator of the immune response; capable of mounting effector responses against intestinal pathogens, while keeping immune tolerance to antigens from commensal bacteria, food and self-proteins [14, 17].

The gut microbiota encompasses trillions of bacteria, archaea, fungi, protozoa and viruses that establish a predominantly symbiotic relationship with the host [15]. It varies among individuals, as it is affected by several factors including genetics, nutrition, medication, age, environmental exposures, among others [18, 19]. Despite this, it has been documented that an adult ‘normal’ gut microbiota is predominantly colonized by bacteria belonging to the Firmicutes and Bacteroidetes phyla, while bacteria from the Proteobacteria, Actinobacteria, Verrucomicrobia and Fusobacteria phyla are found in lower proportion [18]. The genes of the microbial community (known as the microbiome) encode more than eight million bacterial proteins that work as an extension of our own genome [20], by performing hundreds of metabolic, neuro-endocrine and immunologic functions.

The gut microbiota is essential for the appropriate development of the immune system. Axenic mice (germ-free) or gnotobiotic mice (colonized with defined bacterial species) exhibit marked immune deficiencies such as lower number of intestinal Peyer patches and germinal centers, reduced numbers of intestinal CD4⁺ T lymphocytes and IgA secretory plasmatic cells [21–23]. Importantly, these altered immune responses do not only affect the GALT, but also systemic responses as evidenced by quantitative and functional impairments on circulating T helper 17 (Th17) cells and regulatory T cell subsets (Tregs) [22, 24, 25]. The gut-associated microbiota regulates the GALT directly, through signaling of pattern recognition receptors (PRRs) expressed on immune cells that recognize conserved motifs on bacteria (known as pathogen-associated molecular pattern, PAMPs); and indirectly, through secretion of hundreds of metabolites that act as ligands on host receptors thereby modulating intestinal- and extra-intestinal cell physiology [22, 26]. As an example, short chain fatty acids (SCFA) are metabolites produced by the microbial fermentation of dietary fiber that exert potent immunomodulatory roles through its binding

Table 1 Metagenomic studies of gut microbiota composition in RA patients

References	Country	Study groups (n)	Technique	Main findings
Scher et al. [9]	United States	Early RA-UTX ^a (n = 44) Established-RA ^b (n = 26) PsA (n = 16) Healthy controls (n = 28)	16S rRNA gene sequencing and metagenomics	In early RA-UTX versus healthy controls: ↑ <i>Prevotella copri</i> ↓ <i>Bacteroides</i> , Clostridia and Lachnospiraceae In established-RA versus healthy controls: ↔ Composition and abundance of gut microbiota
Zhang et al. [13]	China	RA-UTX (n = 77) Healthy controls (n = 80) RA-DMARDs (n = 21) Paired subgroups: RA-UTX (n = 17) Healthy relatives (n = 17)	Metagenomics	In RA-UTX versus healthy controls: ↑ <i>Clostridium asparagiforme</i> , <i>Gordonibacter pamelaeeae</i> , <i>Eggerthella lenta</i> , <i>Lachnospiraceae bacterium</i> , <i>Bifidobacterium dentium</i> , <i>Lactobacillus</i> sp., <i>Ruminococcus lactaris</i> ↓ <i>Veillonella</i> , <i>Haemophilus</i> , <i>Klebsiella pneumoniae</i> , <i>Bifidobacterium bifidum</i> , <i>Sutterella wadsworthensis</i> , <i>Megamonas hypermegale</i> ↔ Bacterial composition according to RA duration DMARD treatment partially reestablished dysbiosis
Chen et al. [12]	United States	Established-RA ^b (n = 40) Control subjects ^c (n = 32)	16S rRNA gene sequencing	In established-RA versus control subjects: ↑ <i>Collinsella</i> , <i>Actinobacteria</i> and genus <i>Eggerthella</i> , <i>Actinomyces</i> ↓ Microbial diversity and <i>Faecalibacterium</i>
Breban et al. [44]	France	Initial cohort: Established-RA ^b (n = 17) ASp (n = 49) Healthy controls (n = 18) Confirmatory cohort: Early RA-UTX (n = 11) ASp (n = 38) Healthy controls (n = 51)	16S rRNA gene sequencing	In established-RA versus healthy controls: ↑ Phylum Tenericutes, Synergistetes and Proteobacteria, including <i>Desulfovibrionaceae</i> , <i>Succinivibrionaceae</i> and <i>Enterobacteriaceae</i> and genus <i>Klebsiella</i> ↓ α-diversity, <i>Prevotellaceae</i> , <i>Paraprevotellaceae</i> and <i>Bifidobacteriaceae</i> In early RA-UTX versus controls and ASp: ↑ <i>Enterobacteriaceae</i> , <i>Lactobacillus</i> species, <i>Corynebacterium variabile</i> , <i>Staphylococcus aureus</i> , <i>Facklamia</i> and <i>Paraprevotallaceae</i> ↓ <i>Ruminococcaceae</i> and <i>Bacteroidaceae</i>
Maeda et al. [10]	Japan	Early RA-UTX ^d (n = 17) Healthy controls (n = 14)	16S rRNA gene sequencing	In early RA-UTX versus healthy controls: ↑ <i>Prevotella</i> in 6 patients (79% of OTUS corresponded to <i>P. copri</i>)
Picchianti-Diamanti et al. [47]	Italy	RA-UTX ^e (n = 11) RA-MTX (n = 11) RA-ETN (n = 10) RA-ETN + MTX (n = 10) Healthy controls (n = 10)	16S rRNA gene sequencing	In RA-UTX versus healthy controls: ↔ α-diversity and β-diversity ↑ Bacilli and Lactobacillales ↓ <i>Faecalibacterium</i> , <i>Faecalibacterium prausnitzii</i> , <i>Flavobacterium</i> and <i>Blautia coccoides</i> In RA-ETN versus RA-UTX: ↑ <i>Cyanobacteria</i> ↓ Class Deltaproteobacteria and family Clostridiaceae In RA-MTX versus RA-UTX: ↓ Enterobacteriales
Alpizar-Rodriguez et al. [11]	Switzerland	Preclinical RA ^f (n = 83) FDR controls ^g (n = 50)	16S rRNA gene sequencing	In preclinical RA versus controls: ↔ α-diversity and β-diversity ↑ Prevotellaceae and associated genus (<i>Prevotella</i> spp., <i>P. copri</i>) ↑ Lactobacillaceae

