

1 **The microbiota and autoimmunity: their role in**
2 **thyroid autoimmune diseases**

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23 Abstract

24 Since the 1970s, the role of infectious diseases in the pathogenesis of Graves' disease
25 (GD) has been an object of intensive research. The last decade has witnessed many
26 studies on *Yersinia enterocolitica*, *Helicobacter pylori* and other bacterial organisms
27 and their potential impact on GD. Retrospective, prospective and molecular binding
28 studies have been performed with contrary outcomes. Until now it is not clear whether
29 bacterial infections can trigger autoimmune thyroid disease. Common risk factors for
30 GD (gender, smoking, stress, and pregnancy) reveal profound changes in the bacterial
31 communities of the gut compared to that of healthy controls but a pathogenetic link
32 between GD and dysbiosis has not yet been fully elucidated. Conventional bacterial
33 culture, *in vitro* models, next generation and high-throughput DNA sequencing are
34 applicable methods to assess the impact of bacteria in disease onset and
35 development. Further studies on the involvement of bacteria in GD are needed and
36 may contribute to the understanding of pathogenetic processes. This review will
37 examine available evidence on the subject.

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39 Keywords: Bacteria, Graves' disease, Hashimotos's thyroiditis

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46 Abbreviations

47	AITD	autoimmune thyroid disease
48	Anti-Tg	Antithyroglobulin
49	CagA	Cytotoxin-associated gene A
50	CD	Celiac disease
51	DES	Dry eye syndrome
52	ELISA	Enzyme-linked immunosorbent assay
53	GD	Graves' disease
54	GF	Germ free
55	GO	Graves' orbitopathy
56	HAT	Hashimoto thyroiditis
57	HLA	Human leukocyte antigen
58	HP	<i>Helicobacter pylori</i>
59	HPA	Hypothalamic-pituitary-adrenal axis
60	HUVEC	Human umbilical vein endothelial cells
61	IBD	Inflammatory bowel disease
62	IFAA	Immunofluorescent antibody assay
63	NOD	Non-obese diabetic
64	PBMC	Peripheral blood mononuclear cells
65	PM	Pretibial myxedema
66	RA	Rheumatoid arthritis
67	SCFA	Small chain fatty acid
68	SHIME	Simulator of human intestinal microbial ecosystem

69	SPF	Specific pathogen free
70	T1D	Type 1 diabetes
71	TGF- β	Transforming growth factor beta
72	TIM	TNO (gastro-) intestinal model
73	TPO	Thyroperoxidase
74	Tregs	Regulatory T cells
75	TSAb	TSH receptor stimulating antibodies
76	TSBAb	TSH-stimulation blocking antibody
77	TSHR	TSH receptor
78	rIUBT	Radiolabeled urea breath test
79	WB	Western blot
80	YE	<i>Yersinia enterocolitica</i>
81		

82 1. Introduction

83 Bacteria and bacterial antigens have long been considered as possible culprits in
84 inducing autoimmune disease. Using the example of rheumatic fever, the link between
85 bacteria and disease was established at the end of the 19th century by Triboulet and
86 Coyon [1] and several decades later, experimental and clinical data indicated that
87 autoimmunity in rheumatic fever is induced in response to group A streptococci [2].
88 Current mechanisms suggested to lead to autoimmune processes after a bacterial
89 infection include molecular mimicry, epitope spreading, bystander activation and
90 cryptic antigens [3].

91 In autoimmune thyroid diseases (AITD), especially Hashimoto thyroiditis (HT) and
92 Graves' disease (GD), evidence for possible bacterial involvement in onset and
93 progression is based solely on retrospective measures of bacterial antibodies in AITD
94 patients (Table 1). These include the bacteria *Yersinia enterocolitica*, *Helicobacter*
95 *pylori* and *Borrelia burgdorferi* (Figure 1). As in rheumatic fever, several tissues are
96 targeted by the autoimmune response in GD (mainly thyroid, but also adipose tissue,
97 skin and bone) and the whole body is affected by the hyperthyroid state. There has
98 been limited examination of the possible connection between AITD and the microbiota
99 using only serological methods. Other methods such as *in vitro* models, 16S rRNA
100 gene sequencing, next generation sequencing and high throughput sequencing have
101 already been applied to investigate the role of bacteria in other autoimmune diseases,
102 but not in AITD. These platforms allow characterization of the microbiota from AITD
103 affected areas (eyes) or areas adjacent to them (nose, mouth, skin) and contributing
104 factors such as genetic predisposition and environmental factors associated with AITD.
105 The role of microorganisms in the development of AITD is still controversial and not
106 fully elucidated. An understanding of the precise mechanisms of interaction between

107 bacterial agents in inducing thyroid autoimmunity could result in the development of
108 new strategies for prevention and treatment.

109 This review aims to summarize current knowledge on the role of the microbiota in
110 thyroid autoimmunity and will focus on the bacterial component.

111

112 2. Autoimmune thyroid disease

113 Autoimmune disorders are a broad range of related diseases in which inappropriate
114 immune responses of the body arise against its own cells, tissue and organs, resulting
115 in inflammation and damage. This response may affect only a particular tissue/organ
116 of the body (such as in autoimmune thyroiditis) or may be systemic (such as systemic
117 lupus erythematosus). In autoimmunity, the balance between proinflammatory and
118 regulatory mechanisms, as a requirement for sufficient tolerance of the body against
119 its own cells, is no longer maintained. Autoimmune reactions are characterized by the
120 appearance of autoreactive B and T cells, which can be activated via other cells and
121 which are very specific. Several different AITDs exist, but Hashimoto's thyroiditis (HT)
122 and Graves' disease (GD) are the most prevalent [4]. Approximately 5% of the
123 population is affected with HT and the disease is usually diagnosed in the fourth to
124 sixth decade of life [5]. Graves' disease is the underlying cause of 50 to 80% of
125 hyperthyroidism and affects approximately 0.5% of the population [6, 7]. The incidence
126 of GD is around 0.5 per 1000 annually in England [8] and 0.77 per 1000 in women and
127 0.14 per 1000 in men, respectively, in Scotland [9]. AITDs are the most common organ-
128 specific autoimmune diseases and affect more women than men, with a female-to-
129 male ratio from 5 to 10 [10].

130 Hashimoto's thyroiditis, also known as autoimmune or chronic lymphocytic thyroiditis,
131 is characterized by infiltration of the thyroid gland by inflammatory cells, subsequent
132 atrophy of the thyroid tissue [11] and production of antithyroid antibodies, especially
133 against thyroperoxidase (anti-TPO), antithyroglobulin (anti-Tg) and TSH-stimulation
134 blocking antibody (TSBAbs, although these are rare). The destruction and fibrous
135 replacement of the follicle cells lead to hypothyroidism. HT is significantly more
136 frequent in individuals suffering concurrently from other autoimmune diseases like type
137 1diabetes (T1D) or rheumatoid arthritis.

