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REVIEW

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Butyrate-producing human gut symbiont, *Clostridium butyricum*, and its role in health and disease

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ABSTRACT

Clostridium butyricum is a butyrate-producing human gut symbiont that has been safely used as a probiotic for decades. *C. butyricum* strains have been investigated for potential protective or ameliorative effects in a wide range of human diseases, including gut-acquired infection, intestinal injury, irritable bowel syndrome, inflammatory bowel disease, neurodegenerative disease, metabolic disease, and colorectal cancer. In this review we summarize the studies on *C. butyricum* supplementation with special attention to proposed mechanisms for the associated health benefits and the supporting experimental evidence. These mechanisms center on molecular signals (especially butyrate) as well as immunological signals in the digestive system that cascade well beyond the gut to the liver, adipose tissue, brain, and more. The safety of probiotic *C. butyricum* strains appears well-established. We identify areas where additional human randomized controlled trials would provide valuable further data related to the strains' utility as an intervention.

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Clostridium butyricum, a butyrogenic gut symbiont

C. butyricum, a butyrate-producing, spore-forming anaerobic bacterium, is found in a wide variety of environments, including soil, cultured milk products, and vegetables. It is also present in the human gut: it is detected in 10–20% of the adult human population and is often one of the earliest colonizers in infants.[1,2] In the human gut, where it is considered a 'symbiont[3]' (living together with the host), *C. butyricum* has a fermentative lifestyle and can consume undigested dietary fibers and generate short-chain fatty acids (SCFAs), specifically butyrate and acetate. Butyrate is one of the dominant fermentation end-products and is produced by *C. butyricum* via the butyrate kinase (*buk*) pathway.[4] SCFAs produced by microbial organisms in the colon are known to have myriad and important effects on host health, including modulating intestinal immune homeostasis, improving gastrointestinal barrier function, and alleviating inflammation.[5] As such, butyrate-producing organisms like *C. butyricum* have become attractive candidates to test for beneficial effects in a host. Genomic analyses are increasingly identifying novel bacterial strains with health-promoting potential that are distinct from classic probiotics (*Lactobacilli* and *Bifidobacteria*).

C. butyricum is a species that encompasses various known strains, some of which have genes equipping them to produce toxins.[6] However, genomic analyses confirm that other strains do not have these genes nor other markers of pathogenesis potential, and that these nonpathogenic strains have excellent potential to benefit host health through several mechanisms. Certain strains of *C. butyricum* have been used as a probiotic[7] for decades. Strain MIYAIRI 588 (or MIYARI 588; CBM 588), first isolated from the feces of a healthy human by Dr. Chikaji Miyairi in 1933, and later from soil in 1963, is a commercially-available, over-the-counter probiotic widely used in Japan, Korea, and China for the treatment of (antimicrobial-associated) diarrhea.[8,9] Strain CBM 588 is also authorized under the regulation of the European Parliament and of the Council as a novel food ingredient.[10] Its widespread use is enabled by its safe, nonpathogenic and nontoxic profile:

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studies have shown that it is sensitive to antibiotics, devoid of pathogenic markers, and lacks clostridial toxin genes.[11]

Dysbiosis in the gut microbiome has been implicated in multiple diseases, including gastrointestinal, neurological, and metabolic disorders, as well as cancer. Across many of these disorders there is evidence to suggest that *C. butyricum* is a promising therapeutic candidate. Although the precise mechanisms explaining these health benefits are not fully elucidated, current evidence suggests that *in vivo*, localized production of SCFAs is important for conferring health benefits. There are numerous pathways by which acetate and butyrate impact immune homeostasis and the physiology of the gut barrier. It is also suggested that *C. butyricum* may modulate the composition of the gut microbiome, possibly increasing certain beneficial bacterial taxa such as *Lactobacillus* and *Bifidobacterium.*[12–15]

Here we review studies pertaining to *C. butyricum* supplementation and host health, with an emphasis on the mechanisms of action of the probiotic. We have divided the review into two broad sections: (1) conserved interactions between *C. butyricum* and host physiological and immunological pathways, and (2) health outcomes in humans and in murine models of human disease.

C. butyricum supplementation: mechanisms of action and target pathways

The following sections detail well-supported mechanisms by which *C. butyricum* impacts host health with implications in multiple disease states: namely, effects of *C. butyricum* on gut microbial composition, gut barrier function, and immunological and inflammatory pathways. The interaction between *C. butyricum* and the Akt signaling pathway – a multifaceted regulator relevant to cancer, diabetes, and cardiovascular and neurological diseases – is also discussed.

C. butyricum *and modulation of the gut microbiome*

Multiple murine disease models have been employed to examine the effect of *C. butyricum* on gut microbiome composition, using a variety of techniques (Table 1). PCR amplification of the V3, V4, or V5 regions of the 16S rRNA gene from fecal samples, and subsequent Illumina deep sequencing, is the most common technique, used across 10 of the 16 studies detailed in Table 1. Three studies profile gastrointestinal microbiota using PCR-DGGE (denaturing gradient gel electrophoresis), three employ a culture-based approach, and four use qPCR to quantify either specific taxa or specific functions

Culture and qPCR-based measurements support increases in specific taxa in response to *C. butyricum* supplementation, especially *Bifidobacterium* spp. and *Lactobacillus* spp.[13,16,17] Additionally, culturing and qPCR consistently support a decrease in *Enterococcus* and/or *Enterobacteriaceae.*[13,18,19] Interestingly, two studies[20,21] demonstrate via qPCR an increase in butyrate producing genes in the collected stool microbiota (both butyrate kinase (*buk*) and butyryl-CoA:acetate CoA-transferase (*but*)). As the *C. butyricum* genome contains only *buk*, the observed increase implies a concomitant increase in other butyrate-producing microorganisms.

While several studies indicate no effect of *C. butyricum* supplementation on metrics of community composition (diversity indices, clustering in PCA/PCoA analysis),[14,22,23] others show a pronounced effect on diversity and/or a distinct community composition post supplementation.[12,15,20,21,24,25] Some of these differences might be methodological, for example PCR-DGGE data from models of antibiotic-associated diarrhea indicate increased diversity following *C. butyricum* supplementation while, in similar models, illumina 16S rRNA gene sequencing data do not. Profiles obtained with DGGE are limited in both their ability to detect lower abundance organisms and to provide higher taxonomic resolution. Even with 16S rRNA gene sequencing, however, community-level shifts in diversity should not be given undue importance as a wide number of factors are known to contribute including animal genotype, litter, housing, diet, and vendor.[26–28] Additionally, the different *C. butyricum* strains tested, the different animal models employed, as well as the generally small number of animals used in most studies, should lead researchers to interpret observed *C. butyricum*-driven changes in community composition with caution.

Table 1. The effect of *C. butyricum* supplementation on gut microbiome composition across murine models of disease.

