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► **To cite this version:**

Quinten Ducarmon, Franziska Grundler, Yvon Le Maho, Françoise Wilhelmi de Toledo, Georg Zeller, et al.. Remodelling of the intestinal ecosystem during caloric restriction and fasting. Trends in Microbiology, 2023, 31 (8), pp.832-844. 10.1016/j.tim.2023.02.009 . hal-04237362

HAL Id: hal-04237362

<https://hal.science/hal-04237362>

Submitted on 11 Oct 2023

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Review

Remodelling of the intestinal ecosystem during caloric restriction and fasting

Quinten R. Ducarmon,¹ Franziska Grundler,² Yvon Le Maho,^{3,4} Françoise Wilhelmi de Toledo,² Georg Zeller,^{1,*} Caroline Habold,^{3,*} and Robin Mesnage ^{2,5,*}

Benefits of fasting and caloric restriction on host metabolic health are well established, but less is known about the effects on the gut microbiome and how this impacts renewal of the intestinal mucosa. What has been repeatedly shown during fasting, however, is that bacteria utilising host-derived substrates proliferate at the expense of those relying on dietary substrates. Considering the increased recognition of the gut microbiome's role in maintaining host (metabolic) health, disentangling host–microbe interactions and establishing their physiological relevance in the context of fasting and caloric restriction is crucial. Such insights could aid in moving away from associations of gut bacterial signatures with metabolic diseases consistently reported in observational studies to potentially establishing causality. Therefore, this review aims to summarise what is currently known or still controversial about the interplay between fasting and caloric restriction, the gut microbiome and intestinal tissue physiology.

Background

Gut microorganisms rely largely on energy derived from the human diet to perform metabolic functions essential for their survival, and the gut microbiome composition therefore rapidly responds to the food we eat [1]. A decrease in the nutrient flow in the gastrointestinal tract, caused by **caloric restriction** (see [Glossary](#)) or **fasting**, can thus have far-reaching effects on gut microbes and our physiology. From an evolutionary perspective, long periods of fasting are physiological: many animal species and some human communities alternate between periods of fasting and **hyperphagia** according to seasonal variations in food availability or because of religious beliefs (e.g., Ramadan).

Caloric restriction (a reduction in caloric intake) and fasting (the voluntary abstention from eating) are terms sometimes wrongly used interchangeably for various diets which include modest to severe reductions in caloric intake, with different macronutrient profiles, of varying durations and frequencies [2]. The metabolic switch from utilising glucose to stored energy (i.e., glycogen and fat) for energy generation is associated with improvements in various clinical conditions, such as chronic inflammatory disorders and metabolic syndrome. This works, at least partly, by lowering risk factors such as high blood glucose, high cholesterol levels, or high systolic blood pressure [2,3]. Fasting also has potent effects on the immune system [4], including in the gut, but the complexity of these effects is evidenced by different forms and lengths of fasting having distinct and sometimes opposite effects on the immune system [4].

Despite the increasing amount of knowledge acquired in the past decades about the nutritional dependence between gut microbes and host tissues, how fasting relates to intestinal health is still unclear. Although the number of publications is rapidly increasing, some report seemingly contradictory results such as studies concluding that fasting could either disrupt [5] or enhance [6] **colonisation resistance** against exogenous pathogenic bacteria. Other studies concluded

Highlights

Remodelling of the gut and microbiome adaptations to fasting are evolutionarily conserved and comparable in humans and animals (including in hibernators).

During fasting, bacteria utilising host-derived substrates proliferate at the expense of those relying on dietary substrates.

Human studies have focused on faecal samples, thereby neglecting intestinal tissue remodelling.

During fasting, the intestinal mucosa is renewed concomitantly to changes in immune function and a decreased inflammatory state.

Whether the gut microbiome can mediate systemic effects of fasting is still unclear.

Animal studies have limited relevance since rodents with a high metabolic rate can rapidly enter starvation.

Knowledge gaps include how the microbiome is rebuilt after food re-introduction and how overall caloric restriction and specific macronutrients contribute to this process.

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that fasting can mitigate [7] chemotherapy-induced gastrointestinal mucositis or worsen it [8]. A large number of studies have been performed in experimental animals, in laboratory and wild-life settings (including hibernation), with results that are not always easy to reconcile with human studies. It is often not clear if experimental animals are in Phase 2 or 3 of the fasting metabolism, as small model organisms can rapidly enter situations of **starvation** due to their high metabolic rate and low body weight (Figure 1A). In humans, few randomised controlled trials were performed after the administration of very-low-calorie diets [5] or calorie restrictions [9].