Table 1 (continued)

References	Country	Study groups (n)	Technique	Main findings
Chiang et al. [45]	China	Established-RA ^b (n = 138) Healthy controls (n = 21)	16S rRNA gene sequencing	↓ α -diversity in RF negative patients versus controls Differences in β -diversity among RA versus controls Differences in β -diversity among seropositive versus seronegative RA ↑ Verrucomicrobiae and <i>Akkermansia muciniphila</i> in RA versus controls ↑ Gammaproteobacteria in RA-high versus RA-low (cytokines TNF- α e IL-17A) ↑ <i>Collinsella</i> and <i>Akkermansia</i> in active-RA versus inactive-RA
Sun et al. [86]	China	Established-RA ^b (n = 66) Healthy controls (n = 60)	16S rRNA gene sequencing	↓ α -diversity and β -diversity in RA versus controls ↑ <i>Bacteroides</i> and <i>Escherichia-Shigella</i> in RA versus controls ↓ <i>Lactobacillus</i> , <i>Alloprevotella</i> , <i>Enterobacter</i> and <i>Odoribacter</i> in RA versus controls
Kishikawa et al. [43]	Japan	RA ^h (n = 82) Healthy controls (n = 42)	Metagenomics	In RA versus controls: ↔ α -diversity and β -diversity ↑ Genus <i>Prevotella</i> (<i>P. denticola</i> , <i>P. marshallii</i> , <i>P. disiens</i> , <i>P. corporis</i>) ↑ <i>Porphyromonas somerae</i> , <i>Gardnerella vaginalis</i> , <i>Bacteroides sartorii</i> ↓ Gene ID: R6FCZ7 (redox function, belonging to <i>Bacteroides</i> genus) ↑ Biological pathways involved on metabolism Relationship between the bacterial metagenome and the human genome

Symbols: ↓ significant reduction; ↑ significant increase; ↔ no significant change

ASp ankylosing spondylitis, *DMARDs* disease-modifying anti-rheumatic drugs, *ETN* etanercept, *FDR* first degree relatives, *MTX* methotrexate, *PsA* psoriatic arthritis, *RA* rheumatoid arthritis, *RF* rheumatoid factor, *UTX* untreated patients

^aDefined as > 6 weeks and < 6 months of diagnosis, without treatment (DMARDs, biologics or glucocorticoids)

^bDefined as > 6 months of treatment under therapy with DMARDs, biologics and/or glucocorticoids

^cIncluding RA first-degree relatives (n = 15) and healthy controls, paired by age and gender (n = 17)

^dDefined as > 6 weeks and < 2 years from diagnostic, without treatment with DMARDs, biologics or steroids

^eMean disease duration of 6.4 years

^fEULAR criteria: systemic autoimmunity (anti-CCP⁺ or RF⁺) and/or signs and symptoms associated with possible RA

^gFirst-degree relatives of RA patients, without autoantibodies and RA symptoms

^h73% had RA duration of < 1 year; 71% were untreated patients, the remaining were under DMARD therapy

and activation of membrane G-protein-coupled receptors (e.g., GPR41, GPR43 and Olfr78), as well as the activation of histone acetyltransferases and deacetylases or transcription factors that regulate lymphocyte gene expression [26, 27]. SCFAs modulate cytokine production, the development of Treg cells, stimulate production of antimicrobial peptides, promote intestinal integrity, and in general, are recognized to induce tolerogenic and anti-inflammatory immune responses, but under certain circumstances, they can induce proinflammatory responses [27–29]. As an

example, butyrate was shown to induce IL-23 secretion by dendritic cells [30] thereby inducing Th17 cell differentiation, a subtype involved on RA pathogenesis.