Disease	Study	Probiotic	Dose (daily)	Treatment Duration	Study size	Treatment groups	Community analysis method	Community analysis *general metrics*	Community analysis *specific taxa*
	Kong *et al.* 2011	*C. butyricum*	10^4 CFU (low-dose); 10^6 CFU (medium); 10^8 CFU (high)	14 days	n = 10/group	vehicle control, low-dose, medium-dose, high-dose	culture-based		*Bifidobacterium* spp. increased in medium/high-dose groups; *Clostridium perfringens* below detection in all *C. butyricum* groups (vs. above in control)
	Long *et al.* 2018	*C. butyricum* Sx-01; *L. salivarius* C-1-3	$4x10^8$ CFU (*L. salivarius*); 4×10^8 CFU (*C. butyricum*); 2×10^8 CFU each (combination)	14 days	n = 20/group	vehicle control, *L. salivarius* (LS), *C. butyricum* (CB), combination	16S rRNA PCR (V3-V4) and Illumina sequencing	increase in Bray-Curtis dissimilarity index between *C. butyricum* group and other 3 groups	LEfSe (Linear discriminant analysis effect size) shows *Erysipelatoclostridium* and *Coriobacteriaceae, Coriobacteriales* and *Coriobacteria* responsible for differences in *C. butyricum*-treated groups
antibiotic – associated diarrhea (AAD)	Ling *et al.* 2015	*C. butyricum* CGMCC 0313.1; *B. infantis* CGMCC 0313–2	$1.2x10^9$ CFU (C. butyricum); 2×10^{10} CFU (*B. infantis*); 4×10^7 CFU each (combination)	5 days; 15 days	n = 10/group	healthy control, AAD, AAD+saline, AAD+*C. butyricum*, AAD+*B. infantis*, AAD+combination	PCR-DGGE; qPCR on select taxa	DGGE profiles different for AAD vs. healthy; *C. butyricum* and combination treatments increase diversity of DGGE profiles and revert total bacterial counts to normal after 15 days	*C. butyricum* restores *Bacteroides/Prevotella, Clostridium* clusters XI and I, *F. prausnitzii, Bifidobacterium* and *Lactobacillus*, and decreases *Enterococcus* group
AAD	Hagihara *et al.* 2018	CBM588	$3.4x10^8$ CFU/kg	4 days	n = 5/group	healthy control, Clindamycin (AAD), AAD+*C. butyricum*, AAD+combination	16S rRNA PCR (V3-V4) and Illumina sequencing	alpha-diversity not different between clindamycin and combination groups (both decreased); alpha-diversity not different between control and *C. butyricum* groups (both stable)	*Bifidobacterium, Coprococcus*, and *Bacteroides* increased in the combination group
AAD	Hagihara *et al.* 2019	CBM588	$3.4x10^8$ CFU/kg	4 days	n = 5/group	healthy control, clindamycin (AAD), AAD+*C. butyricum*, AAD+combination	16s rRNA PCR (V3-V4) and Illumina sequencing		*C. butyricum* increases *Actinobacteria* (mainly *Bifidobacterium*) post day 10; PICRUSt predicts similar carbohydrate, lipid and amino acid pathways; combination shows increase in starch/sucrose metabolism and steroid hormone biosynthesis vs. clindamycin
AAD	Hagihara *et al.* 2019	CBM588	$3.4x10^8$ CFU/kg	4 days	n = 5/group	healthy control, clindamycin (AAD), AAD+*C. butyricum*, AAD+combination	16S rRNA PCR (V3-V4) and Illumina sequencing	alpha-diversity not different between clindamycin and combination groups, nor between control and *C. butyricum* groups; PCoA (weighted Unifrac distance) shows no change in community composition between *C. butyricum* and control group; clindamycin group separate from the control group; combination separates from the clindamycin group at day 4	combination shows increase in *Bifidobacterium, Lactobacillus* and *Lactococcus* vs. clindamycin
ulcerative colitis (UC)	Zhang *et al.* 2009	*C. butyricum* CGMCC0313.1	$4.6x10^8$ CFU (*C. butyricum*); 0.2 g (mesalamine); 0.1 mmol (sodium butyrate)	21 days	n = 10/group (UC); n = 8 (healthy control)	healthy control, UC, UC+*C. butyricum*, UC+mesalamine, UC+sodium butyrate	culture-based		model shows decrease in *Bifidobacterium* and *Acidobacterium*, and increase in *Colibacter, Fusobacterium* and *Clostridium; C. butyricum*, mesalamine and sodium butyrate reverse both trends
Type 1 diabetes	Jia *et al.* 2017	*C. butyricum* CGMCC0313.1	2.5×10^8 CFU/kg	42 weeks	n = 28 (NOD); n = 25 (NOD+CB)	NOD, NOD+*C. butyricum*	16s rRNA PCR (V4) and Illumina sequencing; qPCR (*buk, but*)	PCoA shows change in community structure in *C. butyricum* group	*C. butyricum* shows increase in *buk* and *but* genes and decreased *Bacteroidetes, and* increased *Firmicutes, Clostridia* and *Clostridiales*; other changes in specific taxa

(*Continued*)

Table 1. (Continued).

Disease	Study	Probiotic	Dose (daily)	Treatment Duration	Study size	Treatment groups	Community analysis method	Community analysis *general metrics*	Community analysis *specific taxa*
Type 2 diabetes	Jia *et al.* 2017	*C. butyricum* CGMCC0313.1	2.5×10^8 CFU/kg	5 weeks (db/db); 13 weeks (HFD-induced)	n = 8/group (db/db); n = 8–12/group (HFD)	db/db+ vehicle, db/db +*C. butyricum*; healthy control, HFD, HFD+*C. butyricum*, HFD+sodium butyrate	16s rRNA PCR (V4) and Illumina sequencing; qPCR (*buk, but*)	PCoA shows change in community structure in *C. butyricum* group and increase in diversity (Shannon)	C. butyricum shows increase in *buk* and *but* genes, increase in *Bacteroidetes*, *Clostridia* and *Clostridiales*, and decrease in *Firmicutes*; other changes in specific taxa
Acute pancreatitis (AP) and severe AP (SAP)	Pan *et al.* 2019	CBM588; *C. butyricum* CGMCC0313.1	$9.6x10^8$ CFU/kg (MIYAIRI); 5.7×10^9 CFU/kg (CB0313.1); 9.6×10^8 CFU/kg (low-dose CB0313.1)	21 days (pre AP or SAP induction)	n = 7/group	healthy control, AP, AP+MIYAIRI 588, AP+CB0313.1; Healthy control, SAP, SAP+MIYARI588, SAP+CB0313.1, SAP+low-dose CB0313.1	16s rRNA PCR (V4) and Illumina sequencing	PCA shows change in community structure in *C. butyricum* group	*C. butyricum* shows decrease in *Desulfovibrionaceae* and increase *Akkermansia muciniphila, Clostridiaceae* and *Lactobacillaceae*
acute liver injury (ALI)	Lui *et al.* 2017	*C. butyricum*	$5x10^8$ CFU	5 days (pre ALI)	n = 10 (normal); n = 28 (ALI); n = 30 (CB)	healthy control, ALI, ALI+*C. butyricum*	16s rRNA PCR (V4-V5) and Illumina sequencing	increase in diversity (Shannon) in *C. butyricum* vs. model group	*C. butyricum* shows increase in *Clostridiales, Lactobacillales* and *Firmicutes*
vascular dementia (VaD)	Lui *et al.* 2015	*C. butyricum* WZMC1016 (CGMCC 9831)	10^6 CFU (low); 10^7 CFU (medium); 10^8 CFU (high)	6 weeks (post rUCCAO)	n = 12 (VaD and sham groups); n = 15 (CB)	sham control, VaD, VaD+CB low-dose, VaD+CB medium-dose, VaD+CB high-dose	PCR-DGGE	model decreased diversity vs. sham; *C. butyricum* (high-dose) increased diversity; DGGE profile cluster analysis shows sham and medium/high doses of *C. butyricum* cluster together, and away from model and low-dose	
cerebral ischemia/reperfusion (I/R) injury in diabetic mouse	Sun *et al.* 2016	*C. butyricum* WZMC1016 (CGMCC 9831)	10^8 CFU	6 weeks (24 h post I/R injury)	n = 12/group	sham control, I/R, I/R + *C. butyricum*	PCR-DGGE; qPCR on select taxa	DGGE profiles show decreased diversity in model vs. sham; *C. butyricum* restores diversity	model shows decrease in *Bacteroides/Prevotella, Clostridium* cluster XIVab, *F. prausnitzii, Bifidobacterium*, and *Lactobacillus*, and increase in *Clostridium* cluster XI, *Clostridium* cluster I, *Enterobacteriaceae* and *Enterococcus; C. butyricum* reverses both trends
colorectal cancer (CRC)	Chen *et al.* 2020	*C. butyricum* ATCC 19398	$2x10^9$ CFU 3x/week	12 weeks	n = 10/group	basal diet, HFD, HFD+*C. butyricum*	16S rRNA PCR (V3-V4) and Illumina sequencing	model shows decrease in diversity (Shannon/Simpson) vs. basal diet; no effect of *C. butyricum* on diversity and PCoA cannot separate *C. butyricum* from model group	model shows increase in *Firmicutes/Bacteroidetes* ratio vs basal diet; LEfSe links *Ruminococcaceae* and *Eubacterium* with *C. butyricum* treatment
Colitis-associated colorectal cancer (CAC)	Liu *et al.* 2020	*C. butyricum*	$2x10^8$ CFU 3x/week	78 days	n = 10/group	healthy control, CRC, CRC+*C. butyricum*	16S rRNA PCR (V3-V4) and Illumina sequencing	no differences among groups in diversity metrics (Chao1, Shannon, Simpson); PCoA shows no separation among groups	*C. butyricum* shows decrease in *Firmicutes/Bacteroides* ratio; other changes in specific taxa
neonatal development	Miao *et al.* 2018	*C. butyricum* CGMCC0313.1	$5x10^8$ CFU	20 days (lactation, maternal mice); 7 days (post-weaning, offspring)	n = 10/group	control, maternal intervention, offspring intervention, maternal and offspring intervention	culture-based		maternal intervention shows decrease in *Enterococcus, Enterobacter*, and increase in *Lactobacillus, Bifidobacterium* in the offspring vs. control; offspring intervention shows increase in *Lactobacillus* and *Bifidobacterium* vs. control

C. butyricum *strengthens the gut barrier*

The intestinal barrier performs a critical balancing act: maintaining the tolerance of gut microbiota and absorption of nutrients on the one hand, and defense against pathogen invasion on the other. It acts as a selective barrier, preventing the permeation of toxins and antigens, while facilitating the absorption of nutrients, electrolytes, and water. Structurally, the intestinal barrier consists of three layers: the mucus layer, the epithelium, and the lamina propria (Figure 1).[30,31]

The mucus layer represents the primary physical barrier against pathogen invasion. This gel-like layer varies in thickness along the length of the GI tract and is primarily composed of glycoproteins (i.e *mucins*) secreted by *goblet cells* in the epithelium.[32] Mucin 2 (MUC2) is the most abundant and well studied secreted gastrointestinal mucin. Knocking out MUC2 in mice can cause the development of spontaneous colitis and increases the risk of colorectal cancer, highlighting the importance of this barrier for immune homeostasis and overall health.[33,34] Introducing *C. butyricum* has been shown to protect properties of the mucosal layer in two different mouse models. Administration of *C. butyricum* Sx-01 significantly increased colonic mucosal thickness in a murine study of probiotic supplementation for general intestinal health,[25] while administration of CBM 588 increased mucin production (measured as increased expression of MUC2) and significantly decreased epithelial damage in the colonic tissue of mice with clindamycin-induced antibiotic-associated diarrhea.[15] The effect of *C. butyricum* on mucosal health may be explained by its capacity for butyrate production. Indeed, butyrate has been shown to increase mucin production by goblet cells *in vitro* by increasing expression of the MUC genes[35,36] (Figure 2).

