



A systematic review of studies on the faecal microbiota in anorexia nervosa: future research may need to include microbiota from the small intestine

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1 **A Systematic Review of Studies on the Faecal Microbiota in**
2 **Anorexia Nervosa – future research may need to include**
3 **microbiota from the small intestine**

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37 **Abstract**

38 Purpose:

39 Anorexia nervosa (AN) is a serious, poorly understood, and often chronic condition.
40 Deviations in the gut microbiota have been reported to influence the gut-brain axis in
41 other disorders. Therefore, if present in AN, it may impact on symptoms and illness
42 progression. A review of the gut microbiota studies in AN is presented.

43

44 Method:

45 A literature search on PubMed yielded 27 articles; 14 were selected and based on
46 relevance, 9 articles were included in the review. The findings were interpreted in
47 the larger context of preclinical research and clinical observations.

48

49 Results:

50 8 out of 9 included studies analysed microbiota from faeces samples, while the last
51 analysed a protein in plasma produced by the gut. Two studies were longitudinal
52 and included an intervention (i.e., weight restoration), five were cross-sectional, one
53 was a case report, and the last was a case series consisting of three cases.

54 Deviations in abundance, diversity, and microbial composition of the faecal
55 microbiota in AN were found.

56

57 Conclusion:

58 There are currently only a few studies on the gut microbiota in AN, all done on
59 faeces samples, and not all describe the microbiota at the species level extensively.

60 In four studies the Archaeon *M.smithii* was found increased in AN and may be an

61 interesting benchmark biomarker for future studies. It is furthermore proposed that
62 microbiota samples could also be collected from the small intestine, where a major
63 exchange of nutrients takes place and where the microbiota may have a relevant
64 biological impact on AN.

65

66 Key words: Anorexia Nervosa, faeces, microbiota, species, biomarker.

67

68

69 **Introduction**

70 Anorexia nervosa (AN) is a serious and often chronic psychiatric condition [1]. The
71 hallmark feature of AN is a reduction of energy intake relative to energy expenditure
72 leading to low body weight. Potential life-threatening medical complications that
73 affect almost every organ frequently occur contributing to AN having a high
74 standardized mortality ratio of 5.2 [3.7-7.5] [2]. In addition, there are no effective
75 treatments for AN and chronicity is high [3].

76

77 Elucidating biomarkers associated with AN could provide guidance for risk
78 stratification, treatment and identify targets for developing novel pharmacological
79 treatments as well as increasing disease understanding. Studies have begun to
80 explore whether the gut microbiota and its associated microbiome might harbor trait
81 biomarkers for AN.

82 Definitionally, “microbiota” refers to a community of microorganisms, including
83 Bacteria, Viruses, Archaea, and Fungi, and in this review, we have focused on the
84 gut Bacteria and Archaea in AN. The “microbiome” refers to the collective genomes
85 of the present microorganisms [4]. More than 1,000 ‘species-level’ phylotypes exist
86 in a human [5]. The majority of these phylotypes are Bacteria, with *Faecalibacterium*
87 *prausnitzii*, *Roseburia intestinalis*, and *Bacteroides uniformis* dominating in the adult
88 microbiota found in faeces samples [6]. The phylotypes are mostly consistent across
89 individuals, but the relative composition and diversity of organisms can vary
90 markedly. In addition, diet has been shown to influence intestinal dysbiosis
91 influencing both risk of glucose intolerance and cancer development [7,8].

92

93 Gut microbiota not only play a critical role in the development of the gut mucosal
94 immunity [9,10], but also affect the regulation of the hypothalamic-pituitary-adrenal
95 (HPA) axis [11], serotonergic neurotransmission [12], and signaling mechanisms
96 affecting neuronal circuits involved in motor control and anxiety in mice [13]. This
97 pathway has been described as the gut-brain axis [14]. The mechanism of this
98 interaction is not fully elucidated, and there are as yet no dedicated studies to
99 explore or intervene with this gut-brain axis in AN.

100

101 Given the long periods of starvation associated with the core psychopathology of
102 AN, considerable adaptation in intestinal microbiota could occur in people with AN.
103 Alternatively, specific intestinal dysbiosis could predispose to the drive toward
104 negative energy balance in AN. Intestinal dysbiosis is known to have an impact on
105 psychological function and mental health including depression and anxiety, both of
106 which are commonly comorbid with AN [15]. AN patients often present with
107 comorbid anxiety (75% lifetime prevalence of anxiety disorder) [16] and more than
108 34% prevalence of depression [17,18]. As such, the gut-brain axis is of particular
109 interest in understanding the psychopathology of AN.