Immune system interaction with the gut microbiota is bidirectional and highly dynamic (reviewed in [17]); for instance, mice lacking MyD88 (a protein involved on the innate immune system sensing of microorganisms) exhibit alterations on the composition and function of their gut microbiota [31], evidencing that quantitative and functional changes on the innate or adaptive immune system may favor

gut dysbiosis. As a result, it remains a challenge to determine if the intestinal dysbiosis frequently detected in RA patients is indeed a causal factor linked to the development of the disease, or rather a consequence of the plethora of immune alterations associated with RA.

Gut microbiota in the etiopathogenesis of RA

Epidemiologic evidence

The relationship between individual infectious agents and the development of RA was noted more than a century ago [6, 32] but until recent years, dysbiosis of mucosal sites is acknowledged as an important element on the model of RA etiopathogenesis [5, 22, 33]. The hygiene hypothesis (a term coined in the early 2000s) was significant in this matter, since it provided epidemiologic evidence of an inverse relationship between the frequency of infections and the frequency of allergic and autoimmune diseases at the population level [34, 35].

At present, there is robust epidemiologic and molecular evidence confirming the role of gut microbiota in the development and regulation of the immune system, and, it has even been suggested that the rise in incidence of immune-mediated diseases observed in the last decades may be due, in part, to microbial deprivation prevalent in the western world [36]. The gastrointestinal tract is essential for the maintenance of homeostatic immune responses and autoimmunity prevention by the generation of key T cell populations such as inducible regulatory T cells (iTregs) and IL17-producing T cells (Th17) [17]. Such precise balance appears to be mostly mediated by the synergistic effects of multiple bacterial strains rather than by single species [32].

Some factors associated with RA etiology such as polymorphisms in *HLA* gene, hormones, age, smoking and stress, all have demonstrated effects on gut microbiota composition [18, 37–40]. Furthermore, some drugs used for RA treatment have antimicrobial properties [41] and it is possible that its therapeutic effects may be partially mediated by modulation of the microbiota. Diet and obesity are associated with substantial modifications of the gastrointestinal bacteria, and, remarkably, both factors are associated with RA risk [19, 42].

Clinical evidence: gut dysbiosis in RA patients

Intestinal dysbiosis, that is, alterations in the composition and/or function of the gut microbiota, is a phenomenon associated with multiple intestinal and extra-intestinal diseases [18, 22]. A surge of metagenomic studies of gut microbiota characterization in RA have demonstrated that most patients

exhibit microbial alterations, such as loss of diversity, altered functional profile or proliferation of specific bacteria mainly at the genus and species level (see Table 1). It should be noted that at present, a signature RA-associated microbial profile has been difficult to prove, probably by differences in the studies such as: (a) the techniques for gut microbiota analysis (e.g., qPCR, 16S rRNA gene sequencing or shotgun sequencing); (b) limitations on the sample size; (c) cohort characteristics (e.g., disease duration, genetic background, disease activity or therapeutic schemes) and (d) environmental exposures and lifestyle-related factors (e.g., geographic location, diet, smoking, physical activity level, among others).

A frequent finding in early RA patients is a significant increase of *Prevotella* genus [43] and *P. copri* species [9, 10, 44] in comparison to healthy controls (Table 1). This bacterium was also found augmented in preclinical stages of RA (i.e., with systemic autoimmunity and/or possible symptoms of RA) [11] but still, no longitudinal studies have been performed to evaluate the contribution of *P. copri* or *Prevotella* spp. to autoantibody production or to assess its role on the transition from preclinical to clinical RA stages. In addition, *P. copri* expansion was not replicated in two large metagenomic studies performed in Chinese RA patients [13, 45] which suggests these findings cannot be generalized.

Another common finding in RA is the proliferation of *Bacillus* and *Lactobacillus*. In early RA, an increase of *Lactobacillus* sp. was reported in fecal samples [46], while in untreated established RA patients, a significant expansion of Bacilli class and Lactobacillales order was found [47]. Similarly, a study by Zhang et al. [13] detected an increase of *Lactobacillus salivarius* in both oral and intestinal RA samples and its abundance was related to disease severity. Paradoxically, collagen induced arthritis (CIA) severity in mice was attenuated by oral administration of *L. salivarius* by decreasing bone erosions and synovial infiltration and augmenting both Treg cells and circulating IL-10 levels [48], whereas an enrichment of *Lactobacillus* genus was reported in CIA susceptible DBA/1 mice before arthritis development [49]. Therefore, the role of these bacteria in RA should be cautiously interpreted since this commensal genus is widely distributed on mucosal epithelium and some of its species are frequently administered as probiotics. In fact, *L. casei* and *L. acidophilus* supplementation in RA patients has shown to decrease inflammatory markers and disease activity [50, 51], while other species such as *L. rhamnosus* and *L. reuteri* did not had significant effects on clinical improvement in active RA patients [52, 53], this highlights the previous observation that physiological effects of probiotics are specific to each bacterial species [47, 54].