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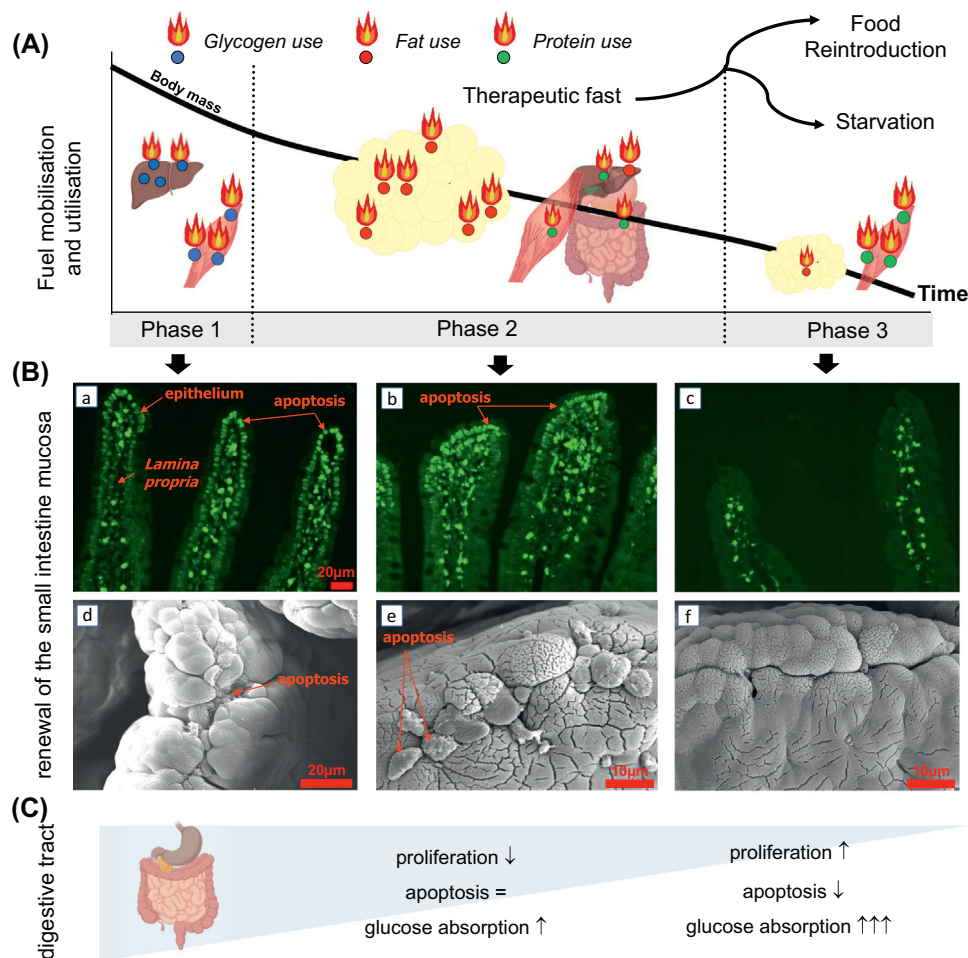


Figure 1. Remodelling of the intestine during the three phases of fasting. (A) During Phase 1, the digestive tract is emptied, and glycogen stores are exhausted [13,118]. This is a short phase, lasting 1 day in humans, and only a few hours in rats. Phase 2 corresponds to fat store mobilisation for energy expenditure, whereas body proteins are mostly spared. Phase 3 is characterised by an increasing protein catabolism mediated by a rise in plasmatic corticosterone levels. Prolonged starvation leads to compromised immune function [119]. In humans with normal weight, Phase 3 is encountered after around 40 days. During therapeutic fasting, people should not go beyond Phase 2. (B) Morphological changes in the intestine between fed and fasted rats can be observed. Localization of apoptosis at the tip of the villi of fed (a) and Phase 2 fasted rats (b), but not in Phase 3 (c) using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Apoptotic cells being extruded into the intestinal lumen are visible in scanning electron microscope (SEM) images in fed (d) and Phase 2 fasted rats (e), but not in Phase 3 (f). Data generated as previously described [17,101]. (C) Phase 2 is a phase of energy sparing with low basal metabolic rate and resting of the small intestine (with decreased cell proliferation and protein synthesis), whereas Phase 3 is characterised in animals by an anticipatory resumption of cell proliferation and arrest of apoptosis to enable immediate macronutrient absorption, especially glucose and peptides, at re-feeding.

Hence, our current understanding of the effects of therapeutic fasting is mostly derived from observational studies [10–12].

In this review we discuss the impact of varying duration and types of caloric and **dietary restrictions**, such as differences between fasting Phases 1 and 2 (Figure 1A) and the later Phase 3, which corresponds to starvation in humans [13]. We first provide a short overview of intestinal remodelling. Changes in the gut microbiome need to be evaluated in the light of remodelling of the intestinal epithelium, because the loss of control over the microbial environment is increasingly shown to be a driver of dysbiosis, rather than the presence or absence of specific microorganisms [14].

Altogether, this review summarises the relationships between caloric restriction, intestinal cellular metabolism and physiology, and gut microbes. This is necessary to better contrast our current understanding of host–microbial symbioses with existing knowledge gaps, thereby identifying promising directions for future research.

The interplay between intestinal tissue remodelling and the gut microbiome

Impacts of the digestive system atrophy

The intestines of mammals appear to share a common response to long periods of fasting, namely, transient atrophy of the intestinal tissue. For example, the mucosal mass was decreased by 50% in rats during fasting [15]. This intestinal atrophy allows for significant energy saving, since maintenance of a functional gut represents 20–30% of the basal metabolic rate of a fed individual [16]. Due to a concomitant decrease in mitosis in crypts and in cell migration rate [17], in addition to an increased rate of **apoptosis** and **autophagy** (Box 1) [18,19], the intestinal epithelium is resorbed during long-term fasting (Figure 1B,C). This profound remodelling of the intestinal epithelium during fasting has long been thought to be the main contributor to changes in the gut microbiome. However, comparative analysis of the response to fasting in vertebrates suggested that the decrease in nutrient availability, but not the structural reorganisation, is the main factor causing changes in the gut microbial ecosystem [20].