138 In 1840 Carl-Adolph von Basedow termed the three typical clinical features in Graves'
139 disease (tachycardia, proptosis, and goiter) as the "Merseburg trias". These symptoms
140 are due to activated thyroid autoreactive CD4+ T cells that infiltrate the thyroid and
141 activate B cells. The latter secrete TSH receptor (TSHR) stimulating antibodies (TSAbs),
142 which in turn induce thyrocyte proliferation and secretion of excess thyroid hormones
143 and lead to hyperthyroidism. The autoimmune response, probably to the TSHR, leads
144 to Graves' orbitopathy (GO) which is characterized by proptosis or bulging eyes, also
145 known as exophthalmos [12].

146 The pathogenesis of AITDs is multifactorial including genetic predisposition for GD
147 (Human Leukocyte Antigen (HLA) Class I molecules C*07 and B*08 as well as HLA
148 Class II molecules DR3 and DRB1*08 [13], CD40, CTLA-4, PTPN22, FCRL3,
149 thyroglobulin and TSHR, reviewed in [14]), pregnancy [4] and a variety of
150 environmental factors (iodine and selenium intake, smoking, acute psychological
151 stress [15-19] (Figure 2). Medication can also influence disease prevalence
152 (amiodarone, certain monoclonal antibodies, interferon alpha, and cytokines). As
153 autoimmune disorders tend to co-exist in the same subjects, celiac disease (CD) is
154 associated positively with orbitopathy in GD patients [20]. In both CD and GO/GD T-

155 helper cells 17 (Th17) play a role in pathogenesis [21-24]. Also, the cytokine IL-15
156 which is involved in the differentiation of Th17 cells and links innate and adaptive
157 immune systems is increased in the sera of Hashimoto thyroiditis (HAT) patients and
158 was detectable in 33% of thyroid-associated ophthalmopathy (another name for GO)
159 biopsies from extraocular muscles [25, 26].

160 Characteristic for AITD are a Th1 pattern of immune response in HT and a
161 predominance of T-helper cells 2 cytokines in GD, indicating a humoral pattern of
162 immune reaction for the latter disease [27]. Furthermore follicular helper T (Tfh) cells -
163 a relatively new subset of antigen-experienced CD4+ T cells found in B cell follicles of
164 secondary lymphoid organs and serving as regulators in the evolution of effector and
165 memory B-cell responses - are found to have an increased frequency in AITD.
166 Therefore, this cell subset might also be important in the pathogenesis of AITD [28].

167

168 3. Function of commensal bacteria

169 In AITD, the major body sites involved, apart from the thyroid, are the eyes and the
170 skin, but nasal and oral microbiota might also be important for GO, considering its
171 proximity to the orbit. Likewise, the gut as the most important reservoir of bacterial
172 metabolism for the host and as the site with the highest numbers of immune cells is
173 discussed in the following. The bacterial communities in these sites may have potential
174 impact on AITD and to assess this, a precise characterization of the bacterial diversity
175 and metabolic profile of commensal bacteria from healthy persons is needed.

176 Due to its high vascularity, good lymphatic drainage, encapsulated position and its
177 generation of hydrogen peroxide for the synthesis of thyroid hormone, the thyroid is
178 remarkably resistant to infection and is rarely infected [29]. Under healthy

179 circumstances, the thyroid should be sterile. Apart from bacterial assessment in
180 suppurative or non-suppurative infection, the authors are unaware of any analysis of
181 bacteria carried out in thyroid tissue from healthy persons or AITD patients [30, 31].

182 When assessing thyroid disease with ocular complications it is logical to focus mainly
183 on the microbiota of the eye. However, there is neither an agreement about a naturally
184 existing resident microbiota on the healthy ocular surface nor about the role resident
185 microbiota may play at this site in ocular surface physiology [32]. Because of the high
186 antimicrobial properties of the ocular surface, the bacterial abundance is innately low
187 and organisms found, although normally classified as commensals, may play a more
188 important role than in other sample sites with less bacterial abundance.

189 The skin is the largest sensory organ of the body and harbors around 113 different
190 bacterial phylotypes and the predominant microbiota on the skin was shown to
191 correlate with lipid content, pH, sweat and sebum secretion [33]. The skin is
192 predestined for complex ecological interactions with the environment and the skin
193 microbiota perform several functions: i) inhibition of pathogen growth through
194 antimicrobial peptides (bacteriocins (reviewed in [34]), microcins (reviewed in [35]) and
195 phenol soluble modulins [36], ii) degradation of proteins associated with
196 *Staphylococcus aureus* biofilm formation [37] and iii) decrease of the skin pH due to
197 hydrolysis of sebum triglycerides by bacterial lipases and esterases [38-40]. The acidic
198 *milieu* is unfavorable for many pathogens like *S. aureus* and *Streptococcus pyogenes*
199 and thus the growth of coagulase-negative staphylococci and corynebacteria is
200 supported [39, 41-43]. Furthermore, commensal bacteria tune the local cytokine
201 production and influence regulatory T cells in the epidermis as well as mast cells [44-
202 47].

203 Despite the close proximity to the skin, the nasal cavity is populated differently and is
204 one of the main reservoirs for *S. aureus*, a commensal organism carried by 20–30% of
205 humans. Colonization is a risk factor for nosocomial infections with this bacterium [48].

206 Gut commensals contribute to the host's well-being in several ways. The microbiota
207 influences both the innate and adaptive immune system by interacting with pattern-
208 recognition receptors such as the toll-like receptors (TLR) which are expressed on cells
209 present in the gut wall, in particular the resident immune cells in the gut-associated
210 lymphoid tissue (GALT). Microbial products activate the TLR and trigger the release of
211 pro-inflammatory (TNF α , IL1 or IL6), anti-inflammatory (IL10) cytokines, or those
212 which determine T lymphocyte phenotypes (IL17, IL23) [49, 50]. Commensal bacteria
213 are able to actively induce regulatory responses in the gut epithelium. Regulatory T
214 cells (Tregs), a subpopulation of T cells which maintain tolerance to self-antigens and
215 prevent inflammatory and allergic responses, are induced via direct sensing of
216 microbial organisms and their metabolites by dendritic cells or T cells. The luminal
217 concentration of the bacterial metabolite butyrate positively correlates with the number
218 of Treg cells in the colon [51] and besides other organisms, *Clostridium* spp. is able to
219 create a transforming growth factor beta (TGF- β) rich environment and this supports
220 Treg cell accumulation [52]. They also perform a number of metabolic functions
221 including food processing, digestion, and the synthesis of different products, e.g.
222 vitamin B12 and short chain fatty acids (SCFA) as a main product of their metabolism
223 [53]. SCFAs serve as an energy source for epithelial cells [54, 55], accelerate colonic
224 transit through stimulation of the gut motility via serotonin [56, 57] and simultaneously
225 regulate the sympathetic nervous system activity directly via the Gi/o protein-coupled
226 receptor GPR41 at the level of the sympathetic ganglion [58]. Among the SCFAs,
227 butyrate in particular modulates immunity and exerts an anti-inflammatory effect. This
228 modulation is due to several effects including butyrate mediated reduction of nuclear

229 factor- κ B and inhibition of histone deacetylase (HDAC) [59, 60]. HDAC prevents gene
230 transcription by keeping chromatin in a compact form and its inhibition by SCFAs alters
231 colonic gene expression and metabolic regulation [61]. Moreover, butyrate induces
232 regulatory T cells in the colonic environment [51, 62, 63].