In summary, although studies performed to date are somewhat heterogeneous, they all demonstrate alterations in gut microbiota of RA patients both in early and established

disease stages. Gut dysbiosis appears to play a key role on the initial stages of RA development [55] although longitudinal studies, especially in preclinical phases of the disease are still necessary to identify early changes of gut microbiota and to explore the mechanisms by which these contribute to RA development.

Molecular evidences

Several mechanisms by which infections or bacterial imbalances at mucosal sites can favor autoimmunity or RA progression have been proposed; in regards to gut dysbiosis these include: (a) autoantigen modification; (b) molecular mimicry between microbial and host epitopes; (c) immune system activation and polarization toward proinflammatory phenotypes; (d) induction of intestinal permeability (Fig. 1).

Post-translational modification (PTM) of self-proteins, in particular citrullination (the conversion of arginine to citrulline on a peptide chain) is a key process on RA pathogenesis. Production of antibodies against citrullinated peptides or proteins (ACPAs) can occur in preclinical stages (before clinical signs or symptoms) in a subset of patients [1, 5]. This modification is mediated by peptidylarginine deiminases (PADs) enzymes during physiological processes such as cell differentiation, apoptosis, gene expression and the formation of neutrophil extracellular traps (NETs) [56]. However, under certain circumstances, increased citrullination or “hypercitrullination” of proteins may occur and promote ACPAs production by generating multiple neo-epitopes for which T and B cell tolerance was not guaranteed [5, 56, 57]. Protein hypercitrullination may occur by over-activation of PAD enzymes, after cell membrane damage, necrosis or NETosis [56] and interestingly, those processes are boosted by environmental factors associated with RA, including smoking and specific mucosa-associated bacteria.

In the oral cavity, two citrullination-promoting bacteria were identified; *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. The first one expresses a prokaryotic enzyme with similar catalytic function to human PADs, named *Porphyromonas* PAD or PPAD, that is able to citrullinate microbial and host antigens including fibrinogen and α -enolase (both frequently targeted by ACPAs) [58]. It has been hypothesized that protein citrullination by PPAD might evoke an immune response that is cross-reactive with self-peptides, leading to loss of immune tolerance and ACPA production [59]. However, this molecular mimicry mechanism for *P. gingivalis* is still subject to debate, as human PADs citrullinate arginine residues within polypeptide chains, while bacterial PPAD preferentially modifies C-terminal arginines [58, 60]. On the other hand, the Gram-negative *A. actinomycetemcomitans*, generates hypercitrullination of host proteins by production of leucotoxin A (LtxA), a bacterial toxin that forms pores on the neutrophil

membrane and induces a calcium-dependent overactivation of intracellular PADs [61]. Therefore, generation of citrullinated antigens by oral bacteria could favor the production of ACPA antibodies [5, 61, 62]. Furthermore, as both microorganisms are linked to periodontal disease, such findings partly explain the epidemiological associations between RA and periodontitis [58].

It is conceivable that citrullination of neo-epitopes by *P. gingivalis* and *A. actinomycetemcomitans* is not only restricted to oral mucosa, but also may occur in distant tissues by translocation of either viable bacteria or its cellular components. Indeed, the entry of oral bacteria to systemic circulation, liver or spleen, has been demonstrated during common dental procedures [63]. Another study revealed that *P. gingivalis* can survive intracellularly in macrophages and DCs; therefore, immune phagocytic cells may act as “trojan horse” spreading the bacteria away from the initial infection site [64–66]. Moreover, detection of DNA from *P. gingivalis* and *P. intermedia* was reported in synovial fluid of RA patients with periodontitis [67, 68]. The mechanisms mediating the entry of bacterial DNA to RA joints are still unclear; however, future studies should demonstrate if bacteria-induced citrullination of proteins can occur at sites other than periodontium. Another remarkable finding that links *P. gingivalis* to RA pathogenesis is that, when orally administered to murine models of arthritis, it increases intestinal permeability and induces significant changes on gut microbiota composition and diversity [69, 70]. These findings highlight the close relationship between oral and gut microbiota and its effects on intestinal and systemic immune responses.

In a recent study, more than 200 citrullinated peptides were identified in colon biopsies from RA patients and healthy subjects [71]. Although this imply that protein citrullination is a general phenomenon in the gastrointestinal tract, it is possible that citrullination in the gut favor the loss of tolerance and RA development in genetically predisposed individuals; while in those who already have RA, it may promote disease severity by amplifying ACPAs production at the intestine. In this context, recent hypotheses highlight that ACPA antibodies are generated in mucosal sites in early stages of the disease [3, 5] based on several findings such as higher frequency of circulating plasmablasts and IgA ACPAs in subjects at risk of developing RA (positive to RF or ACPAs autoantibodies, in the absence of clinical symptoms) in comparison to controls [72]. ACPAs can promote RA pathogenesis by several mechanisms including immune complexes formation and induction of inflammatory responses on innate immune cells after its recognizing by Fc receptors [73], as well as induction of bone erosion by promoting osteoclastogenesis [74].