Epithelial permeability and the integrity of the gut barrier

A variety of microorganisms and their metabolites (e.g., indole derivatives and butyrate) regulate the integrity of the gut barrier through the induction of an immune response [21]. There are controversies as to how fasting affects gut barrier integrity and how gut bacteria are involved in this process. Some studies have suggested that water-only fasting increases gut permeability in a colitis murine model [22] or in rats starved for 72 h [23]. An increase in gut permeability seems to be counteracted by a low calorie intake in humans [10] but also in animal models fed fasting-mimicking diets [22]. By contrast, a body of evidence indicates that caloric restriction in healthy conditions can improve intestinal barrier permeability in mice [24] or humans following a 4-week very-low-calorie diet [25]. During hibernation, the intestinal barrier function seems to be preserved by an increase in the number of tight junctions and occludin phosphorylation [26].

Box 1. Circular economy in the intestine: the activation of autophagy

The induction of autophagy is frequently proposed to be a mechanism by which fasting has anti-ageing effects. The cytoplasm in the basal part of the enterocytes of starved rats contains a large number of autolysosomes, suggesting the activation of autophagy [98]. Autophagy activation is attributed to the inhibition of the nutrient sensing mammalian target of rapamycin (mTOR) pathway [99]. Microbial metabolites can also activate autophagy via stimulation of the immune system. Inhibition of mTOR promotes stem-cell activation, ultimately leading to intestinal regeneration [100]. This regeneration is visible prior to food reintroduction in rats entering Phase 3 (Figure 1B) of the fasting metabolism [17]. In contrast to the reduced cell activity in Phase 2 (Figure 1B), Phase 3 fasting is marked by a significant increase in DNA replication and mitosis in crypt cells and in the cell migration rate [17]. This increased cell turnover rate in Phase 3 of fasting has been interpreted as an anticipation of re-feeding. Although very costly energetically, it should indeed allow for the migration and rapid renewal of enterocytes in the villi, ensuring optimal absorption during re-feeding [101].

Glossary

16S rRNA gene amplicon

sequencing: sequencing of a PCR-amplified region of the 16S rRNA gene (V1–V9), which is conserved in bacteria and archaea, in order to infer taxonomic classifications based on the sequences of those amplicons.

Apoptosis: programmed cell death (essentially cell suicide) which results in the efficient clean-up of damaged cells and is part of normal cell turn-over in an organism to maintain homeostasis.

Autophagy: programmed destruction of cell components, such as organelles and cytoplasmic components. This process takes place in lysosomes and allows cells to re-use these components.

Caloric restriction: dietary intervention that restricts total caloric intake.

Colonisation resistance: the mechanisms employed by the gut microbiome that contribute to resistance against colonisation by exogenous microorganisms.

Dietary restriction: dietary intervention that restricts specific macronutrients.

Fasting: voluntary or season-dependent abstinence or strong limitation of caloric ingestion for a limited period of time. This can be for religious or therapeutic purposes, or in animals in accordance with circadian and seasonal rhythms. The two main forms of fasting are intermittent fasting (some hours over the night, daily) and long-term fasting (some days or weeks, generally once a year). Different forms of nutritional strategies displaying some of the effects of fasting reduce the caloric intake, one or several macronutrients, and the eating time window. The duration and frequency of fasting can vary. Some fasting programmes allow the ingestion of a limited amount of calories in the form of juices or soups (200–250 kcal/day in the Buchinger-Wilhelmi fasting programme) while some do not (water-only fasting). A higher daily caloric intake, over 500 kcal per day, is generally not considered as fasting.

Gluconeogenesis: the process of glucose synthesis from noncarbohydrate precursors.

Hyperphagia: uncontrolled, excessive appetite and eating.

Metagenomic shotgun sequencing: sequencing all the DNA in a particular sample, which allows for both taxonomic profiling and functional profiling of the microbiome.

It is sometimes postulated that mucin consumption by *Akkermansia muciniphila* might negatively impact the integrity of the gut barrier, exacerbate inflammation, and heighten risk for infection [27]. However, mucin foraging by *A. muciniphila* was found to stimulate host mucin production and ultimately restore mucus layer thickness in diet-induced obese mice [28]. Wildlife studies suggest that physiological mechanisms ensuring a protection of the gut barrier have emerged during coevolution between gut microbes and animals (Box 2). For instance, mucin production increases after 1 month of hibernation in squirrels as measured by MUC2 expression [29].

Adaptation of the intestine to food reintroduction

In general, studies in humans are focusing on the caloric restriction period but tend to neglect the food reintroduction phase. In a study on 15 men doing a 10-day fasting, the levels of inflammatory cytokines increased after food reintroduction, suggesting a reactivation of the postprandial immune response [10]. This is also observed in winter fasting in hibernating squirrels [30]. The dietary strategy for food reintroduction seems key to managing this inflammatory period, as suggested by studies showing that the reduction in inflammation and pain observed in rheumatoid arthritis patients persists if the food reintroduction consists of a vegan diet [31].

Microbiota: all the living members of a microbiome.

Starvation: limitation of caloric ingestion causing suffering and leading to organ damage when endogenously stored fat is exhausted.

Torpor: a physiological state with reduced metabolism and body temperature.

Box 2. Lessons from wild animals

Hibernating mammals endure long periods of fasting, from a few days in food-storing species that re-feed between two episodes of **torpor**, like chipmunks and hamsters (i.e., a spontaneous phase of hypometabolism and hypothermia), to several months in fat-storing species like marmots and ground squirrels, which rely solely on their adipose tissue reserves to cover their energy needs in such periods; reviewed in [102]. These fat- and food-storing species differ in their digestive responses to these different feeding behaviours during hibernation, but in both cases, optimal nutrient absorption is observed upon refeeding [103,104].