233 The gut microbiota also protects the indigenous bacterial community against invasion
234 by new and potentially harmful bacteria (colonization resistance) [64-68]. In this context
235 secreted IgA may play a role via a process termed 'antibody mediated
236 immunoselection' (AMIS) which shapes the composition of the microbiota. It has been
237 suggested that AMIS could be exploited by using antibodies to manipulate the
238 microbiota and treat conditions caused by dysbiosis [69].

239 The gut microbiota impacts the central and enteric nervous systems [70] e.g. by
240 producing neurotransmitters such as gamma-aminobutyric acid, serotonin, dopamine,
241 noradrenaline and acetylcholine [71-75]. In turn neurotransmitters produced by the
242 host can directly influence the composition of the gut microbiota [76-78], which may be
243 relevant to the significantly higher levels of anxiety and depression reported in GD
244 patients compared with those with goiter [79].

245 It is logical that the gut microbiota will influence autoimmune conditions such as CD
246 [80-82] and inflammatory bowel disease [83-89] and indeed Di Giacinto and colleagues
247 suggested an amelioration of colitis severity using probiotic bacteria which induced an
248 immunoregulatory response involving TGF- β -bearing regulatory cells [90]. In their
249 large multicenter study of new-onset pediatric CD, using samples from different sites,
250 Gevers and colleagues observed a correlation of specific bacteria with disease status
251 and especially the distinct microbial signature of the rectum at the disease onset offers
252 unique potential for early diagnosis [91]. In CD, Tregs are induced by gliadin in situ
253 [92]. Perhaps more surprising are the reports illustrating an impact of the gut microbiota

254 on autoimmune diseases targeting more distant sites e.g. T1D [93-99], rheumatoid
255 arthritis [100-103] and the in vivo model of multiple sclerosis [104-106]. In the NOD
256 mouse model the incidence of disease is maximal in the germ-free (GF) population
257 whereas specific pathogen free mice are protected [96, 107]. The protective microbiota
258 can be transferred from dam to pup [108] and the normally high T1D female-to-male
259 sex bias in specific pathogen free (SPF) NOD mice can be equalized through fecal
260 microbiota transplantation from male to female mice and its corresponding effects on
261 testosterone levels [109, 110]. In the development of T1D in infants, gut microbiome
262 analysis reveals a decrease in diversity once specific T1D autoantibodies were
263 detectable but before the clinical onset of disease. This is accompanied by signs of
264 intestinal inflammation through increased fecal human β -defensin 2 levels [111].
265 Interestingly, Vatanen and colleagues detected a connection between *Bacteroides*
266 species-rich microbiota and simultaneously high T1D susceptibility in a human
267 population potentially arising from a distinct microbiota-derived type of
268 lipopolysaccharide with immunoinhibitory properties [112]. For a more general review
269 on autoimmune-microbiota interactions the reader is referred to the following
270 references [113, 114].

271 When compared to the human gut and other body sites, the oral cavity ranks second
272 in total microbial load [115] and each bacterial species occupies highly specific niches
273 differing in both anatomic location (such as the lips, cheek, palate, periodontal cavity
274 and tongue) and nutrient availability [116]. The oral microbiota is regularly transferred
275 to adjacent habitats via saliva, although only 29 out of 500 microbial taxa recovered
276 from the mouth are cultivated from faecal samples [117]. Besides breaking down
277 nutrients, the function of the oral microbiota is to modify the local pH or redox potential,
278 the formation of biofilms and quorum sensing to coordinate these biofilms and gene
279 expression [118].

280

281 4. Techniques

282 This section discusses the methods used to assess the impact of bacteria in the onset
283 and aggravation of autoimmune thyroid disease. For articles on mouse models (Banga
284 and colleagues reported a model of GO based on genetic immunization using human
285 TSHR A-subunit plasmid and close field electroporation. Induction of prolonged
286 functional antibodies to the TSHR results in chronic disease with progression to GO-
287 like disease [119]) and segmented filamentous bacteria in thyroid autoimmunity please
288 read elsewhere [120-123].

289 4.1 Culture-dependent techniques

290 4.1.1 Conventional culture

291 Until now, it has not been possible to cultivate and isolate bacteria directly from patients
292 with AITD either from blood or tissue. However, culturing fecal microbial communities
293 from healthy donors, under strict anaerobic conditions, enables capture of a
294 remarkable proportion of the gut microbiota and preserves the distinctiveness of each
295 donor's microbiota [124]. These efforts resulted in the discovery of new taxa [125] and
296 far more genetic potential to form spores than previously assumed [126]. Toft and co-
297 workers screened 107 fecal samples of GD patients for *Y. enterocolitica* (YE), but did
298 not find an increased prevalence of YE. The isolation rate was very low (<1%) and
299 similar to that observed in the local population with diarrheal illness [127]. A reliable
300 animal model for GD/GO that reproduces all the aspects of the disorder has not been
301 available, but very recently, Banga and colleagues reported a new mouse model of
302 GO based upon immunogenic presentation of human TSHR A subunit plasmid by close
303 field electroporation. Induction of prolonged functional antibodies to the TSHR results

304 in chronic disease with progression to GO [119]. In patients with dry eye syndrome
305 (DES), a condition resulting from GO [128], Graham and colleagues performed a
306 comparison of the bacterial community of the ocular surface in DES patients using
307 conventional culture techniques and 16S rRNA gene PCR [129]. Coagulase negative
308 staphylococci were found in both patients and controls, with an increase in culture
309 positivity and mean numbers of bacteria in dry eye. The amount of identified bacterial
310 genera and species was extended with molecular methods including potentially
311 pathogenic bacteria such as *Klebsiella* spp. and repeated sampling and testing of a
312 subset of patients revealed similar results.

313 4.2 Models (in *vitro*)

314 Using the example of intestinal autoimmune diseases like CD or inflammatory bowel
315 disease (IBD) and other autoimmune diseases like primary biliary cirrhosis [130] the
316 use of *in vitro* models in GD can generate knowledge and better understanding of the
317 disease although all of the models have their limitations and thus do not always
318 correlate in detail with pathophysiological conditions in a human body.