Moreover, the gut-associated microbiota likely generates other PTMs of the host proteome that can lead to production

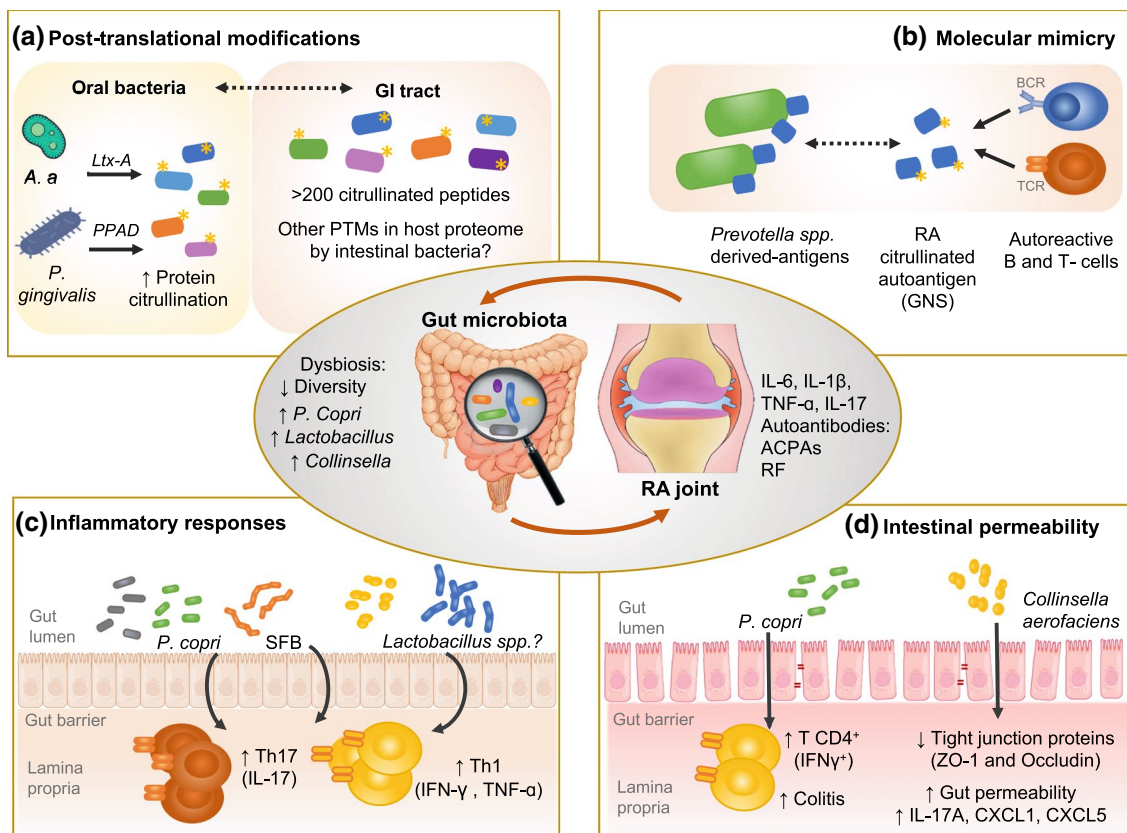


Fig. 1 Molecular mechanisms by which altered-gut microbiota contributes to rheumatoid arthritis (RA) etiopathogenesis. The gut microbiota of RA patients is characterized by reduced diversity and proliferation of particular bacteria. These alterations may impact systemic immune responses and RA by several mechanisms such as: **a** Post-translational modification (PTM) of host proteins; oral bacteria such as *Aggregatibacter actinomycetemcomitans* (*A.a*) and *Porphyromonas gingivalis* augment protein citrullination by leucotoxin-A (Ltx-A) production and *Porphyromonas*-PAD (PPAD) enzyme activity, respectively. More than 200 citrullinated peptides are detected in colon and it is possible that some of these and other PTMs in host proteome are mediated by intestinal bacteria. Arrow indicates the dynamic relationship between gut and oral microbiomes. **b** Molecu-

lar mimicry; the intestinal genus *Prevotella* spp. has antigens that are structurally similar to an RA-citrullinated autoantigen, *N*-acetylglucosamine-6-sulfatase (GNS), which activates T and B cell responses in about 50% of RA patients. **c** Inflammatory responses; in experimental arthritis, *P. copri*, segmented filamentous bacteria (SFB) and *Lactobacillus* species have demonstrated effects on CD4⁺ T cells, specifically by increasing the numbers of IL-17⁺ Th17 cells and activating Th1 cell responses. **d** Intestinal permeability; in RA murine models, *C. aerofaciens* treatment was shown to promote intestinal permeability by increasing inflammatory mediators and chemokines (IL-17A, CXCL1, CXCL5) and reducing the expression of tight junction proteins (ZO-1 and occludin), whereas *P. copri* colonization increased CD4⁺ (IFN γ ⁺) T cell numbers and induced colitis in mice

of other autoantibody systems in RA; for instance, anti-acetylated protein antibodies (anti-APAs) were recently described in approximately 35% of early RA patients and displayed a specificity from 65 to 85% [75]. Acetylation is a reversible enzymatic process that consists on the addition of acetyl groups to free amines of lysine residues. Bioinformatic studies show that most bacteria carry genes predicted to encode lysine acetyltransferases and lysine deacetylases [76]. The microbiome was shown to affect host acetylation patterns in mice [77] and strikingly, certain dietary components can modulate lysine acetylation levels in vivo [78]. Although the precise mechanisms that lead to production of anti-APAs in RA and its biologic and clinical relevance remains to be elucidated, these studies provide an interesting

link between diet, gut microbiota and protein acetylation as a target for antibody response in RA.