110

111 The intestinal microbiota is involved in both weight gain and weight loss as well as
112 with energy extraction from the diet in both humans and animals [19,20]. Differences
113 in the composition of the intestinal microbiota between obese and lean individuals
114 have been consistently described, potentially illustrating differences in energy
115 extraction efficiency between obese and lean individuals [21,22]. Furthermore, in an
116 activity based mouse model of AN Jésus et al. demonstrated increased permeability
117 in the colon, i.e. “gut leakiness”, in anorexic mice, however the authors also found

118 that the gut leakiness was more related to malnutrition than exercise[23]. In another
119 study examining the role of exercise on gut permeability, Pals et al. found that
120 exercise increases intestinal permeability measured with the lactulose and
121 rhamnose differential urinary excretion test [24]. In contrast to this, a study by
122 Monteleone et al. found reduced urinary recovery of lactulose in AN patients
123 reflecting a reduced permeability in the small intestine, where breakdown and
124 absorption of lactulose take place [25].

125 Changes in the intestinal permeability may be caused by AN pathophysiology,
126 however, the current results on gut permeability in AN are conflicting. The potential
127 altered gut permeability in AN may underlie the low-grade inflammation and
128 increased risk of autoimmune diseases found in eating disorders [26]. Moreover,
129 starvation has a significant impact on the gut microbiota, and a diet based on animal
130 products used for re-nutrition, may stimulate the growth of Bacteria that triggers
131 inflammation [27].

132 The small intestine, notably the ileum, contains a microbial flora and the major
133 breakdown of food and metabolism take place there making the small intestine an
134 area of interest in AN, where restrictive dietary intake is characteristic of the eating
135 disorder. This region is driven by rapid uptake, fast transit times, excretions of
136 digestive enzymes and bile salts, which collectively put a selective driving force on
137 resilient microbes with effective survival strategies that differ from those found in the
138 colon [28]. The microbiota found in ileal effluent, which has a uniquely personal
139 composition [29], differ from that found in faeces samples with lower overall
140 diversity, fast changing profiles, and increased relative abundances of species
141 within the orders *Lactobacillales* and *Clostridiales*, and below detection limits of
142 Archaea [30-32]. In addition, studies have shown that the microbiota is highly

143 specific for different gut compartments and even differs within compartments, i.e.
144 the colon [29,33-35].

145

146 The aim of this article is to conduct a review of the evidence of differences in the
147 faecal microbiota in AN compared to healthy controls. This could provide clues to
148 the pathophysiology of AN, index biomarkers, and generate new ideas for treatment
149 development. In addition, guidance for future research is provided.

150

151 **Method**

152 **Protocol for the review**

153 The protocol is available as a table online at this URL:

154 <https://drive.google.com/open?id=0B1bvPK36OIXANVZGNXduSU85RWs>

155 **Eligibility criteria**

156 In view of the rather few studies done, all articles were included, except reviews,
157 animal studies, and studies with no relevance to the intestinal microbiota in AN and
158 the gut-brain axis (Figure 1).

159 **Data sources**

160 The PubMed database in the US National Library of Medicine was searched to
161 identify any relevant studies on August 27, 2017.

162

163 **Search Strategy**

164 The following search terms were included: “Anorexia nervosa” and “microbiota”. The
165 reference lists of studies were also handchecked for additional articles of interest.

166

167 **Study selection**

168 We only included original scientific publications, where the human microbiota were
169 analysed in persons with AN. Excluded were review articles, commentaries,
170 preclinical and animal studies and all other types of non-scientific original
171 publications. One case study was also excluded as it examined fungi from a faeces
172 sample instead of Bacteria and Archaea, of which the two latter are the focus point
173 of this review (Figure 1).

174

175 **Data collection process**

176 Given the heterogeneity in methods and the limited number of publications, results
177 were evaluated as presented in the source publications (Table 1). No additional
178 analysis were made to the original presentations of data and the corresponding
179 results.

180

181 **Risk of Bias**

182 Risk of bias was assessed by HFS and JMS by reviewing the study designs, the
183 methods used, any selection mechanisms presented, and the consistency of results
184 presentation. Risk of bias is included in the above linked protocol.

185

186 **Synthesis of results**

187 The full texts were then retrieved and read in full by two authors (HFS and JMS)
188 independently to determine whether the studies met inclusion criteria, and HFS
189 wrote the manuscript with extensive help from the fifth author (JMS). A second
190 author (CK) and a third author (JT) provided important revisions on science and

191 content to the manuscript, and a fourth author (NH) provided expert input on the
192 microbiota and its role in the metabolism and absorption of nutrients, and a critical
193 view on the limitations of the few studies done so far. As raw data were not
194 extracted, there was no handling of data or combining of results.