319 4.2.1 Cell lines

320 Monolayers of intestinal cell lines are composed of a single cell type and lack the
321 variety found in the intestine, e.g. goblet cells and paneth cells and their crosstalk with
322 other cells of the body. Nevertheless, Caco-2 cells are widely used to study CD and
323 IBD and increase understanding of pathogenesis. In CD, Caco-2 cells exposed to
324 gliadin proliferate, display actin rearrangements and inhibition of spontaneous
325 differentiation [131]. In addition they have demonstrated how 1) patients' serum
326 antibodies modulate the epithelium and 2) bifidobacteria inhibit the toxic gliadin effects
327 [132, 133]. Other intestinal cell lines e.g. T84 and HT29 express IBD related cell

328 surface molecules (CD40) after treatment with cytokines [134]. Caco-2 cells have been
329 used to investigate the beneficial effect of different commensal gut bacteria on anti-
330 inflammatory G protein–coupled receptors expressed by intestinal cells [135] and also
331 probiotics were co-cultured with this cell line: Mattar and co-workers showed that
332 *Lactobacillus casei* up-regulates mucin gene expression [136].

333 The combination of Caco-2 cells in a transwell system with dendritic cells or THP-1 (a
334 human monocytic cell line derived from an acute monocytic leukemia patient) provides
335 a more physiological setting. It allows measurements of cytokine production and tight
336 junction protein expression in response to commensal or pathogenic bacteria as well
337 as CD triggering gliadin [137-139].

338 Patient-derived T-cell lines and clones from the site of inflammation or peripheral blood
339 are widely used in CD and provide information on T cell activation in the lamina propria
340 to specific antigens and T cell infiltration into the intestinal epithelium [140-142]. In GD,
341 Roura-Mir and colleagues analysed lymphocytes from peripheral blood and thyroid
342 lesions ex vivo to investigate the role of CD1-restricted T cells, which are able to
343 present self and foreign lipid antigens to T cells. They suggested a possible effector
344 function of CD1-restricted T cells in tissue destruction [143]. In a cell proliferation assay
345 with peripheral blood mononuclear cells (PBMCs) cellular reactivity to Yersinia outer
346 membrane proteins (YOP) encoded by a 72-kilobase virulence plasmid of YE was
347 present in GD patients and controls whereas intrathyroidal lymphocytes obtained from
348 GD patients demonstrated marked proliferation in response to the released proteins
349 [144].

350

351 4.2.2. Mucosal biopsy organ culture

352 Culturing mucosal biopsies, with their histological architecture intact, allows *in vivo*
353 processes to be studied in controllable conditions outside the body (*ex vivo*). In the
354 past, Ussing chambers have been widely used to monitor net ion transport across living
355 epithelium in mice and humans [145, 146]. Another approach is to study intestinal
356 biopsies of IBD patients, in which apical to basolateral polarity is maintained by a “glued
357 cave cylinder” to facilitate stimulation of each border in turn [147]. In CD, the
358 importance of IL-15 was demonstrated in a culture of duodenal biopsy [148], whilst an
359 organ culture demonstrated an impaired mucosal immune response to gliadin in T1D
360 [149]. Ogino and colleagues showed that in Crohn’s disease, CD14(+) CD163(low)
361 cells, from the intestinal lamina propria of patients, induce the differentiation of naive T
362 cells into Th17 cells and by doing this contribute to the pathogenesis of CD and
363 possibly other Th17-associated diseases [150].

364 4.2.3 Flow models

365 Chambers of flow systems allow co-culture of different cell types in separate chambers;
366 proteins and signals produced by one cell type can flow through the system to have an
367 effect on another cell type, as would happen in the body. To date perfusion flow studies
368 have not been applied to autoimmune disease, but given their utility it is only a matter
369 of time until they are used to study AITD.

370 However, there are many examples of the use of flow in the culture of different
371 mammalian cells [151-153].

372 4.2.4 3D cell culture systems

373 In addition to the use of flow to better mimic conditions *in vivo*, there is also a growing
374 trend moving away from culture on 2D surfaces and into 3D scaffolds and gels. In a
375 model for *H. pylori* infection, primary gastric glands were grown in Matrigel as a 3D

376 spheroid; morphological features of typical stomach tissue were evident and spheroids
377 survived for greater than 9 months [154]. Collagen gels have been used for the 3D co-
378 culture of rat intestinal sub-epithelial myofibroblasts with a rat intestinal epithelial cell
379 line [155]. A simple 3D co-culture model of the gut used non-transformed human
380 neonatal small intestinal cells and non-transformed human monocyte/macrophages for
381 the study of the interaction of *Lactobacillus* spp. with the gut [156].

382 4.2.5 Intestinal models

383 To rebuild the intestine in a larger format than those mentioned above, *in vitro* models
384 can include short-term batch incubators, single stage reactors through to multistage
385 continuous systems and their evolutions (simulator of human intestinal microbial
386 ecosystem (SHIME), EnteroMix, TNO (gastro-) Intestinal Models (TIM) and PolyfermS)
387 have been developed [157-162]. The more complex models can mimic the microbiota
388 and their fermentation processes in different parts of the human gut and enable
389 evaluation of a wide range of environmental regulators of bacterial activity like
390 substrate availability, pH and growth rates. Advantages of these systems are the lack
391 of ethical issues surrounding sampling the human gastrointestinal tract and
392 surrounding the use of radioactive or toxic substances. Running multi-compartment
393 continuous systems is relatively inexpensive and microbial community development in
394 dynamic models after inoculation with faecal microbiota is reproducible [159]; this holds
395 true even for faecal samples of persons with high/low conversion rates of organic
396 materials into energy sources by bacteria [163]. Obvious disadvantages of the
397 intestinal models are the lack of physiological host environment with epithelial cells,
398 immune cells and mucus. To help counteract this, MacFarlane and colleagues added
399 mucin to their model [164] and combined dynamic models with cell culture systems by
400 adding fermentor vessel effluent onto Caco-2 cells [165]. Other groups investigated

401 the effects of the culture effluent on immune cells in the macrophage cell line U937
402 [166]. Besides inoculation with healthy adult faeces diluted with phosphate buffered
403 saline, Cinquin and co-workers used immobilized infant faeces on gel beads [167].
404 Other authors inoculated their gut models with faecal samples derived from IBD
405 patients or from healthy individuals resulting in an increased production of toxic
406 metabolites by IBD microbiota [83]. Also shortened transit time, which is common in
407 irritable bowel syndrome, has been investigated [168]. By mimicking an overgrowth
408 with *Clostridium difficile* after antibiotic treatment, van Nuenen et al. observed a two-
409 fold increase of toxic proteolytic metabolites which could be neutralized by the addition
410 of different inulins, a group of naturally occurring plant polysaccharides and a
411 functional food that stimulates the growth of healthy bacteria (= prebiotics) [169].
412 Probiotics, prebiotics, their synergistic effects and other dietary components have been
413 studied in intestinal models with the aim of increasing the levels of beneficial microbes
414 [170-174]. In the SHIME model, van den Abbeele and colleagues incorporated mucin-
415 covered, simplified ecosystems (microcosms) and assessed the long term colonization
416 of lactobacilli and their stability under antibiotic treatment with tetracycline, amoxicillin
417 and ciprofloxacin [175]. More recent developments for intestinal models include the
418 Host-Microbiota Interaction module for long-term incubation [176] and the “gut-on-a-
419 chip” [177].