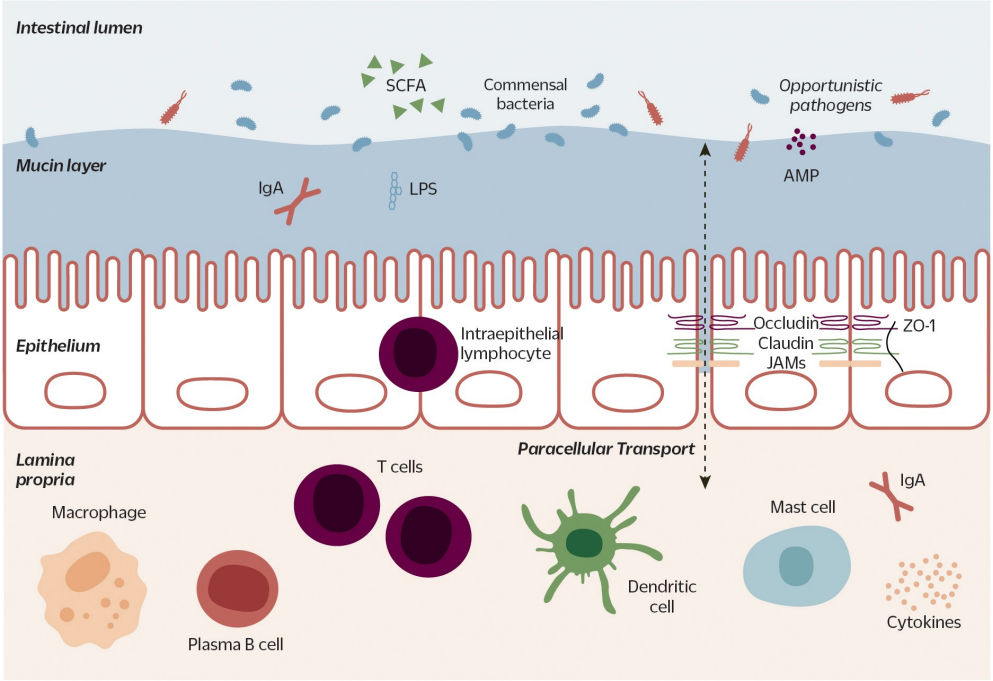

Figure 1. The intestinal barrier separates the intestinal lumen (where microorganisms reside) from host tissues. The gut barrier is composed of three layers: the outermost mucus layer acts as a physical barrier against invading microorganisms; AMPs and antibodies (e.g. IgA) form a chemical barrier. Subsequent to the mucosal layer is the epithelium: a monolayer of epithelial cells mechanically linked by TJ proteins (e.g. claudin, occludin, JAMS, ZO-1). TJs regulate paracellular transport, selectively allowing the passage of small molecules. The epithelium also harbors the mucus-secreting goblet cells (not shown), hormone-secreting enteroendocrine cells (not shown), and several other immune cell types (e.g. intraepithelial lymphocytes). Finally, the lamina propria is a layer of connective tissue beneath the epithelium. It contains the majority of immune cells: innate immune cells such as macrophages, dendritic cells, and mast cells, and adaptive immune cells, such as T cells and antibody-producing plasma B cells. The epithelial and immune cells produce cytokines, or signaling molecules, to communicate across the layers of the intestinal barrier. (Figure adapted from König *et al.* 2016[29]). AMP: antimicrobial peptide; TJ: tight junction; JAMs: Junctional adhesion molecules, ZO-1: zonulin-1

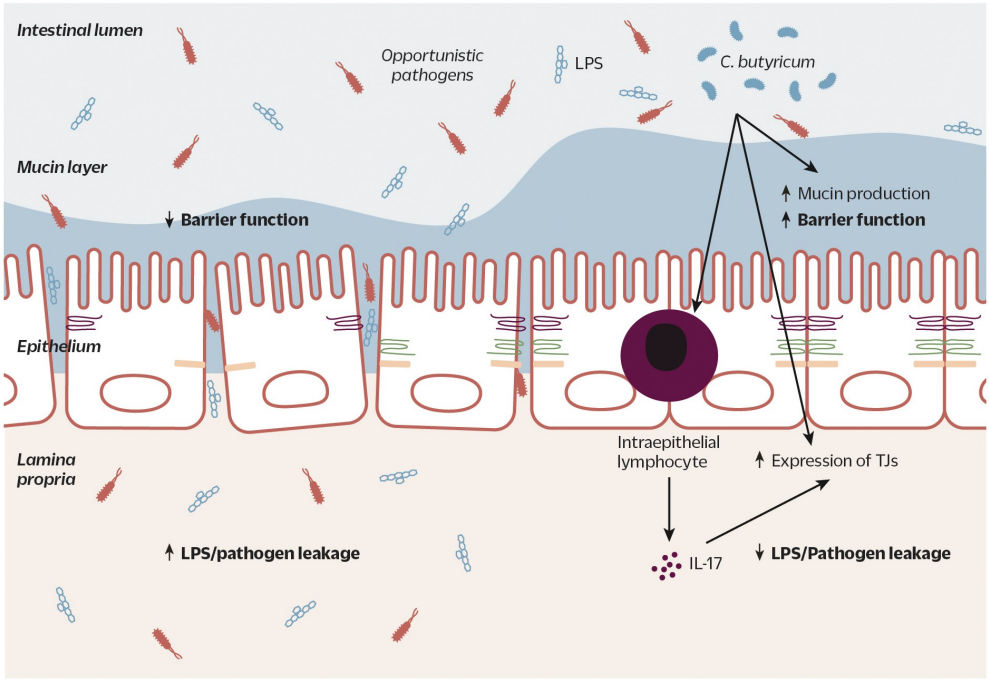

Figure 2. *C. butyricum* strains improve gut barrier function by increasing the thickness of the mucosal layer and increasing expression of TJ proteins. Increased expression of TJ proteins may also result from *C. butyricum*'s stimulation of IL-17 production from intraepithelial T cells. This improved barrier results in decreased permeation of LPS and pathogenic bacteria into host tissue and blood. TJ: tight junction; IL-17: interleukin-17; LPS: lipopolysaccharide

Subjacent to the mucosal layer is the epithelial monolayer (Figure 1). Here, *tight junctions* (TJs) bind epithelial cells together and regulate paracellular transport.[37] Lowered expression of TJ proteins (occludin, claudin, JAMs, ZO-1) increases gut permeability. Increased permeability, in turn, allows bacteria or bacterial components such as lipopolysaccharides (LPS) and other antigens to leak into the lamina propria and incite an inflammatory response. The importance of TJs is seen in occludin knock-out mice, where histopathological abnormalities including chronic inflammation and hyperplasia of the gastric epithelium are observed.[30] As well as influencing mucin production directly, *C. butyricum* has been shown to strengthen barrier function by influencing TJ protein expression across several different disease models, likely hinting at a conserved mechanism of action behind these organisms' efficacy (Figure 2). In a mouse model of antibiotic-associated diarrhea, treatment with CBM 588 increased expression of TJ proteins occludin, claudin-4, and ZO-1.[12,15] Similarly, in a mouse model of severe acute pancreatitis (SAP), two *C. butyricum* strains (CBM 588 and CGMCC0313.1) restored expression of TJ proteins ZO-1, ZO-2, and occludin.[12,38] Following treatment with *C. butyricum*, mice with SAP also had decreased pancreatic levels of *E. coli* and *Enterococcus*, providing further indication of improved barrier function.[12,38] In a mouse model of traumatic brain injury, *C. butyricum* supplementation again induced the recovery of occludin and ZO-1 expression in the brain and colonic tissue of mice.[12,38] As in the SAP murine model, improved barrier function was further highlighted by decreased serum levels of D-lactate, a microbial byproduct and marker of a 'leaky gut'. Finally, in a high-fat diet (HFD)-induced obesity mouse model, *C. butyricum* CGMCC0313.1 supplementation again increased expression of claudin-1 and occludin (measured at both the protein and mRNA level) as well as decreased serum LPS.[39] The replicability in the above described studies strongly supports a role for *C. butyricum* in gut barrier integrity.