One of the mechanisms by which the RA-associated bacteria *P. copri* may contribute to RA etiopathogenesis is by induction of proinflammatory immune responses [10, 79]. Germ-free SKG mice (a genetic model of autoimmune arthritis) inoculated with fecal samples from RA patients enriched with *P. copri*, showed increased numbers of intestinal Th17 cells and developed severe arthritis after exposure to the fungal particle zymosan [10]. While in early RA patients, *P. copri*-derived peptides bound to HLA-DR molecules induced Th1 type inflammatory responses in PBMCs. Furthermore, IgA and IgG anti-*P. copri* antibodies were detected in early and established RA patients, and its levels were correlated with Th17 cytokine profiles, ACPAs and

presence of DNA from *Prevotella* in synovial fluid samples [79]. Unlike *P. copri*, another bacteria from this genus, *P. histicola*, was shown to induce anti-inflammatory responses in murine models of RA [80]. Therefore, physiologic roles for bacteria at the genus level should be cautiously interpreted and not generalized.

On the other hand, epitopes belonging to *Prevotella* spp. exhibit molecular mimicry with a citrullinated autoantigen found in RA joints (*N*-acetylglucosamine-6-sulfatase, GNS), providing a link between mucosal and joint immune response. Both self- and microbial-peptides were predicted to bind strongly to HLA-SE molecules, and were able to activate T and B cell responses in approximately 50% of RA patients [81]. Based on this, the authors speculated that T cells, especially those from patients carrying the SE alleles, are activated at the gut mucosa after recognition of *Prevotella*-derived epitopes and can subsequently migrate to inflamed joints—where there is a high expression of similar autoantigens—thereby mounting a cross-reaction to self-epitopes.

Another mechanism by which gut microbiota alterations can promote autoimmunity or RA progression is by increasing intestinal permeability [9, 12]. In a murine model of AIC, treatment with *Collinsella aerofaciens* (which is abundant in some RA patients) augmented the incidence and severity of arthritis [12]. *C. aerofaciens* promoted intestinal permeability in the epithelial cell line CACO-2 by reducing the expression of tight junction proteins (ZO-1 and occludin) while increasing the expression of the proinflammatory mediators IL-17A, CXCL1, CXCL5 and NF- κ B1 [12]. In a similar way, C57BL/6 mice orally treated with *P. copri* exhibited increased numbers of T-CD4⁺ (IFN γ ⁺) lymphocytes and developed severe colitis after treatment with dextran sulfate sodium [9]. Interestingly, individuals at risk of developing RA had reduced numbers of IFN- γ -producing ILC1 cells, whereas early RA patients showed increased numbers of IL-17-producing ILC3 cells, thus evidencing immunophenotypic alterations at the intestinal level in early stages of disease [82]. Therefore, intestinal bacteria may promote inflammation and permeability of the intestine by modulating the GALT. Loss of gut barrier integrity allows translocation of bacterial antigens to systemic circulation and may consequently activate inflammatory immune responses in extra-intestinal sites [83]. Several butyrate-producing bacteria, such as *Eubacterium*, *Fusobacterium*, *Anaerostipes*, *Roseburia* and *Faecalibacterium*, have shown to promote the maintenance of gut epithelial barrier integrity and anti-inflammatory response both in vitro and in vivo studies [84, 85]; therefore, represent potential candidates for restoring of gut integrity in RA.

Gut microbiota as diagnostic, severity or therapeutic response biomarker

Early RA diagnosis is of significant importance as it allows opportune therapeutic interventions to improve long-term outcomes such as joint damage. In this context, metagenome wide association studies (MWAS) are flourishing tools for the search of microbial biomarkers for disease diagnosis or prognosis [43]. Zhang and colleagues estimated the diagnostic utility of the RA-associated microbiome by using prediction models; they provided a model with an area under the curve of 0.940, a specificity of 0.922 and sensitivity of 0.838. The performance of this microbiome-based diagnostic model for RA was comparable to those based on serum biomarkers [13]. Meanwhile, another study by Scher et al. [9] identified several genes of the microbiome differentially expressed in early RA and pointed out their potential for identifying individuals at risk of developing RA.