420 Intestinal models enable the user to perform mechanistic studies *in vitro* and to develop
421 hypotheses. Nonetheless, intestinal models will always require validation *in vivo* due
422 to the complexity of host-associated environments.

423

424 4.3 Culture-independent techniques

425 4.3.1 Antibody and antigen detection

426 Serological tests like agglutination, enzyme linked immunosorbent assay (ELISA) and
427 Western blot (WB) as well as antigen detection tests in serum and stool or in the form
428 of radio-labelled urea breath test for *H. pylori* detection have been used to explore the
429 possible link between AITD and bacteria by measuring antibodies against bacterial
430 antigens which could induce cross-reactive immune response against self-antigens.
431 Besides genetic predisposition, 25% of the predisposition to Graves' disease is
432 estimated to be linked to environmental factors like infections [178]. Since the 1970s
433 and until more recently, infections with the bacterium YE have been implicated in the
434 pathogenesis of GD caused by increased YE antibody prevalence in GD patients [179-
435 182], but this was not reproduced by all groups [183-185] (see Table 1 and Figure 1).
436 Also prospective studies on this field were undertaken with different outcomes: a case-
437 control twin study and two studies in euthyroid females related to AITD patients
438 with/without follow up revealed no causal relationship between YE infection and
439 autoimmune thyroid disease [184-186]. However, in earlier studies with similar design
440 a higher prevalence of antibodies against YOP was measured [187, 188]. A linear
441 correlation between YE antibodies and antibodies against TSHR, thyroglobulin and
442 thyroid-peroxidase has been described [189]. YE antigens not only display high-affinity
443 binding sites for the hormone TSH and the TSHR Abs from patients with Graves'
444 disease, but also show a sequence homology between its outer membrane porins
445 (Omp) [190, 191] and the TSHR. In their study with mice, Luo and colleagues produced
446 antibodies against the purified extracellular domain of human TSHR and showed that
447 anti-TSHR antibodies reacted with the envelope antigens of YE. When mice were
448 immunized with YE, anti-TSHR-antibodies were induced [192], supporting the concept
449 of molecular mimicry. Hargreaves and co-workers demonstrated that a recombinant

450 Fab germline fragment of a monoclonal TSAb from GD mice doesn't recognize TSHR,
451 but does bind YE outer membrane porins [193].

452 The impact of antibodies to *Helicobacter pylori* (HP) on GD was first observed in 1999
453 [194]. Similar to YE, some groups were able to show a significant increase in HP
454 antibody prevalence and some not [195-200]. In the case of no significant difference
455 in anti-HP-IgG a significant association between AITD and cytotoxin-associated gene
456 A (CagA)-antibodies and between GD and CagA-antibodies was observed [200].
457 Interestingly, Bertalot et al. screened patients after HP eradication and found a
458 reduction in the anti-thyroid peroxidase titre, in anti-thyroglobulin and a partially
459 normalized anti-TSHR titre [201].

460 Besides these two organisms, *Borrelia burgdorferi* and the neurotoxin of *Clostridium*
461 *botulinum* have also been implicated in the context of GD suggesting that antigens
462 cross-reacting with human TSHR share multiple antigenic epitopes with other bacterial
463 antigens [202-205].

464 Glycoproteins of the probiotic bacterium *Bifidobacterium bifidum* were shown to have
465 an immunological similarity with thyroid peroxidase and thyroglobulin, pointing towards
466 a possible role in the pathogenesis of AITD [206]. Nevertheless, several years earlier,
467 Zhou and colleagues ruled out the induction of pathological inflammation in a mouse
468 model of experimental autoimmune thyroiditis due to a bacterium of the same genus,
469 namely *Bifidobacterium lactis* [207].

470 Viruses and their role in AITD have also been discussed and corresponding nucleic
471 acid has been detected via PCR-based methods and immunochemistry [30, 31, 208],
472 but viruses are beyond the scope of this review.

473 Overall, a large number of studies showed epidemiological, serological and molecular
474 evidence that YE and other bacteria are potentially important in the pathogenesis of
475 AITD and GD. None of the studies showed a direct correlation of bacterial infection to
476 the development of AITD and most patients with one of the above mentioned bacterial
477 infections (including those who produce anti-TSHR antibodies) do not develop GD
478 [209]. It might be possible that the ability to produce anti-TSHR antibodies in response
479 to YE antigens homologous to the TSHR persist only in susceptible individuals with the
480 YE antigens acting as a trigger to the disease development. Further studies are needed
481 to get a definite answer.

482 4.3.2 A possible link between microbiota and autoimmune thyroid

483 Over the last decade, sophisticated sequencing techniques and high throughput
484 technologies have become affordable and allowed both characterizations of the
485 microbes living in and on the human host as well as their metabolic functionality. The
486 Human Microbiome Project elucidated the structure and diversity of the healthy human
487 microbiome at almost 20 different body sites and by doing this created a large
488 reference database [210].

489 Alterations in the gut microbiota have already been observed for many diseases
490 ranging from diabetes, alcoholic liver disease and psychiatric disorders to cancer and
491 autoimmune diseases. Numerous studies have been performed within the autoimmune
492 sector regarding the gastrointestinal tract, joints and the neural system, but very little
493 is available for AITD, the most frequent of the autoimmune diseases. Studies
494 examining the microbiome of AITD patients and especially with GD/GO are not
495 available, but many microbiome studies investigating the impact of known risk factors
496 such as genetic risk factors (gender) and environmental risk factors (smoking, stress)
497 have been undertaken (see Figure 2). Also studies on body sites actually or possibly

498 involved in GD/GO like eye, nose, throat and intestine have been performed and all
499 are addressed in this section.

500 *Risk factors to consider*

501 1. Gender and genetics

502 The unequal gender distribution in autoimmune disease has been covered in
503 many publications, but only a few tried to characterize the gut microbiome of
504 females and males to look for differences. Flow cytometry-based *in situ*
505 hybridizations revealed higher levels in *Bacteroides* and *Prevotella* in males
506 than in females with autoimmune disease, but no gender effects could be
507 observed for any other bacteria [211]. Markle et al. entered the topic more
508 deeply with the help of NOD mice [109]. Normally, the incidence of T1D is higher
509 in female NOD SPF mice than in male. Whilst in germ free (GF) mice the
510 incidence is equal between the two genders. Serum testosterone levels were
511 higher in female GF mice than in SPF and higher in male SPF mice than in GF
512 suggesting that colonization by commensal microbes elevates testosterone
513 levels in males and may protect NOD males from developing T1D.
514 Transplantation of the male microbiota to females resulted in altered recipient's
515 microbiota and consequently elevated testosterone levels and changes in
516 metabolite production. Furthermore, the T1D diagnostic parameters islet
517 inflammation and autoantibody production were decreased. Yurkovetskiy and
518 colleagues obtained similar results in the same NOD mouse model before and
519 after puberty: their 16S rRNA gene profiles indicate that the gut microbial
520 communities depend on the gender of post-pubescent mice. After castration,
521 female and castrated male microbiota are more similar to each other than to

522 non-castrated male microbiota. The microbiota differs in males and females
523 after GF mice have been colonized with a female SPF microbiota [110].