195

196 **Results**

197 The search terms “anorexia nervosa” and “microbiota” yielded a total of 27 unique
198 articles. Reviewing the reference lists of all articles did not yield any additional
199 original scientific publications relevant for this review. All 27 articles were screened
200 and assessed for eligibility. 18 of these articles did not meet the eligibility criteria
201 and were excluded (Figure 1). The main study characteristics of the 9 included
202 studies are summarized in Table 1. During revision of this article for publication
203 another study by Mörkl et al. published in November 2017 was included in this
204 article and in Table 1 [36], and thus a total of 10 articles were included. See the end
205 of the “Results” paragraph for a review of the findings by Mörkl et al.

206 Of the 10 studies included in this review, two were longitudinal in design [37,38], six
207 were cross-sectional [39-43,36], one was a case study involving a severe case of
208 AN [44], and the last study was a case series consisting of three cases that was
209 also longitudinal in its design [45]. The diagnostic criteria used for AN were from
210 Diagnostic and Statistical Manual IV (DSM-IV) in four studies [39,37,40,41], from
211 DSM-5th Edition in two studies [45,42], from ICD-10 in one study [36], and not
212 specified in three studies [44,43,38]. All studies except one were published in the
213 last five years [39].

214 The number of AN patients included in the six cross-sectional studies ranged
215 between 9 and 25 [39-43,36], and between 16 and 55 in the two longitudinal studies

216 [37,38]. Three patients with AN were included in the case series [45]. In the
217 longitudinal studies over the course of renourishment, the second time point (T2)
218 was defined as after approximately 14 weeks in one of the studies [38], and when a
219 mean Body Mass Index (BMI) goal of approximately 17.4 kg/m² was achieved in the
220 other study [37]. Healthy controls were included in all studies, and in four of the ten
221 studies, the controls were matched to the AN groups both for age and sex
222 [38,41,42,36]. The 9 studies that examined the intestinal microbiota all examined it
223 from faeces samples.

224 The methods used for assessing quantity and type of species in faeces samples
225 were 16S Revers Transcriptase-PCR based. Morita et al. also included 23S rRNA
226 gene targeted technology. Two studies did not specify whether 16S or 23S rRNA
227 gene targeted PCR was used [40,39]. Additional measures included organic acids
228 including short-chain fatty acids and pH of faeces (chromatography) [38,41], and
229 culture growth and mass spectrometry (Matrix-Assisted Laser Desorption-Ionization)
230 [44], and several measures of body fat including anthropometric assessments and
231 ultrasound measurement of subcutaneous adipose tissue thickness [36]. Three
232 studies evaluated the relation between psychiatric measures and changes in
233 abundance and composition of the microbiota [37,42,36], and one study examined
234 the relation between Caseinolytic peptidase B (ClpB) protein concentrations in
235 plasma and scores on the Eating Disorder Inventory-2 (EDI-2) and the Montgomery-
236 Åsberg Depression Rating Scale (MADRS) [43].

237 Three studies used employees, relatives and family members of the employees as
238 controls [39,42,38]. The other studies recruited controls through public
239 advertisements [43,37,36], through previous studies using a snowball approach and
240 from healthy outpatients [40], or recruited controls through non-disclosed methods

241 [41]. Two studies did not include controls, as the study designs were case studies
242 [45,44].

243 Bias was assessed in all studies, and the results are shown in the linked online
244 protocol. Furthermore, see Table 1 for at description of the quality of technologies
245 used for collection and handling of faeces samples in the different studies.

246

247 ***Microbiota results***

248 Four studies explored the abundance of gut microbiota in AN, and all investigated
249 AN in the acute stage. Two of these studies described a normal abundance of
250 microbiota in AN [40,38], while one found a reduced microbiota abundance in AN
251 [41] and another an altered abundance measured on several microbial phyla,
252 genera, and species [42].

253 Three studies examined the diversity in AN compared to healthy controls. Mack et
254 al. found an overall normal microbial diversity in AN in their weight restoration study
255 at both time point 1 (T1) and time point 2 (T2) [38], and in line with this, Borgo et al.
256 found no significant changes in diversity in acutely ill AN patients compared to
257 controls [42]. The other weight restoration study by Kleiman et al. found a lower
258 alpha (within-sample) diversity at both T1 and T2 compared to controls indicating
259 the number of observed species in the analysed faeces sample [37]. Moreover,
260 Kleiman et al. found a significant association between alpha diversity and
261 depression and eating disorder psychopathology [37]. Changes from T1 to T2 in
262 persons with AN, i.e. beta diversity (between-sample diversity), was also reported
263 by Kleiman et al., however, the alpha diversity remained significantly lower after
264 weight gain than the observed diversity in controls [37]. In the case series, which
265 followed three female patients with acute AN through hospitalization and weight

266 restoration, significant changes in magnitude of composition and diversity on phylum
267 to genus levels were observed, however, these changes were found to be patient
268 specific and not common changes in the three patients [45]. They also measured
269 the resting energy expenditure (REE) and diet-induced thermogenesis in the three
270 patients, which both increased during weight gain, but was not significantly
271 associated with diversity or composition of the gut microbiota.