The changes in gut microbiome composition during fasting are similar to those of hibernating mammals [30], with, for example, increased relative abundance of Bacteroidetes and decreased Firmicutes. This shift between periods of feast and fast can even affect host evolutionary trajectories because the products of microbial metabolism can increase host fitness. For instance, the summer microbiota in bears promotes adiposity by increasing energy harvest from the diet, and also improved glucose tolerance favouring the adaptation to hyperphagia [105]. Furthermore, a recent study in thirteen-lined ground squirrels shows the ability of the gut microbiome to recycle nitrogen from urea, which prevents protein imbalance and preserves lean body mass during hibernation fasting [50]. There are, however, differences across vertebrate classes which could depend on their physiology (e.g., body temperature) or their diets [34]. For instance, the diversity of the gut microbiota of hibernating dwarf lemurs is maintained, in contrast to what is observed in hibernators under temperate latitudes [106]. However, bacterial diversity in these lemurs is reduced during fattening due to blooms of Mollicutes, which specialise in scavenging simple sugars [106].

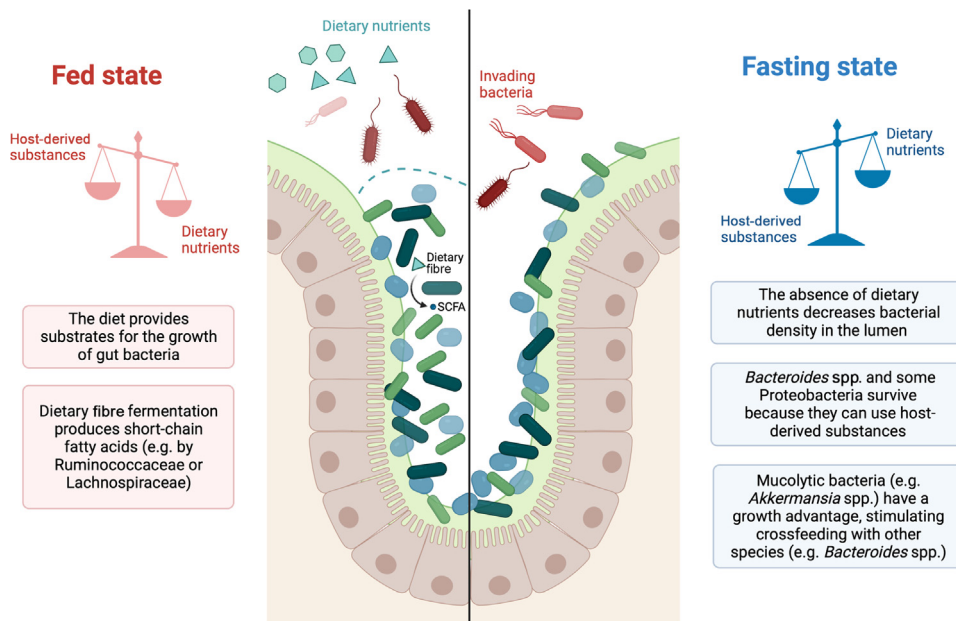
From animals that fast spontaneously in the wild, we learnt that subsequent re-feeding does not cause dysfunction of the digestive system. On the contrary, the absorption of nutrients is even more efficient in some cases. In Phase 3 fasting rats, for example, the gut is preparing for rapid nutrient intake at re-feeding by an increase in the protein level of peptide/H⁺ cotransporter PepT1 and glucose/Na⁺ cotransporter SGLT1. When re-feeding, glucose can then be immediately absorbed at low concentrations through SGLT1, released to the bloodstream via a glucose-6 phosphatase-related pathway, and used as an energy source, whereas peptides should rapidly provide body protein precursors. Increased levels of fatty acid transporter protein 4 (FATP4) and increased synthesis and recruitment of the glucose transporter GLUT2 to the apical membrane of enterocytes upon re-feeding allow for the uptake of large amounts of fatty acids and glucose, respectively. Also, an enhanced gluconeogenic activity is found in the small intestine in fasting Phase 3, when blood glucose is at its nadir [101,107]. This increase in intestinal **gluconeogenesis** is probably enabled by the release of gluconeogenic amino acids coming from catabolism of body proteins. Another remarkable example of the efficiency of the gut function after a fast is that of hibernators. While there is a decrease in gut mass in fat-storing animals [108], gene expression of some gut enzymes and transporters is maintained throughout hibernation [109,110]. In food-storing species, the intestinal morphology is even preserved during hibernation [111], which allows a rapid assimilation of nutrients during the short inter-torpor arousal phases [104]. There is even a positive correlation between the duration of torpor (and therefore of fasting) and digestive efficiency in these species [112]. The speed at which nutrients are assimilated is crucial for these animals because it determines their physical condition when they emerge from hibernation, on which their ability to reproduce as quickly as possible depends.

Decreased lipopolysaccharide (LPS) biosynthesis has been proposed as a link between gut-microbiome-mediated effects of caloric restriction and metabolic improvements, as LPS acts upon the immune system. In mice, gut **microbiota** alterations induced by caloric restriction suppressed LPS biosynthesis [32]. This had further systemic effects, inducing fat browning and reducing fatty liver progression. In a study of 15 men monitored during a 10-day fast with a daily intake of 250 kcal [10], plasma LPS-binding protein, which reflects the exposure to bacterial LPS, was significantly decreased during fasting and remained reduced after food reintroduction. The association between *Escherichia coli* and secretory (s) immunoglobulin (Ig) A (sIgA) levels suggested that the increase in sIgA levels during fasting could be due to the increased abundance in bacteria causing local immune stimulation in the gut [10].