524 In a large study with more than 400 twin pairs, Goodrich and colleagues
525 characterized the gut microbiomes of monozygotic twins and found them to be
526 more similar than those of dizygotic twins [212], Christensenellaceae belonging
527 to the Firmicutes was the taxon with the highest heritability. Evidence from mice
528 also suggests that the genetics of the host strongly influences the microbiome
529 of the gastro-intestinal tract [213] and has also shown that variation in the
530 microbiome influences disease outcomes, e.g. the occurrence of T1D in non-
531 obese diabetic (NOD) mice or the induction of experimental autoimmune
532 encephalomyelitis [106, 108].

533 The intestine is the largest immune organ in the body and is comprised of
534 trillions of commensal organisms and is affected by treatment (antibiotics,
535 corticosteroids) [214, 215] and diets among others.

536 Furthermore, genetic investigations demonstrated a connection between CD
537 and GD [216, 217] and also T1D organ culture studies indicate an unbalanced
538 mucosal immune response to gliadin [149].

539

540

541

542 2. Pregnancy

543 The prevalence of GD in pregnancy is rare and ranges between 0.1% and 1%
544 [218]. In pregnancy, the gut microbiome changes each trimester [219] and

545 pregnant women have increased total bacteria and *Staphylococcus* numbers
546 which seems to be related to increased plasma cholesterol levels. The mothers'
547 body weights also seems to be of importance because reduced numbers of
548 some anaerobes (*Bifidobacterium* and *Bacteroides*) and increased numbers of
549 other anaerobic bacteria (*Staphylococcus*, *Enterobacteriaceae* and *Escherichia*
550 *coli*) were detected in overweight compared to normal-weight pregnant women
551 [220]. In pregnant mice it has been shown recently that changes in the maternal
552 gut microbiota are dependent upon the mother's periconceptual diet but not
553 upon increases in maternal weight gain during pregnancy [221].

554 3. Smoking

555 Smoking alters the oropharyngeal and tracheal environment in smokers
556 compared to non-smokers, but in 2013 Biedermann and co-workers suggested
557 an effect of smoking also on the gut microbiota [222, 223]. The group found an
558 increase of *Firmicutes* and *Actinobacteria* and a non-significant decrease of
559 *Bacteroides* and *Proteobacteria* with simultaneous increase in microbial
560 diversity after smoking cessation. *Bacteroidetes* seems to be the only phylum
561 with a significant change only after 4 weeks of smoking cessation maintained
562 through to eight weeks. Principal component analysis separated the bacterial
563 community composition of the smoking cessation group clearly from the control
564 group, particularly between before and after smoking cessation.

565 4. Stress, anxiety

566 As discussed in an earlier section, stress can modify the microbiota composition
567 and vice versa [68, 70, 77-79].

568 *Body sites to consider*

569 1. Nose

570 Despite the close proximity of the nose to the eyes, the role of the nasal
571 microbiota in the pathogenesis of AITD has not been examined. Most of the
572 studies compared the nasal microbiome of healthy persons [210, 224-227] with
573 those of persons with chronic rhinosinusitis and other nasal inflammatory
574 diseases [228-232]. Partially, these investigations included additional cultural
575 assessment of the microbiome [225, 229, 231] and samples were taken from
576 the depth of the sinus of patients undergoing endoscopic sinus surgery [231,
577 232]. The healthy nasal microbiome consists of mainly staphylococci
578 (coagulase-negative staphylococci, *Staphylococcus aureus*), corynebacteria,
579 propionibacteria and *Moraxella* spp., whereas between the studies of nasal
580 inflammation, there is no apparent consensus [210]. The nasal microbiota differs
581 seasonally and the diversity decreases within the first year of life [233]. Also
582 Graves' disease tends to vary seasonally with more frequently relapses in spring
583 and summer [234]. Further studies are needed to reveal possible relationships
584 between the microbiota and disease progression.

585 2. Eye

586 Clinically recognized GO occurs in about 50% of GD patients and therefore a
587 comparison of the eye microbiota in these patients would be helpful, but has not
588 been done yet [12]. In a mouse model of autoimmune uveitis, it was recently
589 shown that activation of retina-specific T cells is dependent on gut microbiota-
590 dependent signals [235].

591 3. Skin

592 1.5% of GD patients suffer from pretibial myxedema (PM) and other GD related
593 skin disorders [236]. Characteristic for PM are skin thickening especially in the

594 pre-tibial area, but the disorder can also occur in other areas. No study focused
595 particularly on the skin microbiota in patients with GD and subsequent skin
596 disorders, although there is evidence that the skin microbiota varies in primary
597 immunodeficiency [237] and also in skin (affecting) disorders like psoriasis [238,
598 239], atopic dermatitis [240], systemic lupus erythematosus [241] and Morbus
599 Behcet [242].

600 4. Throat

601 Several diseases except AITD have been linked to the commensal bacterial
602 population in the human mouth. In RA, Zhang and colleagues observed a
603 concordance between the gut and oral microbiomes in patients with RA and a
604 dysbiosis which was partially resolved after treatment [103]. Also in Sjögren's
605 syndrome, a systemic autoimmune disorder characterized by lymphocytic
606 infiltrates in exocrine organs, altered bacterial communities have been noticed.
607 Szymula and colleagues showed the ability of peptides originating from oral and
608 gut bacteria activating Sjogren's syndrome Antigen A (SSA)/Ro60-reactive T
609 cells [243, 244].

610 5. Gut

611 In autoimmune thyroid disease the link between microbiota and disease onset
612 or progression has not been elucidated yet. However, possible relations should
613 be pointed out: already in 1988, Penhale and Young found in a rat model of
614 autoimmune thyroiditis that modulation of the gut microbiota results in a
615 significant influence on susceptibility to thyroid autoimmunity [245]. According
616 to them, SPF rats were markedly less susceptible to the induction of
617 experimental autoimmune thyroiditis by thymectomy and irradiation than
618 conventionally reared rats of the same strain. Additionally, the incidence of
619 thyroid lesions indicating thyroiditis as well as measured autoantibodies

620 increased in conventional rats and the offspring of conventional reared mothers
621 were more susceptible to develop autoimmunity. 27 years later, a PCR-
622 denaturing gradient gel electrophoresis with universal primers targeting V3
623 region of the 16S rRNA gene and quantitative real-time PCR revealed a different
624 intestinal microbiota composition in hyperthyroid patients compared to controls
625 whereas hypothyroidism leads to bacterial overgrowth in the small intestine
626 assessed by hydrogen glucose breath test [246, 247]. Both, hyperthyroidism
627 and hypothyroidism often go hand in hand with thyroid autoimmunity. Not only
628 the microbiota composition, but also its enzyme activities have to be considered:
629 glucuronidases responsible for provision of conjugated thyroxine are mostly of
630 bacterial origin [248, 249]. Regarding the ability to produce hormones, the gut
631 microbiota “has the potential to produce hundreds of products. From a
632 morphological and biochemical perspective, it is far larger and more
633 biochemically heterogeneous than any other endocrine organ in man” [250]. T4
634 malabsorption can be due to diverse gut microbiota in patients with CD and
635 lactose intolerance [251, 252]. Similar to patients with T1D, a morphological and
636 functional damage of the intestinal barrier was found [253] [254, 255].