272 Common findings in the acute stages of AN with regard to specific microbiota were
273 that the phylum Bacteroidetes was low in AN in two studies [37,38]. Conversely, two
274 other studies showed that Bacteroidetes also was decreased (or trending towards
275 decreased) in obese individuals [40,39]. The phylum Firmicutes was increased in
276 AN in three studies [39,37,38] and decreased in one study [42].

277 The genus *Methanobrevibacter* and specifically, on the species level, *M. smithii* was
278 increased, when present, in AN patients compared to normal-weight participants in
279 three studies [40,39,38]. Mack et al. detected species belonging to the genus
280 *Methanobrevibacter*, of which *M. smithii* is the most common species in the human
281 gut, in 22 % of patients with AN at T1, which was higher than the proportion of AN
282 patients with *Methanobrevibacter* at T2 (14 %), and *Methanobrevibacter* was found
283 in 15 % of controls [38]. The relative abundance of *Methanobrevibacter* was
284 statistically higher in the 22 % of AN patients at T1 than the 15 % of controls
285 ($p=0.004$). In the study by Million et al. *M. smithii* was detected in 64 % of all
286 participants including AN patients, normal-weight controls, and obese participants
287 (BMI>25), and *M. smithii* concentrations were higher in participants with BMI<25
288 ($p=0.008$) with a trend towards a correlation between a higher BMI and lower *M.*
289 *smithii* concentration ($p=0.08$). In line with this, Armougom et al. detected *M. smithii*
290 in 100 % of the AN patients and 75 % of lean participants and found *M. smithii*

291 statistically increased in AN compared to the lean participants ($p=0.0171$) [39], and
292 Borgo et al. found a significantly higher average of genome copy number of *M.*
293 *smithii* in their AN group compared to controls [42].
294 The case study examined the microbial composition of a faeces sample from a 21-
295 year-old Caucasian woman with a severe case of AN with a BMI of 10.4 kg/m²[44].
296 19 new microbial species never previously observed were found, of which 11 were
297 isolated and sequenced. Of these, 7 species belonged to the phylum Firmicutes, 2
298 belonged to the phylum Bacteroidetes, and 2 belonged to the phylum
299 Actinobacteria. Interestingly, *M. smithii* was not identified in the faeces of the patient
300 in contrast to the other studies that found an increase in this species in the acute
301 stages of AN [39,40,38,42].
302 Apart from that, no clear patterns were detectable with regard to microbiota in the
303 nine selected studies.

304

305 ***Effects of weight restoration***

306 Diversity and richness was initially normal and increased after weight gain in one
307 study [38]. Kleiman et al., reported a lower diversity both at baseline and after
308 inpatient weight restoration [37]. Duration of inpatient stay was defined in one study
309 [38] as 14.0±6.8 weeks (mean±SD) with BMI at admission of 15.3±1.4 and at the
310 end of treatment 17.7±1.4 (mean±SD). In the other study [37] duration of stay was 4
311 weeks (Dr. Ian Carroll, personal communication) and BMI at admission was
312 16.2±1.5 and 17.4±0.9 (mean±SD) at endpoint. In the case series duration of stay
313 varied from 34 to 73 days and, as mentioned before, changes in diversity and
314 composition were largely patient specific and no common trend was observed [45].

315 With regard to specific microbiota, the relative concentration of Bacteroidetes was
316 found low at T1 and further decreased at T2 in AN compared to healthy controls in
317 one study [38]. *M.smithii* and the mucin degrading genera *Verrumcomicrobia* and
318 *Bifidobacteria* were found to be increased in AN at baseline (T1) compared to
319 controls in one study [38], and the study by Kleiman et al., also found lower
320 abundances of Bacteroidetes in AN after weight restoration compared to controls
321 [37]. Firmicutes was increased compared to controls after weight restoration in both
322 weight restoration studies in AN [38,37].

323

324 ***Relation to clinical symptoms***

325 One weight restoration study found an improvement in total gastrointestinal (GI)
326 scores (reflecting complaints) after weight restoration, although most upper and a
327 few lower GI symptoms such as abdominal pain and bowel noises did not change
328 [38], and no correlations between GI symptoms and microbiota measures were
329 found. The other weight restoration study found an association between alpha
330 diversity in AN and levels of depression, anxiety, and eating disorder
331 psychopathology at baseline [37].