Effects of caloric restriction/fasting on intestinal microbial communities

Changes in gut microbiota composition

As early as 1915, it was reported that certain gut bacteria are able to survive on intestinal secretions containing mucus or desquamated cells, even after 31 days of fasting [33]. Research later showed that the gut microbiota switches composition during fasting (Figure 2). As of today, the changes in bacterial composition profiles occurring in human and animal gut microbiota during fasting are well characterised [10,11,30,34] (Figure 2). When food intake is interrupted, bacteria capable of utilising host-derived substrates (mucins, shed epithelial cells) proliferate at the expense of bacteria which rely strictly on dietary substrates for their survival. Long-term fasting caused a decrease in the relative abundance of Firmicutes and an increase in Bacteroidetes and Proteobacteria [10]. Studies have also been conducted to characterise the changes occurring during intermittent fasting. For example, Ramadan fasting caused an increased abundance



Trends in Microbiology

Figure 2. Changes in microbial composition during caloric restriction and fasting. A large number of studies in different animal species have clearly shown that the most severe types of caloric restriction and fasting cause a switch in the composition of the gut microbiota. Abbreviation: SCFA, short-chain fatty acid. Figure created with BioRender.com.

of Lachnospiraceae and Ruminococcaceae [11], findings which are well replicated in animal models of intermittent fasting [12].

Changes in gut microbiome communities can vary considerably depending on the fasting regimen. Frequency of bowel movements and stool consistency are factors which can profoundly impact microbiome composition [35]. The whole gut transit time in the human population is approximately 24 h on average, although large interpersonal variability exists. During long-term fasting, the elimination function of the large intestine does not fully stop. Drenick *et al.* noticed a cessation of stool after 3–4 days in obese individuals, after which only mucus was excreted at 2- or 3-week intervals [36]. During long-term fasting periods that have been scientifically documented, stool evacuation was infrequent, with 37–48 days between stools [37].

Few studies of the gut microbiome during fasting have attained high-resolution taxonomic resolution (at the ranks of species or strains). Thus, the species subsisting in the human gut when nutrient flow is interrupted are still not well identified. The only comprehensive study using shotgun metagenomics [38] found that *Roseburia hominis*, *Erysipelotrichaceae* sp., *Bacteroides nordii*, *Clostridium asparagiforme*, and *Anaerotruncus collhominis* were more abundant, while the species *Coprococcus comes*, *Dorea longicatena*, *Eubacterium rectale*, and *Dialister invisus* were depleted after 5 days with a daily nutritional energy intake reduced to 300–350 kcal. From the large number of Clostridiales changing significantly in abundance, significantly altered microbiome capacity for short-chain fatty acid production was inferred in this study.

Ecological interactions

The persistence of Bacteroidetes under caloric restriction can be explained by their metabolic capacity and flexibility [39]. Bacteroidetes are very flexible glycan foragers that can utilise host-derived substances. *Bacteroides* spp. can rapidly modulate their gene expression profiles to adapt to new nutrient sources, and thus persist in the gut during fasting [10,40]. *Bacteroides* spp. can also effectively switch from a metabolically active mode to a quiescent one when nutrients are lacking [41].

Bacteria surviving in the gut during caloric restriction are generally capable of cooperating for survival. Enterobacteriaceae and Bacteroidales spp. were found to exchange soluble nutrients (i.e., crossfeeding), including mucin- and carbohydrate-derived monosaccharides under malnourished conditions [42]. Although this pathological situation cannot be directly compared with fasting, it could provide evidence explaining why Bacteroidales spp. and Enterobacteriaceae were the most abundant bacterial groups in faecal samples of patients during long-term fasting [10].

Increased mucin foraging can also result in the proliferation of sulfur-reducing bacteria, which can obtain sulfate via cross-feeding mediated by Bacteroides-encoded sulfatases [43]. This is perhaps why fasted hamsters and hibernating ground squirrels have an increased abundance of *Desulfovibrio* [44,45]. The abundance of the hydrogen sulfide (H₂S)-producing microbe *Bilophila wadsworthia* increased during long-term fasting in humans [10]. The resulting increased production of H₂S could explain some of the beneficial effects of fasting. H₂S signals via a post-translational modification termed persulfidation, which is a known molecular mediator of the pleiotropic benefits of dietary restriction, including longevity and stress resistance [46]. One study demonstrated that the H₂S production capacity was increased by late-life intermittent fasting, and that this production was positively correlated with improvements in frailty tasks in mice [47]. However, H₂S production by microbes has also been associated with colorectal cancer and therefore its role could be double-edged (Box 3) [48].