As with increased mucin secretion, *C. butyricum*'s protective effect on the intestinal epithelium may be attributed to butyrate production (Figure 2). Multiple studies note increases in fecal butyrate concentrations following *C. butyricum* supplementation and/or the proliferation of other butyrate-producing bacteria.[12,15,20,21,24,39] Animal studies have shown

beneficial effects of direct supplementation with sodium butyrate against increased intestinal permeability,[40,41] and several *in vitro* studies have identified the signaling pathways involved in butyrate regulation of TJ protein expression.[42,43] Alternatively or in parallel, immunomodulation of the versatile cytokine interleukin-17 (IL-17) by *C. butyricum* may contribute to these effects. Hagihara and colleagues describe an enhanced barrier integrity effect following probiotic administration of *C. butyricum* that also resulted in an increased production of IL-17 by γδ T cells, a specific subset of intraepithelial T cells that act as a part of the first line of defense, in the colonic lamina propria (Figure 2).[15] Although often perceived as pro-inflammatory, IL-17 secreted locally by γδ T cells serves to protect and repair the gut barrier by ensuring expression of TJ proteins.[44] Finally, Hagihara and colleagues have also reported that the protective effect of *C. butyricum* on the intestinal barrier was associated with host metabolic alterations that lead to attenuation of antibiotic-induced gut inflammation.[15,45] *C. butyricum* promoted the production of anti-inflammatory lipid metabolites such as palmitoleic acids, prostaglandin metabolites, and specialized pro-resolving mediators in mouse colonic tissues. Such lipid metabolites, especially protectin D1,[46] contribute to the promotion of anti-inflammatory IL-10 secreting T-cells in the colon. Therefore, *C. butyricum*-mediated modulation of host immunological and metabolic pathways supports yet another protective mechanism of *C. butyricum* in intestinal inflammation.

C. butyricum *modulates immune function and inflammation*

Box 1: Immune homeostasis and the gut microbiome

As described above, the gut barrier separates the host from the trillions of microorganisms that inhabit the gut lumen. To provide an effective defense, the gut barrier harbors a multitude of immune cells and represents the largest immune organ in the human body.[47] The intestinal epithelium monitors the gut microbiota via *pathogen recognition receptors* (PRRs) capable of recognizing *pathogen-associated molecular patterns* (PAMPs) such as lipopolysaccharide (LPS). PRRs distinguish the pathogens from non-pathogens and mediate immune response against the former. The inflammatory mediators released by the epithelium upon detection of pathogens trigger the maturation of *antigen presenting cells* (APCs) such as dendritic cells. Mature APCs then produce signals to activate the differentiation and expansion of appropriate T cell types. Additionally, microbial components and metabolites of both pathogens and commensals can stimulate the release of host-derived antimicrobial peptides (AMP) or secreted immunoglobulin A (IgA) that provide an additional chemical barrier between the luminal microorganisms and the host (Figure 1). Therefore, changes in bacterial composition in the gut can shift the immune system, with effects often extending beyond the gut.[48]

Immune homeostasis – the balance between pro – and anti-inflammatory profiles – is maintained through the proper regulation of immune cells and cytokines. Under homeostasis, the types of immune cells and the cytokines they produce maintain a balance between pro – and anti-inflammatory profiles. Inappropriate activation of the immune response can be self-deleterious, and is an underlying problem of many modern inflammatory diseases: immune disorders such as allergies and autoimmune diseases; inflammatory bowel disease; metabolic diseases such as diabetes and hepatitis; and neurodegenerative diseases. All of these inflammatory diseases have been associated with changes in the composition of gut microbiota. Thus, there currently exists a rationale for probiotic bacteria, such as *C. butyricum*, playing a protective role against infectious gut-acquired pathogens, intestinal injury, and inflammatory conditions by modulation of the host immune system.[16,19,48]

T cells are a part of the adaptive immune system and are major contributors to shaping host immune profiles.[49] Differentiation of naive T cells to effector T cells is controlled by distinct sets of cytokines and transcription factors, typically secreted by innate immune cells including APCs. For example, dendritic cell production of IL-6 or IL-23 induces expansion of T helper (Th)17 cells, while the production of TGF-β in the absence of IL-6 or IL-23 leads to the expansion of *regulatory T cells* (Tregs). Effector T cells are grouped depending on the profile of the cytokines they produce and the effect of each cytokine. Th1 and Th17 responses, driven by interferon-gamma (IFN-γ)

and interleukin (IL)-17, respectively, typically dominate during *inflammatory* disease. IFN-γ and IL-17 mediated inflammations – involving the killing of infected cells and engulfing of invading pathogens – often lead to tissue damage and exacerbation of inflammation.[16,50] On the other hand, Th2 response suppresses Th1 and Th17 response but leads to B cell-mediated antibody production, an important step for the body to prepare immune responses to future infections but also a contributor to allergic diseases when improperly controlled. Tregs are a special group of effector T cells that oppose the inflammatory Th responses and therefore are *anti-inflammatory*. The Treg response restrains the inflammatory response by the production of IL-10, an anti-inflammatory cytokine that suppresses the Th1, Th2, and Th17-mediated inflammation.[51] Restoring the Treg response and maintaining a balance with pro-inflammatory responses may be a critical part of combating inflammatory diseases.

C. butyricum *restores immune homeostasis by promoting Treg responses*

The Treg response suppresses activation of inflammatory responses driven by effector T cells such as Th1, Th2 and Th17 (See Box 1 for background on immune homeostasis). The balance between Tregs and pro-inflammatory effector T cells in the intestine is increasingly recognized as regulated by the gut microbiome.[52] Murine disease models have shown that administration of *C. butyricum* promotes intestinal immune tolerance by increasing the abundance of Tregs, as evidenced by the accumulation of induced Tregs especially in the mesenteric lymph node,[21,50,53] along with the decreased proportion of Th1 and Th17 cells in the disease target organs such as the pancreas,[21,50,53] spleen,[21,50,53] liver,[24,54] brain, and intestines.[21,50,53] In a murine model of colitis, depletion of Tregs partially abolished the preventive effect of CBM 588.[48] The mechanism behind the increased Treg population in groups administered *C. butyricum* has been attributed to increased production of TGF-β, a cytokine that induces Treg differentiation[16,50,55,56] (Figure 3). Several studies have pointed to colonic dendritic cells as the source of *C. butyricum*-induced TGF-β.[57,58] Dendritic cells, as the major antigen presenting cells (APCs), translate the external stimuli to differentiate naive T cells and modulate the adaptive immune response. CBM 588 has been shown to activate Toll-like Receptor 2 (TLR2)-dependent TGF-β expression in colonic dendritic cells, and thus promote Treg differentiation.[57] As a result, mice administered *C. butyricum* were protected from chemical-induced colitis via a dendritic TGF-β and Treg dependent mechanism. *C. butyricum* also suppressed the maturation of dendritic cells and the emergence of an inflammatory phenotype. In a chemical-induced irritable bowel syndrome (IBS) mouse model, *C. butyricum* decreased the proportion of dendritic cells expressing *T cell immunoglobulin and mucin domain 3* (TIM3).[58] TIM3-expressing dendritic cells are positively associated with pro-inflammatory cytokine secretion. Therefore, *C. butyricum* is postulated to promote an anti-inflammatory Treg response by increasing TGF-β secretion, possibly by modulating dendritic cell signaling. Alternatively, butyrate produced by *C. butyricum* may directly facilitate the differentiation of Tregs via histone deacetylase (HDAC) inhibition,[59,60] rather than modulating dendritic TGF-β[57]

An anti-inflammatory cytokine, IL-10, suppresses the effector T cell response in a variety of ways. First, IL-10 signals to APCs to produce TGF-β and therefore triggers Treg differentiation.[61] IL-10 amplifies the suppressive actions of Tregs by inducing further IL-10 production. Additionally, IL-10 suppresses pro-inflammatory cytokine production in various APCs and T cells by inhibiting nuclear factor-kappa B (NF-κB).[62] The anti-inflammatory effect of *C. butyricum* and its role in promoting Treg response is tied to the increased level of IL-10[16,48,50,55,56] (Figure 3). In a murine colitis model, depletion of macrophage-produced IL-10 diminished the protective effect of CBM 588, confirming its indispensable role.[48] *C. butyricum* increases IL-10 levels by activating TLR2 in colonic macrophages. Specifically, CBM 588 lipoteichoic acid (LTA) has been shown to modulate TLR2 signaling and suppress pathogen-induced inflammation and apoptosis *in vitro*.[63,64] Thus, *C. butyricum* promotes an anti-inflammatory Treg response by induction of IL-10 and TGF-β, possibly through activation of a TLR2-dependent pathway (Figure 3).