637 In 2009, Oresic and colleagues investigated the contribution of the gut
638 microbiota to lens and retinal lipid composition. In their comprehensive lipidomic
639 profiling of lens and retina from conventionally raised and GF mice the authors
640 found a decrease of lens phosphatidylcholines in the presence of gut microbiota
641 due to an increased exposure to oxidative stress than in GF mice [256].

642 In summary, the questions dominate the answers concerning the impact of the
643 microbiota in AITD and there is room for future research on this topic.

644

645 5. Conclusion

646 Interactions between the host and the gut microbiota influence host immunity and
647 physiology and therefore are important to maintain intestinal homeostasis. Disruption
648 of these host–microbial interactions due to dysbiosis can alter this balance leading to
649 disease. Currently, very little is known about the impact of bacteria and microbiota in
650 autoimmune thyroid disease. The author and co-authors are engaged in the project
651 “Investigation of Novel biomarkers and Definition of the role of the microbiome In
652 Graves’ Orbitopathy” (INDIGO), which is part of the Industry-Academia Partnerships
653 and Pathways (IAPP) program in People Marie Curie Actions (FP7-PEOPLE-2013-
654 IAPP). The project aims to identify prognostic biomarkers to facilitate early preventative
655 intervention, to investigate the role of the microbiome on disease progression and to
656 assess the impact of probiotics in disease reduction. Hopefully, results will answer
657 these questions and provide insight into the influence of environmental factors on
658 gene–microbe interactions and the potential role of intestinal bacteria in the onset and
659 progression of Graves’ disease.

660

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664

665 Declaration of Interest

666 All authors declare that they have no competing interests.

667

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- 1371

1372 Table 1

Bacterium	Cohort	Antibodies	Method	Antibody prevalence in patients in comparison to controls	Reference
<i>H. pylori</i>	GD and HAT	anti- HP (IgG)	ELISA, rIUBT	no sign. Difference	[257]
<i>H. pylori</i>	AITD	anti- HP, anti-CagA	WB	increase in AITD (p=0.006)	[194]
<i>H. pylori</i>	HAT	anti-CagA (IgG)	ELISA, rIUBT	No sign. difference	[199]
<i>H. pylori</i>	AITD	anti- HP	ELISA	increase (p=0.032)	[195]
<i>H. pylori</i>	GD	anti-CagA (IgG), HP-antigens (stool)	ELISA	increase (p<0.001) with positive AEIA; increase in anti-CagA (p<.005)	[196]
<i>H. pylori</i>	GD and HAT	anti-CagA (IgG), HP-antigens (stool)	ELISA	correlation between HP (p≤0.0001) and Cag-A (p≤0.005) in GD, not in HAT	[197]
<i>H. pylori</i>	AITD	anti- HP (IgG), anti-CagA	WB	no sign. difference in anti-HP-IgG, association between CagA-antibodies and AITD	[200]
<i>H. pylori</i>	Blood donors	anti-HP (not specified)	EIA	increase in donors with thyroid autoantibodies (p=0.018)	[198]
<i>Y. enterocolitica</i>	GD	anti-YE	agglutination (Gruber-Widal)	increase (p<0.005)	[179]
<i>Y. enterocolitica</i>	Thyroid disease	anti-YE	agglutination (Gruber-Widal)	present in 42% of 36 patients with thyroid disease and in none of 77 controls	[182]
<i>Y. enterocolitica</i>	AITD	anti-YE 0:3 and 0:9 (IgM, IgA, IgG), YP	ELISA	anti-YE IgA 0:3 increased (p<0.01), no difference in IgM and IgG	[258]
<i>Y. enterocolitica</i>	GD and HAT	anti-plasmid YE proteins (IgA, IgG)	WB	increase in anti-immunogenic protein, IgA and IgG in GD and HAT (p<0.01; p<0.001)	[259]
<i>Y. enterocolitica</i>	GD and HAT	anti-YE 0:3, 0:5, 0:6 and 0:9	micro-agglutination	anti-YE 0:3 not significantly different, anti-0:5 increase in GD and HAT (p<0.001)	[260]
<i>Y. enterocolitica</i>	GD and HAT	anti-YE 0:3	agglutination (Gruber-Widal)	increase in GD (p<0.01), no significant increase in HAT	[261]
<i>Y. enterocolitica</i>	GD and HAT	anti-YOP2-5	WB, PBMC proliferation assay	YOP2-5 antibodies found in GD (96%), HAT (55,5%) and controls (70,8%)	[144]
<i>Y. enterocolitica</i>	GD and HAT	anti-YE 0:3 and 0:9	agglutination (Gruber-Widal), ELISA	no difference, thyroid therapy didn't change immunoreactivity	[183]
<i>Y. enterocolitica</i>	GD and HAT	anti-YE 0:3, 0:5, 0:8 and 0:9	agglutination (Gruber-Widal)	increase in GD (p<0.05)	[189]
<i>Y. enterocolitica</i>	AITD	anti-YOPs 0:9 (IgA and IgG)	WB	increase of IgA and IgG antibodies against YOPs (p<0.05 and p=0.002, respectively)	[187]
<i>Y. enterocolitica</i>	AITD	anti-YOPs 0:9 (IgA and IgG)	WB	no difference	[184]
<i>Y. enterocolitica</i>	GD	anti-YOPs 0:9 (IgA and IgG)	WB	increase in IgA and IgG (p=0.054 and p=0.043, respectively)	[178]
<i>Y. enterocolitica</i>	GD and HAT	anti-YOPs 0:9 (IgA and IgG)	WB	no difference	[186]
<i>Y. enterocolitica</i>	AITD	anti-YOPs 0:9 (IgA and IgG)	WB	no difference	[185]
<i>B. burgdorferi</i>	GD and HAT	anti-BB (IgG)	ELISA	no difference	[204]
<i>B. henselae</i>	HAT	anti-BH (IgG)	IFAA	increase, case report	[262]

Table 1: Evaluation of anti-bacterial antibody prevalence in patients with autoimmune thyroid disease (AITD), Graves' disease (GD) and Hashimoto thyroiditis (HAT) with different test methods. Abbreviations: cytotoxin-associated gene A (CagA), *Helicobacter pylori* (HP), *Yersinia enterocolitica* (YE), *Yersinia* outer proteins (YOP), enzyme-linked immunosorbent assay (ELISA), western blot (WB), radiolabeled urea breath test (rUBT), immunofluorescent antibody assay (IFAA), peripheral blood mononuclear cells (PBMC).