Box 3. Impact of caloric restriction on intestinal pathogens

Reducing food intake during a gastrointestinal infection is a highly conserved response across species and has been suggested to be host-protective [113], although this is debated. However, much less is known about how fasting affects subsequent pathogen colonisation and infection and its severity. A potential concern is weakening microbiome-mediated colonisation resistance by caloric restriction [5,114]. Through a variety of mechanisms, an intact microbiome can provide colonisation resistance against exogenous microorganisms, thereby contributing to organismal resistance against infections [115]. It has been shown that a fibre-deprived diet can lead to mucus layer thinning due to a switch in microbial metabolism from dietary substrates to host-derived glycans. In turn, this brings enteropathogens into closer proximity to the epithelial layer, which could lead to more severe infections [114]. While a fibre-deprived diet is not the same as a caloric restriction diet in which all nutrients are generally reduced, this mechanism might translate to the caloric restriction setting too, considering the absolute decrease in fibre intake. Supporting this idea, a recent study suggested that a very-low-caloric diet for 8 weeks (800 kcal/day) followed by a conventional low-calorie diet for 4 weeks could lower colonisation resistance against *Clostridioides difficile* [5]. When performing fecal microbiota transplant (FMT) of the post-diet sample into germ-free mice, it was shown that these faecal samples were enriched in *C. difficile* throughout the colonisation experiment as compared to germ-free mice receiving a pre-diet FMT, but that the baseline abundance of *C. difficile* was not different between the groups, indicating lowered colonisation resistance against *C. difficile*. Importantly, these mice did not suffer from a *C. difficile* infection, indicating that the microbiome was still sufficiently functional to prevent symptomatic infection. This report stands in contrast to another *in vivo* study involving fasting and *Salmonella* Typhimurium infection models, which found fasting to strengthen microbiome-mediated colonisation resistance [6]. Using the streptomycin mouse model, fasting for 24 h prior to infection with *S. Typhimurium* only led to very few macroscopic signs of intestinal damage, much less than in the fed counterparts. It was subsequently shown that this was at least partially due to *S. Typhimurium* being unable to invade the intestinal epithelium: when *S. Typhimurium* was administered intravenously, fasting did not provide any protection against systemic infection, suggesting that the protection was gut-specific. In summary, additional research is necessary to fully understand the extent to which fasting modulates microbiome-mediated colonisation resistance and how this depends on specific diets and pathogens.

Even less is known about long-term effects of caloric restriction on host health that are due to microbiome changes. It is conceivable that a relative increase in H₂S-producing bacteria and Proteobacteria during fasting [10] could have long-term detrimental effects, as H₂S-producing bacteria have been repeatedly associated with colorectal cancer development [48,116]. Likewise it cannot be excluded that colibactin-producing *E. coli*, a potentially carcinogenic bacterial strain [117], is among those Proteobacteria species expanding during fasting. While somewhat speculative at this point, the possibility that (temporary) blooms of potentially harmful bacteria during fasting may have unintended negative long-term consequences on intestinal health deserves further study.

While most studies have focused on carbon metabolism to understand how caloric restriction can reshape microbial communities, nitrogen metabolism is no less crucial. Urea produced from metabolising nitrogen-containing compounds by animals can be secreted into the intestine and degraded by the action of bacterial ureases, as demonstrated by the accumulation of urea in the faeces [49]. Nitrogen recycling by gut bacteria was recently even found to be a mechanism by which hibernating ground squirrels can maintain muscle mass [50]. Whether this can happen in humans is still an open question, but it is certainly of clinical interest given the findings regarding muscle mass preservation during long-term fasting [51].

A. muciniphila emerged as a key player in the response to caloric restriction because it can thrive on mucins as an energy source and was observed to increase in relative abundance during hibernation [30], after very-low-calorie diets [5], and during long-term fasting [52]. The presence of higher levels of *A. muciniphila* during caloric restriction in obese adults is associated with greater improvement in glucose homeostasis and blood lipids [9,53].

Far-reaching effects on human physiology

It is widely accepted that the gut microbiome interacts not only with intestinal tissue but also with distant tissues. For example, several studies using animal models have proposed that the gut microbiome can mediate beneficial effects of fasting on diabetes-induced cognitive impairment [54], lipid metabolism [55], and blood-pressure regulation [56]. Ketone bodies resulting from a ketogenic diet enhance the anticancer effects of chemotherapeutic agents and are associated with the proliferation of *Eisenbergiella massiliensis* [57]. Furthermore, ketone bodies produced during fasting can effectively boost antitumor immunity [58,59].

Evidence available to date points to compositional stability of the gut microbiota under modest dietary calorie restriction, as reported for a 20% caloric restriction study of 147 obese adults [9], or for a weight-loss diet study using a low-carb or low-fat regimen [60], both resulting in ~5% weight loss over 1 year. Nonetheless, more severe caloric restrictions could change microbial profiles more durably [61]. It remains to be clarified under which caloric restriction/fasting regimens consistent microbiome changes can be observed, and also whether the microbiota is changing due to changes in dietary composition during calorie restriction or due to decreasing availability of dietary energy sources per se.

The production of short-chain fatty acids (SCFAs) by the gut microbiota plays an important role in the maintenance of health, including on body weight regulation and insulin sensitivity [62]. Studies performed so far suggested that SCFA production decreases during fasting in humans [10] or hibernating animals [63], probably reflecting reduced metabolic activity of the gut microbiota. However, the increased proportion of Firmicutes observed after food is reintroduced has been proposed to explain the increase in SCFA production observed in comparison to baseline after Ramadan fasting [64]. This seems also true for propionate, the level of which increases after intermittent fasting concomitantly to the relative abundance of *A. muciniphila* [65], a known propionate producer.

Although the gut microbiome is impacted by fasting in the aforementioned studies, it remains an open question whether it can be a mediator in the effects of fasting on the prevention of diseases in humans. Most studies reporting a link between the gut microbial taxa and diseases do not establish causal relationships. Many changes observed in the gut microbiome in diseased individuals are common to multiple diseases [66], and may as well reflect changes in the gut microbiome caused by the disease as causal contributions of the microbiome to disease development. However, we expect that elucidating which of the physiological effects of caloric restriction are mediated by the gut microbiome will be an intense area of research in the coming years. Towards this goal, it was recently shown that the microbiome can mediate antitumour effects of caloric restriction [67].