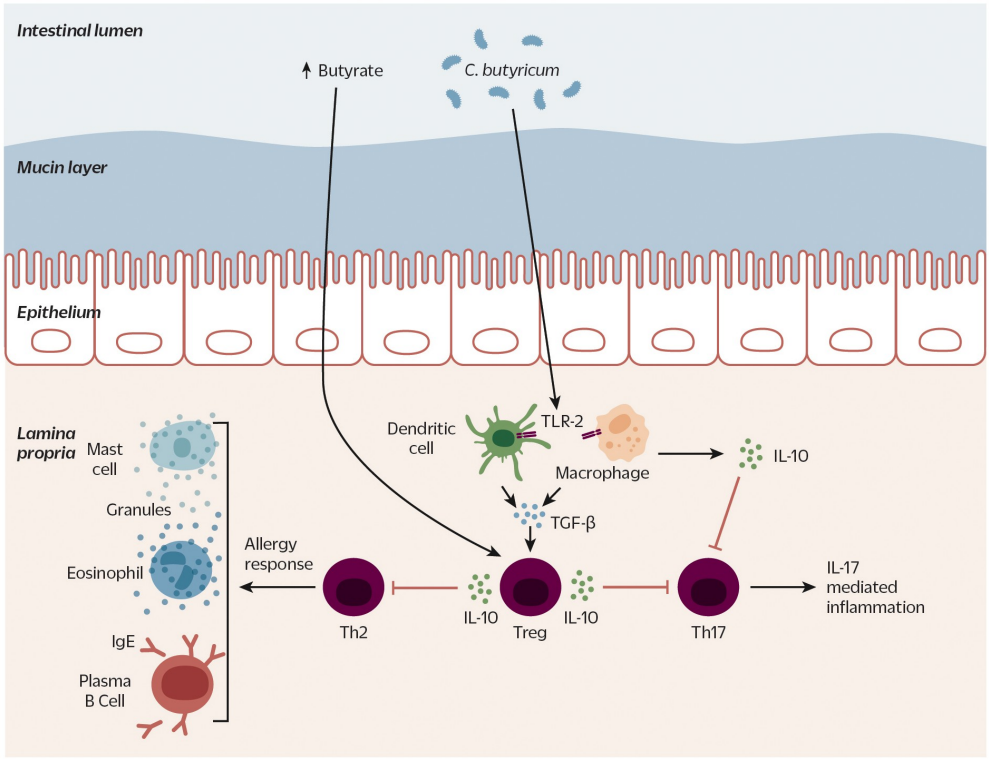

Figure 3. *C. butyricum* strains modulate the immune system to present a tolerant and anti-inflammatory signature. Possibly via butyrate signaling and/or an LTA-activated TLR2-dependent pathway, *C. butyricum* stimulates TGF-β and IL-10 secretion from APCs (dendritic cells and macrophages), Tregs and intestinal epithelial cells. Increased levels of butyrate, TGF-β, and IL-10 contribute to Treg differentiation. An increased population of Tregs and IL-10 inhibit differentiation of Th17 cells that induce IL-17-mediated inflammation, and Th2 cells that induce an allergic response from eosinophils, mast cells, and plasma B cells. Dotted lines indicate indirect effect. LTA: Lipoteichoic acid; TLR2: Toll-like receptor 2; TGF-β transforming growth factor beta; APC: antigen presenting cell; IL-10: interleukin-10; Treg: regulatory T cell; Th17: T helper 17; Th2: T helper 2

C. butyricum has been shown to suppress Th1 and Th17 mediated inflammations, presumably via the increased Treg response (Figure 3). Uncontrolled Th1 and Th17 responses are the culprits behind autoimmune and other inflammatory diseases.[65,66] Treatment with *C. butyricum* in several inflammatory disease models decreased levels of the pro-inflammatory cytokines of Th1 and Th17 cells. Rats with oxazolone-induced colitis receiving either *C. butyricum* CGMCC0313.1 or sodium butyrate displayed improved pathologies in the colon compared to the no treatment group, including significantly decreased serum levels of IL-23, suggesting a possible anti-inflammatory mechanism behind the improved colitis.[16] In a model of food allergy-induced diarrhea, treatment with *C. butyricum* CGMCC0313.1 prior to milk protein challenge significantly reduced the incidence and severity of diarrhea, and concomitantly decreased serum levels of IL-17.[50] The beneficial effects of *C. butyricum* extended beyond the gastrointestinal tract; *C. butyricum* CGMCC0313.1 treatment improved pancreatic damage and decreased the Th1 and Th17 cells in the pancreas in a model of type 1 diabetes.[21] Similarly, in a model of nonalcoholic fatty liver disease (NAFLD), CBM 588 alleviated liver damage while decreasing levels of IFN-γ and IL-17 in the liver and the ileum.[54]

Allergic diseases are driven by improper activation of the Th2 response.[13,67] Th2 cytokines induce B cells to produce immunoglobulin E (IgE) (*i.e.*, antibodies) and activate allergy-responsive immune cells (eosinophils, basophils, and mast cells). Because of its role in inducing memory and repair, the Th2 response is typically considered anti-inflammatory. However, in the context of allergic disease overproduction of Th2 cells can trigger inappropriate immune responses that can be damaging to the host. *C. butyricum* administration has been shown to alleviate allergic responses, again possibly by the modulation of Tregs (Figure 3). In a mouse model of asthma, *C. butyricum*

CGMCC0313.1 reduced airway hyperresponsiveness and lung inflammation by suppressing the Th2 response and increasing anti-inflammatory IL-10.[68] In asthmatic lungs, mast cells infiltrate the airway and *degranulate*, releasing inflammatory molecules and proteases that result in tissue swelling.[69] In lungs from mice administered *C. butyricum*, the number of infiltrating immune cells, level of Th2 cytokines (IL-4 and IL-5), markers of mast cell degranulation, and levels of IgE were all decreased. Similarly, in a food allergy mouse model, *C. butyricum* CGMCC0313.1 treatment resulted in attenuation of allergic reaction, along with an increased measure of forkhead box P3 (Foxp3), an indication of increased Treg.[50] Pre – or post-treatment with *C. butyricum* reduced the severity of diarrhea and anaphylaxis, as well as circulating mast cell proteases, IgE, and Th2 cytokines IL-4, IL-5, and IL-13. Increased abundance of Tregs in the intestine (mesenteric lymph node) and the associated cytokines IL-10 and TGF-β in the serum were noted. Thus, *C. butyricum* attenuates the Th2 response-driven allergic reaction and the associated inflammation by mediating the Th2/Treg balance.

In summary, *C. butyricum* plays an immunomodulatory role in the intestinal epithelium, by mediating tolerogenic APC signaling to promote the Treg response in the presence of pathogenic or pro-inflammatory stimuli. Such modulation results in suppression of pro-inflammatory effector T cell responses, such as Th1, Th2, or Th17. An alternative, non-mutually exclusive explanation for these effects may be direct suppression of Th response by IL-10 production from the intestinal epithelial cells or APCs (Figure 3).

Activation of Akt pathway by C. butyricum

Akt, also called Protein Kinase B (PKB), is a serine/threonine-specific protein kinase that is involved in multiple cellular processes such as cell survival, proliferation, apoptosis, and metabolism. The Akt signaling pathway is highly regulated, and its dysregulation is linked to neurodegenerative and metabolic diseases, as well as cancer.

Cellular apoptosis, or programmed cell death, underlies many complications of diseases, especially neurodegenerative disorders. Activation of Akt releases phosphorylated Akt (pAkt) to translocate to the membrane, where it influences downstream factors to promote cell survival or inhibit apoptosis. *C. butyricum* administration prevents unwanted neuronal apoptosis and brain damage via activation of the Akt pathway (Figure 4a).[19,38,70] For example, *C. butyricum* supplementation restored levels of cerebral pAkt in murine models of diabetic ischemia/reperfusion,[19] vascular dementia,[70] and traumatic brain injury,[38] alongside improvements in cognitive impairment and/or histopathological evidence of ameliorated neuronal apoptosis and brain damage. Upstream molecules such as brain-derived neurotrophic factor (BDNF)[19,70,71] and glucagon-like peptide-1 (GLP-1) were also increased.[38,71] Downstream of Akt phosphorylation, the mitochondria mediates apoptosis via several pathways, one of which is regulated by B cell lymphoma 2 (Bcl-2) and Bcl-2 associated x protein (Bax).[72] In a model of vascular dementia, *C. butyricum* CGMCC 9831 treatment contributed to preventing apoptosis through an Akt-dependent mechanism as evidenced by increased concentrations of the anti-apoptotic Bcl-2 and decreased the pro-apoptotic Bax.[70] Furthermore, caspase-3, a protease activated by Bax and a central mediator of cell death, was suppressed as a result of CGMCC 9831 treatment in a mouse model of cerebral ischemia/reperfusion injury.[73]

GLP-1 is an upstream ligand of the Akt pathway, participating in, among other things, gut-brain communication.[74] *C. butyricum* supplementation increased GLP-1 secretion from the L cells within the intestinal epithelium, putatively due to increased butyrate production triggering the activation of G-protein-coupled receptors 41 and 43 (GPR41 and GPR43).[20,71,75] Therefore, modulation of GLP-1 signaling may be a mechanism by which *C. butyricum* CGMCC 9831 in the gut exerts anti-apoptotic neuroprotection in the model of traumatic brain injury[19,38,70] (Figure 4a). Similarly, in a model of chronic stress-induced depression, *C. butyricum* CGMCC 9830 administration reversed depression-like behavior, increased levels of hippocampal BDNF, and also increased intestinal levels of GLP-1.[71] Thus, *C. butyricum*-mediated anti-apoptotic effects may be due to increased SCFA, increased GLP-1 signaling, and the consequent activation of PI3K/Akt pathway (Figure 4a).