332 With regards to correlations between psychiatric symptoms measured on the BDI
333 scale and specific microbiota, Borgo et al. found a negative correlation between
334 *Clostridium spp.* and depression score, and, in addition, a negative correlation
335 between faecal butyrate concentration and depression and anxiety scores [42].

336 Breton et al. examined the role of ClpB protein concentrations in plasma and its
337 correlations to clinical symptoms in 24 patients with restrictive AN, 29 patients with
338 bulimia nervosa, 13 patients with binge-eating disorder, and 29 gender-matched
339 controls [43]. ClpB protein is produced by *Enterobacteriae* such as *Escheria coli* and

340 has been found as a conformational mimetic of alfa-Melanocyte Stimulating
341 Hormone (alfa-MSH), which is thought to be involved in satiety and anxiety [46].
342 Indeed, Breton et al. found that ClpB protein concentrations correlated positively
343 with alfa-MSH-reactive IgG for all patients with eating disorders and an increase in
344 ClpB protein concentrations was found in plasma in eating disorder patients
345 compared to plasma in controls, and that ClpB protein concentrations were
346 significantly correlated with several subscales on the Eating Disorder Inventory-2
347 (EDI-2) for all patients with eating disorders and the Montgomery-Åsberg
348 Depression Rating Scale (MADRS) total score and specifically the anhedonia score
349 for AN patients ($p < 0.05$). The study adds evidence to the potential role of ClpB
350 protein produced by *Enterobacteriae* in the gut and its impact on the brain and
351 psychopathology in eating disorders.

352

353 Mörkl et al. examined the faecal microbiota from five groups; 18 inpatients with AN,
354 20 athletes, 22 overweight participants, 20 obese participants, and 26 normal-weight
355 controls [36]. They found a lower alpha diversity in AN and obese participants
356 compared to other groups, and that the athletes had the highest alpha diversity. In
357 addition, they found that greater levels of depression measured on the BDI
358 correlated with a lower alpha diversity, when all groups were included in the analysis
359 ($p = 0.032$). Beta diversity was associated with several parameters, and beta diversity
360 was significantly associated with several different measures of body fat ($p < 0.05$),
361 smoking status ($p = 0.002$), and cholesterol-HDL ratio ($p = 0.024$).
362 The phylotype Coriobacteriaceae was the only enriched phylotype in AN compared
363 to other entities. Collectively, the authors concluded that their results were evident of
364 a gut dysbiosis in AN.

365

366 **Discussion**

367 Individuals with AN are known to have a highly variable weight gain during
368 therapeutic renourishment, and the factors that contribute to this variability are
369 currently unclear [47]. Factors such as increased (and secretive) physical activity
370 [48] and diet-induced thermogenesis have been proposed [49]. Thereby, it is
371 reasonable to hypothesize that AN patients may have a microbial imbalance in their
372 gut that in part contributes to the poor response to weight gain observed during
373 treatment.

374

375 Overall, there are few studies investigating the microbiota in AN, all are
376 experimental, and they differ with regard to design and results. In this review, we
377 have attempted to identify both common and divergent features in order to inform
378 future investigations.

379 Regarding abundance of microbiota, two studies found a normal abundance of
380 microbiota in AN [40,38], while two studies found a reduced and altered abundance
381 of microbiota [41,42]. The diverging results make an interpretation difficult.

382 In four studies, the diversity of the microbiota was compared to controls and
383 explicitly described. Two of these studies found a normal microbial diversity in AN
384 and in the weight restoration study by Mack et al. diversity further increased after
385 weight gain [38,42]. The third study found a lower alpha diversity compared to
386 healthy controls that was still significantly lower upon weight gain [37], and Mörkl et
387 al. also found a low alpha diversity in AN patients and in obese individuals. There
388 was, however, a major difference between the two weight restoration studies in the
389 duration of follow-up, differing by approximately 10 weeks. This may have had an

390 impact on the ability of the microbiota to adjust to environmental factors. However,
391 with only these four studies, it is too early to draw any firm conclusion on the
392 diversity of the microbiota in AN.