Comparison with pathological conditions

Comparing microbiome changes across pathological conditions and dietary interventions in healthy individuals may yield important clues as to shared host–microbe circuits. A meta-analysis including nine studies on the gut microbiota and anorexia nervosa in patients between 2009 and 2017 [68] showed changes in microbial composition that included a reduction in the abundance of Firmicutes concomitant with an increase in Bacteroidetes and Enterobacteriaceae [68]. Several studies on total parenteral nutrition – a situation where critically ill patients are systemically supplied with nutrients while the enteral lumen remains deprived of food – also revealed a decrease in the Firmicutes-to-Bacteroidetes ratio, in humans [69], and in a rat model [70]. In a recent study examining the interplay between glucose metabolism and the gut microbiota in 256 participants with type 2 intestinal failure, it was demonstrated that total parenteral nutrition can induce insulin resistance by reducing the levels of Lactobacillaceae and indole-3-acetic acid (IAA) levels, which in turn increases serum LPS concentrations [71]. Although any connection would be speculative, it is perhaps interesting to note that the increase in the serum levels of another gut-bacteria-derived tryptophan metabolite, indole-3-propionic acid, was linked to the ability of intermittent fasting to promote nerve regeneration and repair via the recruitment of neutrophils [72]. These observations in pathological conditions can resemble situations of starvation (Phase 3, Figure 1A) – although there may be important differences in concomitant drug administration, uncontrolled inflammation, or other degenerative processes linked to disease aetiology, which are not observed during fasting in healthy individuals.

Technical aspects of study design and analysis of microbiome studies

Some of the contradictory findings and issues that have arisen around the effects of fasting on the gut microbiome can be pinpointed to technical differences concerning study design and methods for microbiome profiling and statistical analysis, which are especially critical in human studies [73,74]. These technical differences also complicate meta-analyses, which can be valuable in establishing robustness of microbiome-fasting associations across studies. To inform investigators' choices on the design, analysis, and reporting of gut microbiome studies, community recommendations are becoming available [75]. However, most of these only partially cover longitudinal intervention studies, which, amongst others, we discuss later.

Microbiome profiling techniques and current bottlenecks

A prime consideration for any human microbiome study concerns the trade-off between sample size (statistical power) and sequencing depth within a finite study budget [76]. Traditionally, most nutritional intervention studies have measured taxonomic profiles using **16S rRNA gene amplicon sequencing**. This method, however, is limited in taxonomic resolution to genus level and thus only sufficient for detecting broad compositional changes. With the decreasing cost of sequencing, **metagenomic shotgun sequencing** (sequencing of all DNA in a sample) is becoming more widely adopted. Importantly, this technology allows investigators to attain functional information and species- or strain-level resolution. To offset the cost of deep metagenomic sequencing for large-scale (longitudinal) studies, shallow shotgun sequencing can be an alternative [77]. Although shallow shotgun sequencing can provide reliable species-level classifications and also functional information, it is limited in quantifying lowly-abundant taxa or gene functions and in constructing metagenome-assembled genomes (MAGs).

Study design, confounders, and the need for repeated sampling

Another crucial consideration for many nutritional intervention studies is whether one wants to primarily investigate the resulting effect of an intervention, or whether one wants to more broadly study community dynamics during the intervention period (e.g., fasting) and community resilience thereafter (during food reintroduction). In the former scenario, as little as two sampling timepoints (before and after intervention) may suffice, while in the second scenario it will be important to more densely sample participants longitudinally. The gut microbiome is known to be affected by many external factors, including diet, medication, and timing of sampling due to the microbiome's rhythmicity [78,79]. The latter is important as most tissues display a rhythmicity of gene expression in response to time-restricted eating [80]. Considering these factors in designing a microbiome study can help to avoid spurious associations due to confounding [81,82]. In case group sizes are sufficiently large, randomisation of study participants would be a straightforward solution in many settings. However, as this is often not feasible in microbiome studies, study participants could alternatively be matched between groups for lifestyle and clinical covariates to reduce their confounding influence. If lifestyle and clinical data are recorded accurately, it can also be (partially) accounted for in the analysis (post hoc), but statistical methodology for longitudinal studies is less well established and generally less flexible to allow for such adjustments than for cross-sectional analysis [83,84]. In longitudinal studies, participants can, in principle, serve as their own controls, but depending on the study design, baseline matching helps to ensure that intervention effects can more easily be compared between groups.

One crucial aspect that remains understudied, and on which consensus is lacking, is the number of baseline samples per individual. Multiple baseline samples can be instructive to assess normal intra-individual, day-to-day variation, which could subsequently be considered when estimating the intervention effect [85]. Grempi *et al.* [85] did estimate a preintervention intra-individual variability by calculating beta-diversity metrics and could associate this variability with intervention

success. It is, however, not clear how many baseline samples are necessary to accurately determine day-to-day variation in a person's gut microbiome. Poyet *et al.* suggested that averaging between five and nine timepoints may be optimal for estimating abundances on healthy individuals with stable gut microbiomes [86], but the benefit of such a larger number of baseline samples for (nutritional) intervention studies remains unclear.

Sample size calculation and effect size estimation

For the appropriate design of any clinical trial, power and sample size calculations are usually needed. Whereas robust tools are available to do this for 'traditional' continuous and noncontinuous data [87], these methods cannot readily be applied to microbiome data, which are far more complex than single continuous measurements such as blood pressure. Several methods have been developed for sample size calculations in microbiome studies, for example based on PERMANOVA for cross-sectional studies, which can inform on global shifts in community composition. However, these methods are not readily applicable to longitudinal studies or if changes in relative abundances of microbial taxa are to be estimated [88].