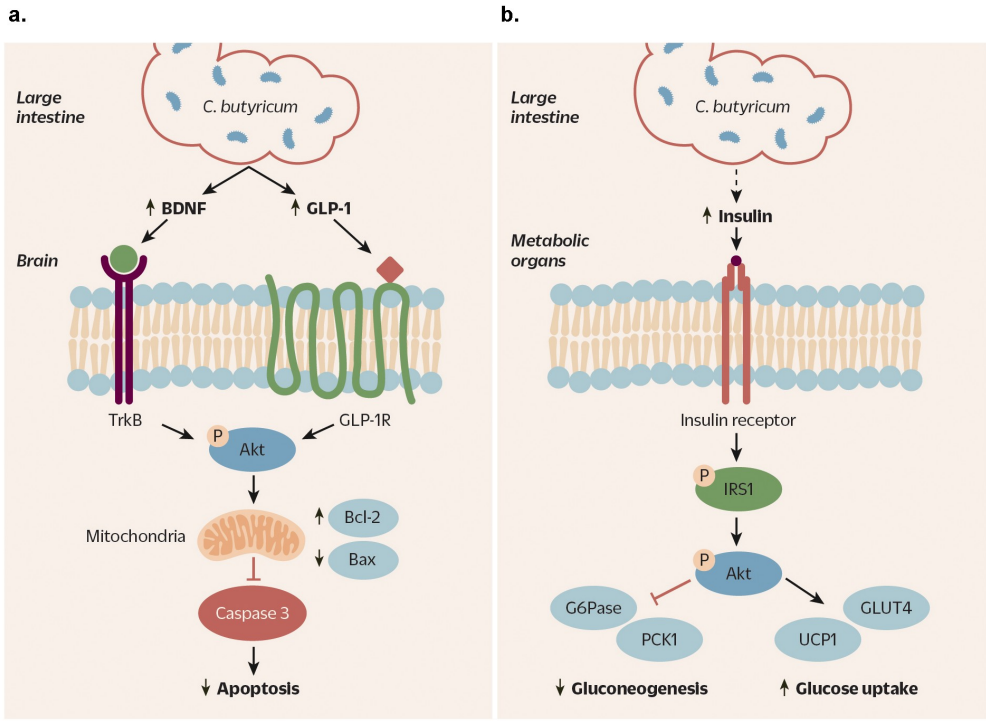

Figure 4. Signal transductions of *C. butyricum*-activated Akt pathways and their impacts in neurodegenerative disorders and metabolic diseases. A) Treatment of various neurodegenerative animal models with *C. butyricum* activates the Akt pathway in the brain via increasing BDNF and GLP-1. BDNF binding to its receptor, TrkB, or GLP-1 binding to GLP-1 R in the brain activates Akt via phosphorylation. Downstream of phosphorylated Akt, elevation of Bcl2 and downregulation of Bax in the mitochondria leads to inhibition of caspase-3-mediated apoptosis. As a result, subjects treated with *C. butyricum* are protected from neuronal death and damage. B) *C. butyricum* treatment of diabetic models, presumably via increased GLP-1 signaling and insulin sensitivity, results in increased phosphorylation of IRS-1 and the downstream activation of Akt in metabolic organs such as the liver, adipose tissue, and skeletal muscle. Activation of Akt pathway suppresses gluconeogenesis by increasing expression of G6Pase and PCK1, and induces glucose uptake by upregulation of GLUT4 and UCP1. Therefore, *C. butyricum* treatment of diabetic individuals may be effective in improvement of insulin sensitivity and glucose homeostasis via activation of Akt pathway. BDNF: brain derived neurotrophic factor; GLP-1: glucagon-like peptide 1; TrkB: tropomyosin receptor kinase B; GLP-1 R: glucagon-like peptide 1 receptor; Bcl2: B cell lymphoma 2; Bax: Bcl2 associated x protein; IRS-1: insulin receptor substrate 1; G6Pase: glucose-6-phosphatase; PCK1: phosphoenolpyruvate carboxykinase 1; GLUT4: glucose transporter type 4; UCP1: uncoupling protein 1

The physiological roles of the Akt pathway are not limited to cellular apoptosis. Also upstream of Akt is insulin receptor substrate 1 (IRS-1), an adaptor protein crucial in insulin signaling. Dysregulation of IRS proteins and the subsequent failure to activate the Akt pathway can lead to insulin resistance. In a diabetes mouse model, *C. butyricum* CGMCC0313.1 or sodium butyrate supplementation increased levels of GLP-1 in the intestine and in the serum.[20] GLP-1 is an insulinotropic hormone (*i.e.*, stimulates insulin secretion), and thus the serum insulin level was increased as compared to untreated mice. Accordingly, the IRS-1/Akt pathway was activated, and improved insulin signaling was evidenced by improvements in glucose homeostasis and adipose tissue metabolism, and alleviated pancreatic inflammation. The observed benefits are driven by decreased gluconeogenesis within the liver––through downregulation of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase 1 (PCK1)––concomitant with increased glucose uptake by insulin targeted tissues, mediated by glucose transporter type 4 (GLUT4) and uncoupling protein 1 (UCP1) (Figure 4b). Therefore, *C. butyricum*, presumably via butyrate, may reduce insulin resistance through GLP-1-induced activation of the IRS-1/Akt pathway.

Benefits of *C. butyricum* supplementation across disease endpoints

The mechanisms described above show potential for beneficial effects of *C. butyricum*. From a host

perspective, what matters is the ability of probiotic strains to actually confer a measurable health benefit. The following sections detail evidence for the host health effects of *C. butyricum* strains – first in animal models of human disease, and then in human clinical trials. Taken together, these studies demonstrate specific health benefits of *C. butyricum* supplementation in a variety of immune-linked diseases. The safety of *C. butyricum* is also borne out in these animal and human studies.

Evidence from murine models of disease

C. butyricum *and gastrointestinal health*

Several studies in animal models have demonstrated *C. butyricum*'s ability to reduce the incidence of antibiotic-associated diarrhea (AAD) and antibiotic-induced gut dysbiosis.[8,14,18,76] In an antibiotic-associated diarrhea mouse model, administration of a probiotic mix containing *C. butyricum* and *Bifidobacterium infantis* for 15 days restored colonic mucosal architecture and fecal microbial diversity, and decreased systemic inflammation.[18] Similarly, *C. butyricum* alone effectively prevented colonic damage when administered in combination with antibiotics.[14,15]

Studies have also identified *C. butyricum* as a preventative intervention for gut pathogen infection. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes diarrhea and hemorrhagic colitis in humans. Takahashi *et al.* demonstrated that EHEC O157:H7 growth, toxin production, and adhesion to epithelial cells is inhibited *in vitro* by CBM 588.[77,78] Prophylactic *C. butyricum* administration to germ-free mice prior to EHEC O157:H7 infection prevented death completely, while administration post infection increased the survival rate from 0% in non-treated controls to 50%.[77] *C. butyricum* may also play a preventative role in *Clostridium difficile* infection. *C. difficile* is a common pathogen responsible for a large proportion of AAD cases, and in a human study *C. butyricum* administration was associated with decreased fecal levels of *C. difficile* as well as the toxin it produces.[79] More recently, Woo and colleagues demonstrated that *in vitro* co-culture of *C. difficile* with CBM 588 decreased the pathogen's toxicity in a dose-dependent manner.[80] Oka *et al.* used a non-lethal rat model of *C. difficile* infection to show a reduced incidence of diarrhea with CBM 588 supplementation.[81]

C. butyricum supplementation has also demonstrated a positive effect across several murine models of colitis, ulcerative colitis (UC), and irritable bowel syndrome (IBS).[16,48,56,58,82,83] CBM 588 culture supernatant (termed "*C. butyricum* derivative") reduced mucosal damage and diarrhea in a dextran sulfate sodium (DSS)-induced colitis model in rats.[82] CBM 588 probiotic supplementation prevented DSS-induced colitis in mice, an effect mediated by modulation of IL-10.[48] Zhao and colleagues also found protective effects in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced mouse model of IBS: *C. butyricum* supplementation reduced intestinal visceral hypersensitivity and mucosal inflammation.[58] *C. butyricum* supplementation has also been investigated in three types of gastric ulcer models: alcohol-induced, restraint cold stress, or pyloric ligation; supplementation decreased gastric mucosal injury alongside improvements in oxidative stress and inflammatory status.[84]

C. butyricum *in the management of metabolic diseases*

Metabolic diseases including diabetes (type 1 and type 2) and liver diseases are considered systemic conditions, as they arise from and continue to affect dysregulated inter-organ crosstalk. Even though *C. butyricum* and other gut microbes are physically located in the gut lumen, their indirect effects extend from gut barrier integrity to immune modulation and endocrine regulation, consequently influencing metabolic signals throughout the body (Figure 5). In this section, *C. butyricum*'s potential in improving energy metabolism in the pancreas, adipose tissue, and liver is explored in animal models of human metabolic diseases.