393

394 With regard to specific changes in microbiota species, there were some findings that
395 may indicate the trend of change in AN. The phylum Firmicutes was found
396 increased in AN in three studies [39,37,38], and Bacteroidetes was low in AN in two
397 studies [37,38], although potentially conflicting findings were made in two other
398 studies, where Bacteroidetes was found to be decreased (or trending towards
399 decreased) in obese individuals [40,39] and Firmicutes was found to be decreased
400 in one study [42]. Another study found an increase in the phylum Coriobacteriaceae
401 [36]. However, these findings may also reflect that it is dysregulation per se, which
402 may be at a similar level and direction in AN and obese individuals, and not specific
403 BMI scores, that is related to the composition of the microbiota. Interestingly, the
404 genus *Methanobrevibacter*, specifically *M.smithii*, was also increased, or trending
405 towards an increase when present in patients with AN or participants with BMI<25 in
406 four studies [40,39,38,42].

407 *M. smithii* is involved in the breakdown of polysaccharides from vegetable sources
408 and the finding of this specific Archaeon could illustrate an adaptation to a typical
409 diet rich in vegetables and fruits in persons with AN. However, methanogenic
410 Archaea, such as *M. smithii*, have also been linked to constipation, a common
411 complaint in patients with AN, which statins have been shown to alleviate by
412 suppressing the growth of methanogens [39,50-52]. The evidence of *M. smithii* in
413 faeces from constipated patients necessitate further investigation of whether this

414 finding in AN patients is only related to constipation or also related to AN
415 psychopathology as a potential biomarker.
416 There were additional changes found in the different studies, but no clear additional
417 patterns were detectable with regard to specific microbiota in the nine selected
418 studies. 5 out of the 9 studies that examined the microbiota in faeces samples
419 reported microbial changes on the species level extensively, while the remaining
420 four studies reported mainly findings on phylum to genus level, which may be too
421 broad [38,45,37,36]. Differences in microbial species may better reflect changes that
422 are related to specific biological effects [53].

423

424 With regard to the effect of weight gain on specific microbiota, Firmicutes was
425 increased after weight restoration in both studies in AN [38,37]. Furthermore, the
426 Bacteroidetes was found low before weight gain and decreasing in AN in one of the
427 studies [38], while the other found a decreasing level of Bacteroidetes after weight
428 restoration [37]. Only in one of the weight restoration studies were *M.smithii* and the
429 mucin degraders *Verrumcomicrobia* and *Bifidobacteria* increased before weight
430 gain, a finding that was not replicated after weight gain in AN [38]. The only
431 significant finding after weight gain, as interpreted from the two studies, was an
432 increase in the phylum Firmicutes. To conclude on specific microbiota differences in
433 the acute stages of AN compared to controls, only one finding on the species level
434 remains substantial and concrete; an increased concentration of the Archaeon
435 *M.smithii*. However, on the phylum level Firmicutes was consistently shown to be
436 overrepresented and specific species results from this phylum could be expected in
437 the future.

438

439 9 of 10 studies included in this review collected faeces samples i.e. reflecting mainly
440 the colorectal microbiota. Differences in the microbiota in the distal parts of the gut,
441 when taken from faeces samples, may not relate in a meaningful way to all relevant
442 biological functions, since faeces mainly contains a mixture of Bacteria from the
443 various compartments of the colon and some Bacteria from the distal ileum, and
444 therefore faeces samples are proxies for the gut microbiome rather than displaying
445 true host-microbe interactions [54]. In the large intestine resorption of fluid and the
446 forming of faeces take place and Bacteria are kept at a distance from the epithelial
447 cells by the mucus coat [55,56]. Theoretically, another interesting location for
448 microbiota sampling in AN, in addition to faeces samples, would be the small
449 intestine, notably the ileum, where there is a microbial flora and where the major
450 breakdown of food and absorption of nutrients take place. Furthermore, Bacteria
451 and Archaea from the small intestine are subjected to a harsh environment with fast
452 transit time, digestive enzymes, and bile, and therefore largely contrast the
453 environment in the colon, demanding more resilient inhabitants in the small intestine
454 with different survival strategies, and these microbes are also subjected to
455 breakdown through the digestive tract [28]. Any reported findings in faeces might
456 therefore not represent the microbiota in the small intestine. A study by Vandeputte
457 et al. showed that faecal microbial richness was decreased in female patients with
458 faster intestinal transit time measured with Bristol Stool Scale (BBS) as a proxy of
459 intestinal transit time[57]. Thus, the patients with diarrhoea had the least diverse
460 faecal microbiota, and different enterotypes existed in patients with different BBS
461 scores. In loose stool the *Prevotella* enterotype dominated, while the
462 Ruminococcaeae-*Bacteroides* enterotype, which includes the genus
463 *Methanobrevibacter* with the main species *M. smithii*, dominates in harder stool.