Power calculation conceptually depends on a prespecified effect size. For clinical and dietary microbiome intervention studies, it is as of yet unclear what appropriate effect size measures are, and expected effect sizes highly depend on the intervention. Nutritional intervention studies have generally shown mild effects on the microbiome as compared to, for example, antibiotic administration [89]. In the case of caloric restriction, the effect size will also be strongly influenced by the severity of the interventions. For example, Sowah *et al.* studied mildly decreased caloric intake (~20%) with the distribution of macronutrients changing only slightly, mostly because of a relative increase in protein [9]. This was likely the reason why, in this well-powered study, no significant effects of caloric restriction on the gut microbiome were observed. However, if caloric intake is completely ceased or reduced to only several hundred calories per day, much stronger effects were observed [10,38].

Sampling methods during fasting regimens

Microbiome profiling from faecal samples, as performed by most studies, likely most closely reflects the luminal microbiome of the colon or rectum and may not be fully representative of the mucosal microbiome [90]. During severe caloric restriction or starvation, in contrast to less-stringent nutritional intervention studies, individuals will have strongly reduced bowel frequency, and stool production can even stop completely after 3–4 days [36]. This reduction in stool production likely also leads to a decrease in bacterial cell mass, which in turn poses a problem for relative abundance estimation. For example, relative abundance of mucolytic bacteria is reported and well accepted to increase during fasting, but it is conceivable that their absolute abundance remains similar, while nonmucolytic bacteria decrease in their absolute abundance. In these more extreme cases of fasting, an approach that additionally estimates bacterial density, such as quantitative microbiome profiling [91], will likely yield more meaningful quantification of microbial abundances and mitigate the risk of analysis artefacts resulting from compositionality of relative abundance estimates [92]. To study the gut microbiome with high temporal resolution when bowel movements are infrequent, rectal swabs may be an alternative to faecal samples [93]. Lastly, as the enrichment of mucolytic bacteria during severe fasting is generally observed, sampling the intestinal mucosa will be of great interest to understanding mucosal microbial changes during fasting.

Accurate reporting of food intake

Assessing dietary intake is notoriously difficult, and the chosen method may heavily influence the results obtained [94]. Methods that depend on self-reporting through, for example, 24 h recalls, or food-frequency questionnaires, are known to often underestimate food intake [95]. Ideally, one

would objectively measure adherence to a specific diet using biomarkers capable of measuring the (reduced) intake of the interventional food groups, such as plasma alkylresorcinols in the case of whole-grain and rye intake [96], but these are not always available. An alternative in the case of long-term fasting is to strictly control the dietary intake in a clinical setting. This is, however, not always feasible, and, for example, smartphone image-based dietary assessment apps may be interesting low-cost alternatives [97].

Concluding remarks

In this review we describe the complex interplay between caloric restriction, the gut microbiome, and intestinal tissue. While we highlight that much remains unknown on the topic of intestinal health in mammalian superorganisms during caloric restriction and fasting, several overarching responses were identified. First, the overall response to fasting in the gut microbiome is conserved in humans and animals, with an increased reliance on host-derived substrates when dietary substrates are lacking. Second, the profound remodelling of intestinal tissues during fasting – leading to a renewal of the mucosa, changes in immune function, and a decreased inflammatory state – are thus probably key in determining the effects of fasting on the gut ecosystem. This leads to the hypothesis that therapeutic effects of fasting on the gut ecosystem might originate in the ability of host tissues to regain control of the microbial environment. Although the field has rapidly moved forward in recent years, confusion and controversy originating from the lack of a standardised nomenclature should be resolved, and study designs and analyses should be more harmonised. More work is especially needed to better characterise the period after fasting, with the aim of gaining a deeper understanding of intestinal remodelling during food reintroduction and to monitor long-term effects on the gut microbiome (see [Outstanding questions](#)).

Acknowledgments

We thank members of the Zeller group for fruitful discussions. This work received funding from EMBL, the Federal Ministry of Education and Research (BMBF grant no. 031L0181A to G.Z.), the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation no. 395357507 – SFB 1371 to G.Z.) and the Health + Life Science Alliance Heidelberg Mannheim through state funds approved by the State Parliament of Baden-Württemberg (Postdoctoral Fellowship to Q.D.).

Declaration of interests

R.M., F.G. and F.W.T. are employees of the Buchinger Wilhelmi Development and Holding GmbH, Überlingen.

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Outstanding questions

The different forms of fasting are poorly defined. Nomenclature needs clarification.

Stool production is significantly reduced during long-term fasting. What happens in different intestinal compartments? Studies using mucosal biopsies or non-invasive technologies to sample the gut microbiome are missing. This could include measurement of the mucus layer. Does it get thicker with an improved barrier function?

Fasting seems to affect bacteria in a species-specific manner. Future metagenomic analyses should reach species- or strain-level resolution. What about fungi and viruses? What about other microbiomes (e.g., oral)?

New studies are needed to understand the relative roles of overall caloric restriction and specific macronutrients on the gut microbiome.

How does long-term fasting affect both colonisation and infection resistance against enteropathogens? Would fibre supplementation aid in supporting a healthy mucus layer during this period?

The food reintroduction period is neglected as most studies focus on the fasting period. How long do fasting-induced changes persist after food reintroduction? Can different dietary strategies influence which bacteria repopulate the gut? Can prebiotics or live biotherapeutic products influence this process?

It is not clear which systemic physiological effects of fasting occur independently of, and which are mediated by, the microbiome. In addition, limited information is available to understand the effects of enemas or other therapies frequently added to fasting interventions, or if changes during fasting can affect the toxicokinetics of medications.

Translation from animals to humans can be problematic, as the human gut is different from that of rodents. What experimental design appropriately reproduces human fasting without putting the experimental animals in the proteolytic starvation phase? What are corresponding time scales?

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