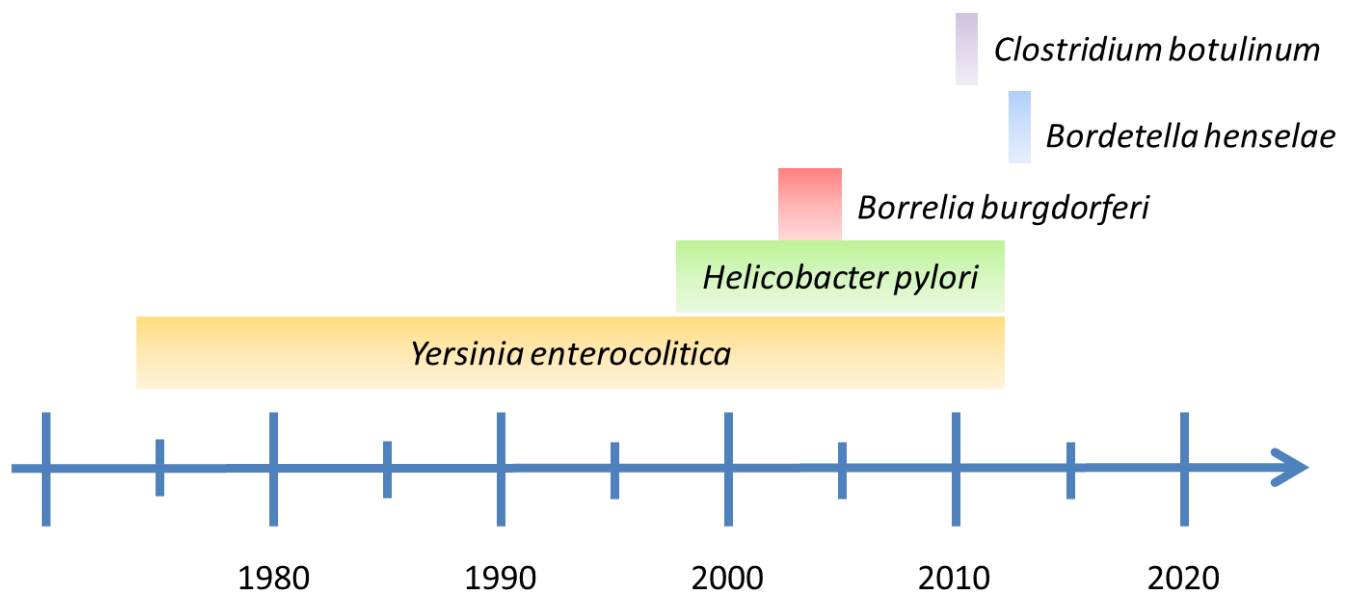


Figure 1

Figure 1: Role of bacteria in autoimmune thyroid disease: publications on this topic focusing on the five most cited bacterial organisms.

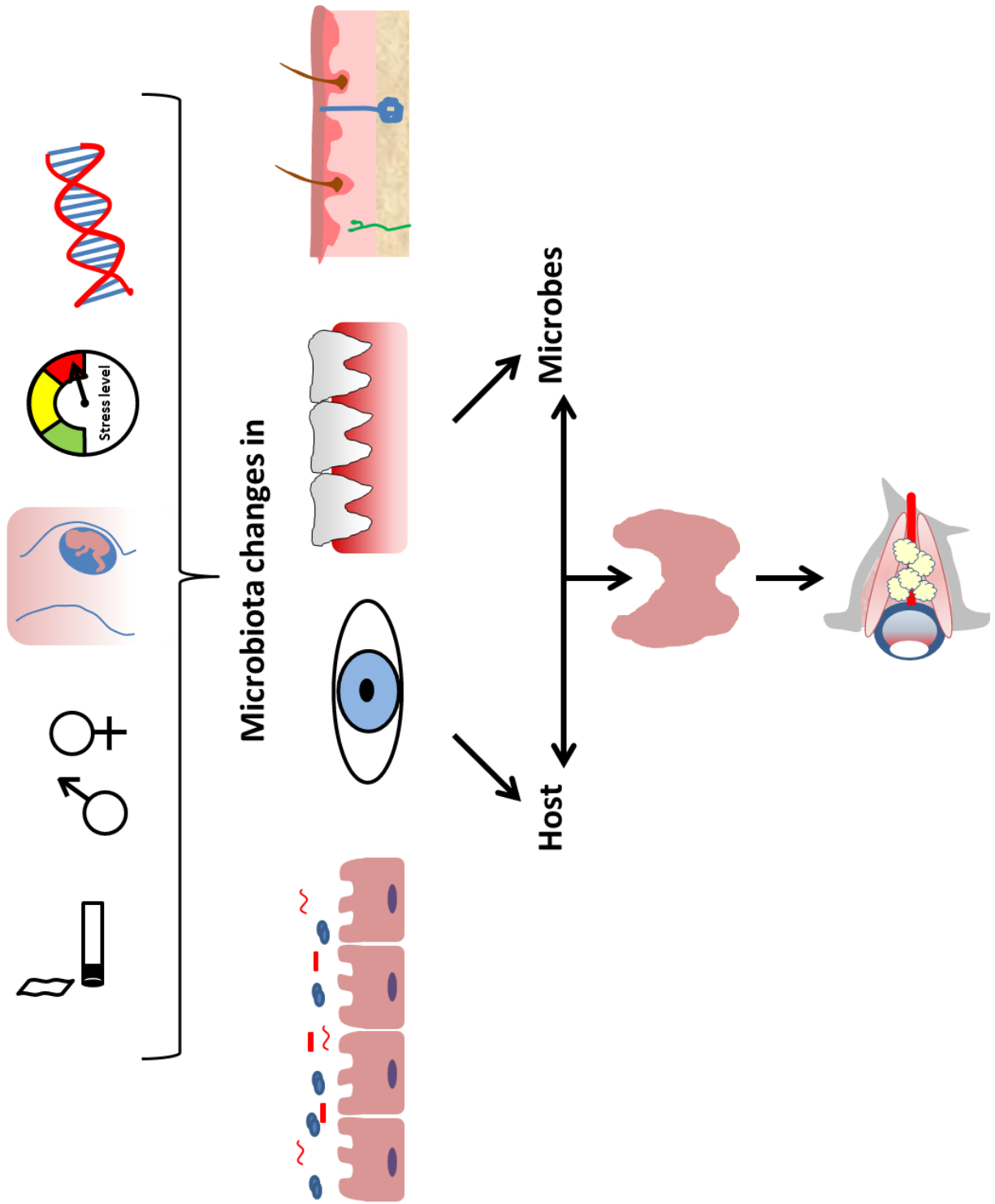


Figure 2

Figure 2: Overview of the main environmental factors influencing Graves`disease.
Environmental factors affect bacterial populations at different body sites and therefore potentially contribute to the development of Graves' disease and orbitopathy.