464 This supports the repeated findings of *M. smithii* in AN patients and suggests *M.*
465 *smithii* to be perhaps correlated mainly with transit time, which is frequently
466 decreased in AN, rather than with AN psychopathology. Interestingly, Archaea
467 including *Methanobrevibacter* and *M. smithii* have been found to be below detection
468 limit in ileal effluent from ileostomy patients [30] suggesting this specific Archaeon
469 might dominate mainly in the colorectum and less in the small intestine.

470 Vandeputte et al. concluded that transit time may be a selective force on microbial
471 life strategies. In line with this, different transit times within the individual GI tract
472 may promote growth of different species and offer less diversity in compartments
473 with faster transit, i.e. the small intestine. In line with this, several studies have found
474 significant differences in microbiota composition between different compartments in
475 the GI tract in patients with ileostomy, autopsy patients, and patients undergoing
476 both gastro-duodenoscopy and colonoscopy [58,30-32], and studies have found
477 separate clusters of bacteria at both family and species taxonomic levels, when
478 comparing colonic and rectal mucosal samples with faeces in healthy control
479 persons and patients with IBS [35,29,33,59,34]. Thus, different microbial
480 compositions exist within different intestinal compartments and even between
481 faeces and rectal mucosal samples indicating the importance of intestinal mucosal
482 biopsies for microbiota analysis in specific compartments.

483 From an immunological point of view, the small intestine is also an interesting
484 location as the microbiota is important for immunological homeostasis and
485 susceptibility to immune-mediated diseases and disorders through the Peyer's
486 patches and other parts of the gut-associated-lymphoid-tissue (GALT), which are
487 prominent in the small intestine and constitute a major line of defence against
488 pathogens in the GI tract [60,61].

489 To retrieve samples from the small intestine, one will need to use pinch or
490 submucosal biopsy from endoscopy [54], alternatively using more novel
491 technologies, such as capsule endoscopy, which need further research before
492 implementation [62].

493 However, despite the several studies that point at the small intestine as an
494 immunologically and metabolically important anatomical location for AN, faeces
495 samples remain the easiest to collect and should continue to be the standard
496 approach to analyzing the gut microbiota until the use of more minimally invasive
497 approaches, such as capsule endoscopy, are further developed.

498

499 The mechanistic link between gastrointestinal illnesses and psychiatric disorders
500 has been well-established[63]. Raevouri et al. found an increased prevalence of
501 autoimmune disorders in patients with eating disorders, which could possibly be
502 caused by alterations in the gut microbiota, while another study found that early life
503 stress altered the microbiota, the systemic immune responses, and resulted in an
504 elevated HPA-axis function in a rat model [26,64]. It has also been established in a
505 systematic review that more than 50 % of patients with IBS also meet the criteria for
506 mood disorders[65].

507 The relation between weight gain and clinical symptoms were assessed in two
508 studies [38,37] and one weight restoration study found that total GI scores
509 (reflecting complaints) were improved by weight gain, although individual symptoms
510 did not change [38], while the other weight restoration study described an
511 association between within-sample alpha diversity and levels of depression, anxiety,
512 and eating disorder psychopathology in AN at baseline [37]. Borgo et al. also found
513 a negative correlation between depression and *Clostridium spp.* ($p=0.089$) and an

514 inverse correlation between faecal butyrate concentration and depression
515 ($p=0.0379$) and anxiety ($p=0.0206$) scores in AN patients compared to controls,
516 while Breton et al. found correlations between ClpB in plasma and several
517 subscales on the EDI-2 in all eating disorder patients and with MADRS total score
518 and anhedonia score in AN patients when compared to controls (both $p<0.05$) [42].
519 These findings may support that it is the gut-brain-axis that is underlying, or at least
520 involved, in these symptoms in AN, and ClpB in plasma potentially provides an
521 interesting link between the gut and the brain. The gut–brain axis has been
522 described as a bidirectional communication network that monitors and integrates gut
523 functions and connects them to cognitive and emotional centres of the brain. This
524 network includes the central, autonomic, and enteric nervous systems, in addition to
525 the neuroendocrine, enteroendocrine, and neuroimmune systems [66]. Furthermore,
526 the network might mediate both the effects of genetic and environmental factors on
527 brain development and function, and has been proposed to be involved in the
528 aetiology of several psychiatric disorders [67]. Albeit early findings, which require
529 validation in repeated and larger studies before any clear conclusions can be made,
530 they point in a direction for the design of future microbiota studies, which should
531 include the assessment of psychiatric symptoms in AN over time.