The potential of some intestinal bacteria as disease activity or severity biomarkers is supported by their relationship with clinical parameters of RA. For instance, the phylum of Euryarchaeota was directly correlated with disease activity and was proposed as an independent risk factor for RA by multivariate analysis [47], whereas *Prevotella-2* and *Alloprevotella* were positively correlated with the inflammatory biomarker C-reactive protein [86]. A recent study identified an increased abundance of Gammaproteobacteria and less abundance of Bifidobacterium in patients with elevated levels of inflammatory cytokines (TNF- α and IL-17) than those with lower levels, and *Collinsella* and *Akkermansia* abundance was higher in patients with active RA than those with inactive RA [45]. In another study, the abundance of *Haemophilus* spp. showed a negative correlation with serum anti-CCP and RF antibodies levels, while expansion of *Lactobacillus salivarius* was higher in the active disease RA group than in the other groups [13]. Since these studies reveal variations in gut microbiota composition in subsets of RA patients with distinct clinical features, such findings may have clinical implications for the development of new therapeutic strategies focused on modifying specific intestinal microbes or their metabolites.

Osteomicrobiology is an emerging research field that studies the relationship between microbiota and bone metabolism [87]. Bacterial antigens activate the immune system and can induce osteoclast differentiation and bone erosion by production of TNF- α , IL-17 and RANK [88]. In a mice model of CIA, *P. gingivalis* oral infection was shown to produce oral dysbiosis, higher bone loss and joint destruction [64]. However, to date there are no studies in RA patients demonstrating the impact of gut alterations on bone catabolism or radiographic progression.

Meanwhile, pharmacomicrobiomics is focused on studying the relationship between gut microbiota and the therapeutic response, absorption, distribution and metabolism of drugs [89]. Despite it has been reported that DMARD treatment partially reestablishes RA gut dysbiosis [13], and significant differences on gut microbiota composition were reported when comparing patients under different treatment schemes [47] (see Table 1), several aspects remain to be explored in relation to RA drugs and gut microbiome, for example, how gut microbial composition and function impact the bioavailability, toxicity or therapeutic response, which is highly heterogeneous among patients.

In summary, the usefulness of the microbiome as diagnostic, severity or therapeutic response biomarker remains scarcely explored. It should be considered that currently, the study of the microbiome is restricted to a few laboratories and its analysis requires highly trained personnel. Therefore, in the long-term, it will be necessary to replicate findings in other populations, as well as perform larger longitudinal studies and evaluate its usefulness to the clinical practice considering benefit–cost analysis.

Implications and potential of the gut microbiota for RA treatment

The gut microbiome is highly dynamic and responds rapidly to intrinsic and extrinsic stimuli of the host. Hence, it can be modulated by strategies such as the use of antibiotics, prebiotics and probiotics intake, fecal microbiota transplant (FMT) or simply, through dietary intake [18]. In recent years, the pharmaceutical industry has shown substantial interest on the development of therapeutic products based on bacterial-cocktails or on microbiota-derived molecules for the treatment of chronic diseases associated with gut dysbiosis.

Use of probiotics in RA

Evidence about the effects of supplementation of RA patients with bacterial strains that provide health benefits, namely probiotics, is modest in general terms, as the majority of studies performed to date are limited in sample size and are heterogeneous with respect to supplementation duration, the administered strain or the clinical and inflammatory outcomes measured. There is evidence of significant reductions on clinical, inflammatory biomarkers and metabolic profiles on RA patients after supplementation with *Lactobacillus rhamnosus* GR-1, *Lactobacillus reuteri* RC-14 or *Lactobacillus casei* 01 [50, 52], while other clinical trials did not show significant changes on clinical activity parameters [52, 53]. A recent study in adjuvant-induced arthritis (AIA) rats showed that treatment with a single bacterium (*L. casei*

ATCC334) was able to suppress the induction of arthritis and protect bones from destruction in AIA rats by restoring the microbiome dysbiosis in the gut [90].

In general terms, probiotics supplementation in RA patients has proven to be secure but further research is still required to provide a solid rationale regarding which strains are more appropriate, taking into consideration the clinical features of the patients. Few studies have explored the effect of probiotic supplementation on the management and prevention of RA comorbidities such as depression, infections, body composition, metabolic alterations and oxidative stress [2, 91]; therefore, this creates an area of opportunity that should be further addressed in the future. Finally, characterization and evaluation of probiotics at strain level, not only genus and species, is a relevant aspect since it may yield completely opposite effects [54]. For example, *E. coli* includes strains that are commensal, pathogenic or carcinogenic, while *P. histicola* -in contrast to *P. copri*- has been identified as a potential probiotic in RA, as it induces an expansion of Treg cells and suppresses the development of arthritis in experimental models [80].