Obesity and type 2 diabetes

C. butyricum has potential anti-diabetic effects as murine type 2 diabetes studies have reported that *C. butyricum* supplementation ameliorates abnormalities in host metabolism and microbial butyrate production, which is shown to be deficient in humans with this condition.[85] Jia *et al.* examined

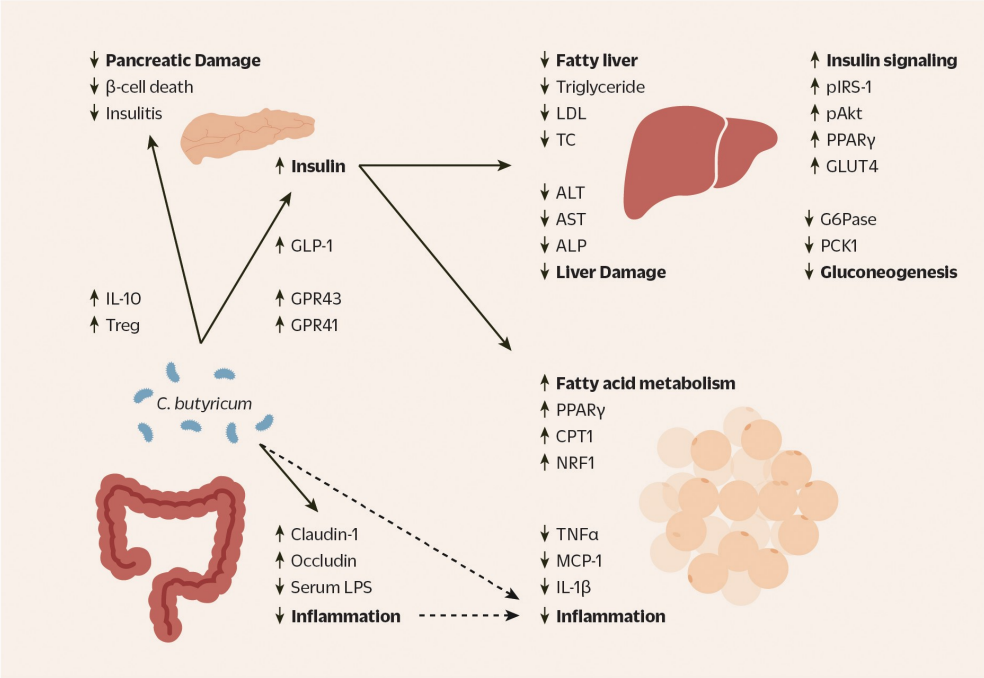

Figure 5. The proposed beneficial effects of *C. butyricum* strains in the gut, pancreas, liver, and adipose tissue of individuals with metabolic disease. *C. butyricum* increases the concentration of SCFA as well as the abundance of butyrate-producing bacteria in the gut. The increased secretion of GLP-1 in the colon and the bloodstream can be attributed to the increased SCFA receptor signaling via GPR41 and GPR43. GLP-1 has pleiotropic effects across many organs in reducing appetite, slowing gastrointestinal motility, decreasing gluconeogenesis and increasing glucose uptake, and most importantly, increasing secretion of insulin from the pancreas. As such, in the liver *C. butyricum* increases insulin signaling (pIRS-1, pAkt, PPAR-γ, GLUT4) and decreases gluconeogenesis (G6Pase and PCK1). Serum markers of hepatic lipid storage such as triglyceride, LDL, and TC are decreased, indicating improved lipid metabolism. Concurrently, the markers of hepatic damage (ALT, AST and ALP) are decreased as well. In the adipose tissue, *C. butyricum* upregulates genes involved in mitochondrial fatty acid oxidation, indicating enhanced fatty acid metabolism: PPAR-γ, CPT1α, and NRF2. Finally, pro-inflammatory cytokines TNF-α, IL-1β, and MCP1 are decreased in the adipose tissue. Such anti-inflammatory effect may be associated with the direct effect of *C. butyricum*-produced metabolites such as butyrate, indirect effects of butyrate such as increased GLP-1 signaling and insulin secretion, and/or the decreased gut inflammation due to healthier gut epithelium evidenced by increased tight junction proteins claudin-1 and occludin and increased levels of serum LPS. Additionally, the population of Tregs and the level of anti-inflammatory cytokine IL-10 are increased due to *C. butyricum*, contributing to the reduced inflammation in the other organs, allowing proper insulin secretion and energy metabolism. SCFA: short chain fatty acid; GPR41/43: G-protein-coupled receptor 41/43; GLP-1: glucagon-like peptide 1; pIRS-1: phosphorylated insulin receptor substrate 1; pAkt: phosphorylated Akt; PPAR-γ: peroxisome proliferator-activated receptor-gamma; GLUT4: glucose transporter type 4; g6pase: glucose-6 phosphatase; PCK1: phosphoenolpyruvate carboxykinase 1; LDL: low density lipoprotein; TC: total cholesterol; ALT: alanine transaminase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; CPT1α: carnitine palmitoyltransferase-1α; NRF2: nuclear factor erythroid 2-related factor 2; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1 beta; MCP1: monocyte chemoattractant protein 1; LPS: lipopolysaccharide; Treg: regulatory T cell; IL-10: interleukin 10

both leptin receptor-deficient ($lep^{db/db}$) and high-fat diet (HFD) + streptozotocin induced diabetic mice and Shang *et al.* examined HFD-induced diabetic mice.[20,39] Both studies noted *C. butyricum* supplementation reduced weight gain and fat accumulation, and improved glucose tolerance and insulin sensitivity. Parameters of improved metabolism included increased serum insulin and decreased fructosamine and G6Pase supporting glucose metabolism, and increased respiratory exchange ratio (RER) indicating better fat oxidation.[20,39] Authors invoke promotion of mitochondrial function as a part of the metabolism-boosting effect of *C. butyricum*, supported by increased markers of mitochondrial metabolism in the adipose tissue, as well as the activation of the insulin-mediated hepatic peroxisome proliferator-activated receptor gamma (PPAR-γ).[20,39] In addition, both studies showed decreased systemic and localized inflammation in the adipose tissue, pancreas, and/or colon with *C. butyricum*. Jia and colleagues also noted a *C. butyricum*-induced decrease in hepatic and pancreatic damage, and Shang *et al.* additionally saw improved gut barrier function post supplementation.

Finally, *C. butyricum* supplementation across both studies lead to improvements in the HFD-induced reduction of fecal SCFAs, and Jia and colleagues further measured increased expression of microbial butyrate-production genes, as well as increased mRNA levels of host colonic GPR41 and GPR43 with treatment.

Type 1 diabetes

Type 1 diabetes involves autoimmune damage to the pancreatic β-cells (responsible for insulin production and secretion), leading to insulin deficiency. Both dysregulation of the gut immune system and decreased gut butyrate production are implicated in the pathology of type 1 diabetes.[86,87] Thus, Jia and colleagues investigated the possible effects of *C. butyricum* in the development and progression of type 1 diabetes.[21] *C. butyricum* supplementation of non-obese diabetic (NOD) mice both delayed the onset and reduced the incidence of diabetes.[21] As detailed earlier in the mechanisms section, this study demonstrated the role of *C. butyricum* supplementation in the modulation of Treg development and migration as the basis of improving insulitis, the main culprit in T1D. *C. butyricum* induced and propagated more Tregs from the gut, and suppressed pro-inflammatory IFNγ and IL-1 cytokines in the pancreas. Thus, it is hypothesized that the improvements noted may stem from an increased Treg response and immune tolerance, decreasing the destructive autoimmunity in the pancreas. In addition, *C. butyricum* supplementation again increased expression of microbial genes involved in butyrate production, and trended toward increased fecal butyrate levels. Interestingly, direct oral administration of sodium butyrate led to an earlier onset of T1D compared to control mice. The efficiency of orally administered butyrate in increasing colonic butyrate levels is unresolved and researchers in this study did not see an increase in fecal butyrate levels with oral sodium butyrate supplementation.