532

533 There were a number of potential weaknesses in all studies included. All studies
534 included were conducted in adults except the study by Mack et al. and Patient C in
535 the case series[45], and AN usually has an onset in the adolescence, which may
536 imply that the results were the effects of long standing undernutrition, and/or a
537 selected diet, i.e. as caused by mainly external factors and less from any internal
538 inherent morphological or structural deviation. However, it may also be inherent

539 morphological or structural deviations that explain differences between adolescents
540 and adults with AN, or why the disease has persisted into adulthood. It is also
541 possible that observed abnormalities in the microbiota in adults with AN are absent
542 in their adolescent counterparts as a result of different hormonal levels, or it may
543 relate to other maturational effects. Another limitation of the studies is that all
544 studies have included females only, neglecting the fact that 10% of AN patients are
545 males, which make the results hard to extrapolate to a male population with AN,
546 though limiting the population to females only reduces the risk of sex as a
547 confounder in the studies.

548 All of the studies were experimental and albeit several were well designed, the
549 studies were in general not designed to adjust for potential bias. For the sake of
550 compliance with the rules of systematic reviews, we included a bias analysis (see
551 link in methods section to our online protocol). We found that recruitment was
552 selective e.g. based upon available patients at the ward, and that selection of
553 healthy controls was biased in some studies e.g. including available staff or family
554 members of staff. This may imply that the controls used may not reflect changes in a
555 general/normal population, and using control persons that are genetically related
556 might underestimate potential findings as the intestinal microbiota might also be
557 genetically based and certain Bacteria and Archaea might be shared within families.

558 Furthermore, the choice of timepoint for investigation in the cross-sectional studies
559 and other factors such as sample collection procedures, calorie intake and contents
560 of the diet [68], activity level, and medication were not always specified and may
561 account for variation between studies. In addition, transit time, i.e. stool consistency,
562 varies from person to person and has been shown to have a significant impact on

563 microbial composition [57], and only one study accounted for stool frequency
564 measured with the Gastro-Questionnaire [69].
565 In the weight restoration studies, calory intake was only controlled for in the study by
566 Mack et al., apart from the case series, where calory intake was measured
567 extensively throughout hospitalization [45]. Additionally, activity level were in general
568 also not controlled for, which may have influenced bowel movements and energy
569 expenditure. Furthermore, not all studies examined the abundance of microbiota,
570 and the duration of weight restoration in the two large longitudinal studies was not
571 equal [38,37], and in the cross-sectional studies severity and duration of AN were
572 not consistently specified throughout the studies, why these may have differed
573 substantially and contributed to differences in the results [40,39,41-43,36]. Only one
574 individual was studied in the case study [44] and three individuals in the case series
575 [45] and the relevance on a great scale to the characteristics of the microbiota in AN
576 and replicability therefore remain limited.

577 Potential differences in the methods used to determine the microbiota, i.e.
578 differences in sampling, nucleic acid extraction, and analysis techniques etc. can
579 also have contributed to the variability in results [54].

580 Other limitations were that not all studies examined the abundance and diversity of
581 microbiota, why a potential link between overall abundance and diversity of
582 microbiota and AN remain undefined. In addition, different clinical aspects were
583 investigated in the two large weight restoration studies making their results hard to
584 compare.

585

586 **Conclusion**

587 Few studies have examined the microbiota in AN, and all studies thus far have been
588 experimental, and hypothesis generating. Larger, controlled studies will strengthen
589 the validity of the results and should be a clear recommendation for future studies.

590 Future studies should focus more on reporting specific microbial species either
591 through marker gene analysis based on an amplicon of a single gene or through
592 metagenomic sequencing, which attempts to sequence all or most genes in a
593 sample.

594 An issue raised in this systemic review is that faeces samples may not optimally
595 reflect differences in the microbiome that are biologically relevant for AN as they are
596 proxies for the microbiome in the intestinal microbiota rather than reflecting true
597 host-microbe interactions in the various gut compartments. It is proposed that future
598 studies on the microbiota in AN in addition to faeces samples consider collecting,
599 when possible, faeces biopsies from the small intestine, where breakdown and
600 absorption of nutrients occur, and where a large impact of the microbiota on
601 biological functions, and thereby symptoms and signs, is likely to occur. However,
602 analyzing the microbiota from faeces samples remains to date the most convenient,
603 minimally invasive, and easiest obtainable way to analysing the microbiota in AN
604 and potentially finding a biomarker. The intestinal microbiota in AN is an interesting
605 field and has yet to be fully unraveled.

606

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610

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614

615 **Conflict of interest**

616 The authors declare no conflict of interest.

617

618 **Ethical approval**

619 This article does not contain any studies with human participants or animals

620 performed by any of the authors.

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625

626 **Table and figures:**

627

628 - Table 1. Title: Microbiota studies in anorexia nervosa.

629 - Figure 1. Title: Flow diagram of the Study Selection.

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