Diet, microbiota and RA

Epidemiologic associations between intake of specific nutrients or foods (e.g., intake of saturated fats, red meat, alcohol or sugars) with the development of RA remain controversial [92]. An explanation for such inconsistent findings may be that isolated food or nutrients only confer modest effects that are difficult to detect in epidemiologic studies, as in real context, individuals rather consume food groups that are better depicted by dietary pattern analysis [93]. A generally regarded healthy diet pattern is the Mediterranean diet, characterized by high consumption of fruit, vegetables, whole grains, legumes, fish and olive oil, low intake of red meat and moderate consumption of alcohol [94, 95]. However, even the relationship of this diet with reduced RA risk shows conflicting results [92, 94, 96]. A recent large population-based case–control study revealed lower RA risk in women who adhere to a healthy dietary pattern [93]. This can be explained by the immunomodulatory effects of fiber as well as the anti-inflammatory effects of omega-3 fatty acids intake [92]. Conversely, western diets and high intake of saturated fats have shown to promote intestinal permeability and inflammation, thereby allowing the translocation of bacterial proinflammatory components such as lipopolysaccharide (LPS) to systemic circulation [95]. During obesity, the adipose tissue releases numerous biologically active factors; including adipokines such as leptin, and inflammatory cytokines such as TNF- α , IL1- β e IL-6, that may contribute to RA pathogenesis by heightening the inflammatory response,

accelerating metabolic alterations and the development of CV comorbidities [42, 97].

Diet is a source of multiple bioactive compounds with potent immunomodulatory and antioxidant effects; for example, capsaicin derived from chili has shown to bind TRPV1 receptors expressed on T lymphocytes and regulate its activation [98], while curcumin, derived from the Asian spice *Curcuma longa*, exerts potent antioxidant and anti-inflammatory effects through the inhibition of nuclear transcription factor κ B (NF- κ B), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [99]. 1,25-dihydroxyvitamin D, the active form of vitamin D; regulates the expression of hundreds of target genes and has shown to suppress B cell proliferation and antibody production, inhibit the release of Th1 and Th17 cytokines [100] and downregulate proinflammatory cytokine secretion such as IL-1 α , IL-1 β and IL-6 in monocyte-derived macrophages from RA patients [101]. Likewise, dietary fiber promotes intestinal SCFAs generation by gut microbiota, and these metabolites have demonstrated extensive anti-inflammatory and beneficial metabolic effects [95].

In this context, a recent clinical trial reported that dietary supplementation of RA patients with high-fiber bars during 28 days, reduced the number of circulating Treg cells, improved the Th1/Th17 ratio and reduced the levels of CTX-1, a biomarker of bone erosion [102]. Currently, the evidence of specific diets (e.g., vegetarian or restrictive diets) or nutritional supplementation with specific compounds is still limited, but clinical trials are on course to evaluate the effect of anti-inflammatory diets or Mediterranean diets on clinical activity parameters and quality of life in RA patients (NCT02941055; NCT04262505). Therefore, dietary interventions as a complement to RA therapy may be useful not only to help reestablish the dysbiosis associated to the disease, but also to improve the metabolic profile of the patients and to reduce the CV risk, which represents the major comorbidity of RA [1, 3].

Fecal microbiota transplant on RA

FMT is the transfer of fecal bacteria from a healthy donor into the gastrointestinal tract of a recipient in order to change the recipient's microbial composition and confer a health benefit [103]. This procedure is highly efficient for the treatment of intestinal diseases, in specific, infections by *Clostridium difficile*. Although the regulatory aspects of FMT are still on progress on several countries, the FDA already classified it as a procedure in research phase. Nowadays, its therapeutic potential is being explored increasingly on a wide range of chronic- and extra-intestinal diseases, including autism, depression, epilepsy and metabolic syndrome [103]. As for RA, there are no data available regarding its effects on the clinical parameters or disease activity

in the patients; however, a clinical trial is on course to evaluate the efficacy and security of FMT in Chinese patients with RA refractory to methotrexate treatment (registry: NCT03944096).

Conclusions and perspectives

The evidence about the role of gut microbiota on RA is constantly increasing and has led to a better understanding of the etiopathogenesis of the disease. Metagenomic studies performed to date in RA are heterogeneous, but all of them have demonstrated that gut microbiota alterations are present both in early and established stages of the disease. A prevailing challenge is to expand gut microbiota studies to other populations and further explore its potential as biomarker for clinical activity or therapeutic response. Emerging studies are also shedding light on the functional and molecular underlying mechanisms by which gut microbiota contributes to RA; however, several research areas remain barely explored, for instance, the interaction between the genome and the microbiome of RA patients, and the role of other microbiota constituents in RA etiopathogenesis, such as viruses. Since some phages can infect bacteria, it is predictable that virome alterations contribute to intestinal dysbiosis.

Gut microbiota modifications of the RA patient by the use of probiotics, prebiotics, diet or FMT as a complementary approach to drug therapy have the potential to restore gut dysbiosis, improve clinical activity parameters and metabolic profile, and reduce the CV risk, which is one of the major RA comorbidities. As we advance on understanding host–microbial interactions, and we further elucidate the molecular mechanisms by which gut microbiota contributes to RA development, progression or even therapeutic response, it will be possible to develop intervention strategies tailored to each patient, considering their clinical characteristics, genetics and environmental exposures, in order to restore not only gut microbiota composition, but to modulate specific functional and metabolic pathways with therapeutic goals.

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