Liver disease

The potential benefits of *C. butyricum* have also been investigated in liver metabolic dysfunction. Nonalcoholic fatty liver disease (NAFLD) is common in obese and insulin resistant individuals, especially those with type 2 diabetes. Studies of *C. butyricum* supplementation in murine models of NAFLD reported improved hepatic lipid metabolism and immunoregulation. Seo and colleagues investigated the effect of CBM 588 supplementation in a rat model of HFD-induced NAFLD and found *C. butyricum* treatment helped maintain metabolic parameters including body weight, fat mass, and insulin resistance at normal levels while also protecting from liver injury and dysregulation of lipid metabolism.[88] Hepatic accumulation of lipid droplets decreased, alongside decreased hepatic levels of cholesterol, free fatty acids, and phospholipids. Supporting these observations, hepatic gene expression analysis reflected a decrease in triglyceride synthesis and an increase in excretion of excess lipids via the conversion of cholesterol to bile acids. In another study, mice treated with *C. butyricum* B1 exhibited improved symptoms of HFD-induced steatohepatitis, and their liver and intestines showed an anti-inflammatory signature.[54] The authors reported increased levels of butyrate but not other SCFAs in the liver and feces of *C. butyricum*-treated mice, and showed *in vitro* that butyrate effectively induced anti-inflammatory T cell differentiation. Therefore, *C. butyricum* modulates the dysregulation of lipid metabolism and immune response against a prolonged high-fat diet, possibly via butyrate production, resulting in protection from fatty liver disease.

Acute liver injury is one of the main forms of liver disease, caused by oxidative stress and inflammation. Liu and colleagues examined the hepatoprotective effects of *C. butyricum* CGMCC 8808 in a mouse model of acute liver injury and found prophylactic administration of the probiotic increased the survival rate compared to control mice.[24] *C. butyricum* administered prior to the insult also attenuated liver injury, as revealed by histological analyses and by decreased serum markers of liver damage. The hepatoprotective ability of *C. butyricum* was partially attributed to its anti-oxidation and anti-inflammatory effects: administration of *C. butyricum* prior to the event increased the activities of anti-oxidative enzymes (superoxide dismutase and catalase) and the oxidative stress-sensor nuclear factor erythroid 2-related factor 2, and improved inflammatory tone.

C. butyricum *in neuroprotection*

The microbiota-gut-brain axis is a developing area of research that posits microbial influence on the bi-directional communication between the central and enteric nervous systems.[89] Gastrointestinal bacteria have the ability to alter the endocrine and neurotransmitter signaling, as well as incite immune reactions in response to inflammatory neurological conditions. Therefore, probiotics have been suggested as a potential intervention for various neurological disorders. *C. butyricum* engages in cross-functional communication between systems by influencing several immune and metabolic pathways, deterring the progression of detrimental neuroinflammatory reactions.

C. butyricum *reduces neurodegeneration via anti-apoptotic pathway*

As described in a separate mechanisms section above, *C. butyricum* activates an anti-apoptotic Akt pathway. Treatment with *C. butyricum* has been shown to alleviate a number of neurodegenerative disease conditions in murine models, and the effect has been correlated with increased Akt pathway activation and reduced apoptosis. In a diabetic mouse model with induced cerebral ischemia-reperfusion injury, treatment with *C. butyricum* CGMCC 9831 was able to not only lower blood glucose levels, but also reverse histopathological damage to the hippocampal neurons, and improve learning and memory deficits.[19] Similarly, treatment with CGMCX 9831 in a mouse model of induced vascular dementia improved histopathological damage in the hippocampus and consequently spatial learning ability;[70] in a model of traumatic brain injury (TBI), the treatment improved cognitive, fine motor, and sensory functions as well as markers of neurodegeneration.[38] Some of these observed benefits were paired with increased butyrate levels in the brain.[70,73] Across all studies, *C. butyricum* activated anti-apoptotic pathways in the brain and reduced neurodegeneration.

C. butyricum *prevents blood-brain-barrier dysfunction*

In a model of TBI, cerebral edema – an accumulation of extracellular fluid in the brain – was accompanied by neuronal injury and blood-brain-barrier (BBB) dysfunction.[38] The BBB is maintained by endothelial cells and tight junction proteins to prevent nonselective permeation of the blood into the CNS, a function comparable to that of the gut epithelial barrier. Remarkably, *C. butyricum* demonstrated the ability to prevent brain endothelial barrier dysfunction, evidenced by decreased brain water content and the restoration of normal levels of tight junction protein expression. Interestingly, disrupted intestinal barrier function is often a complication of TBI, and indeed *C. butyricum* treatment maintained pre-TBI induction levels of tight junction proteins in the colon and suppressed colonic inflammation. These results suggest a connection between intestinal and brain barrier improvement by *C. butyricum*, although further investigation is necessary. One such connection may be provided by *C. butyricum*'s ability to stimulate the secretion of GLP-1. GLP-1 is neuroprotective in models of brain injury and protects the BBB, potentially via modulation of tight junctions.[90–92] Indeed, *C. butyricum* treatment in the TBI model also increased the expression of the GLP-1 receptor in both the brain and the gut,[38] supporting the notion that *C. butyricum* mediates barrier function via GLP-1 signaling.

C. butyricum *suppresses neuroinflammatory response*

Multiple sclerosis is a chronic neurological autoimmune disease of the central nervous system. In multiple sclerosis, the immune system attacks the sheath around nerve fibers, called myelin. Additionally, inflammatory immune cells infiltrate and cause encephalitis, *i.e.* inflammation in the brain. In a mouse model of multiple sclerosis, treatment with *C. butyricum* significantly improved neuropathological inflammation in the lumbar spinal cord, evidenced by a decreased percentage of lymphocyte infiltration and myelin loss when compared to controls.[53] This alleviation of disease severity was also supported by a lowered incidence of external disability in the *C. butyricum*-treated group.

C. butyricum *against tumorigenesis*

Disrupted epithelial homeostasis, inflammation, and tumorigenesis have a well-established connection.[93,94] In addition, the gut microbiome is critical in the

establishment and development of immune homeostasis,[95] and therefore is implicated in cancer development, progression, and treatment (extensively reviewed elsewhere[96-98]). In this section, we summarize the beneficial effects of *C. butyricum* in animal cancer models.

Colorectal Cancer

Colorectal cancer (CRC) is the third most diagnosed cancer across both genders, and is commonly malignant.[22,99] Multiple lines of evidence heavily implicate the gut microbiome in CRC.[100–102] High fiber diets have been shown to decrease the risk of CRC, hinting at the gut microbiome-CRC connection.[103,104] Further, fecal microbiome profiling of CRC patients shows a decrease in butyrate-producing *Roseburia* and *Lachnospiraceae* when compared to healthy individuals, as well as a significant decrease in genes for microbial butyrate production.[105] On the other hand, certain opportunistic pathogens (e.g. *Enterococcus, Escherichia, Streptococcus*) are associated with an increased risk of CRC in humans[105] and murine mechanistic studies have shown that Enterotoxigenic *Bacteroides fragilis*, adherent-invasive *Escherichia coli* strain NC101, and *Fusobacterium nucleatum* can induce colonic tumorigenesis and promote colitis-associated CRC via pro-inflammatory pathways.[106–109] Moreover, several bacterial metabolites have been implicated in the initiation and/or progression of CRC.[100]

C. butyricum has shown anti-tumorigenic properties *in vivo* across four separate mouse models of CRC. In a 1,2-dimethylhydrazine dihydrochloride (DMH)-induced CRC murine model, researchers concluded that oral treatment with *C. butyricum* restored the DMH-induced weight decline, reduced tumor incidence, and decreased tumor size.[110] Two separate studies of colitis-associated CRC (CAC) showed decreased tumorigenesis and increased survival with *C. butyricum* supplementation.[23,111] Finally, in a HFD-induced $Apc^{min/+}$ mouse model of CRC, *C. butyricum* supplementation reversed the HFD-induced weight gain, and again, decreased intestinal tumor size and burden.[22,111]

Across these studies, several mechanisms behind decreased tumorigenesis are proposed (Figure 6a). *C. butyricum* induced an anti-inflammatory immune signature and improved gut barrier function, both *in vivo* and *in vitro*. Liu *et al.* saw inhibition of NF-κB signaling and a decrease in pro-inflammatory cytokines *in vivo* with *C. butyricum* supplementation.[23] Chen *et al.* showed a reversal of the DMH-induced skew of Th2 and Th17 responses *in vivo*, and decreased expression of TLR4, NF-κB and IL-22 both *in vitro* and *in vivo*.[110] While known for its role in tissue repair and regeneration, IL-22 expression in tumor environments can be detrimental due to its cell proliferation effect.[112] *C. butyricum* cultured medium has been previously shown to significantly reduce TLR4 expression, an effect attributed to the metabolite butyrate.[113] Finally, Xiao and colleagues showed reduced colonic inflammation (measured by myeloperoxidase activity) and improved gut barrier function (in histological parameters *in vivo* and increased transepithelial resistance and decreased permeability *in vitro*).[111]

Additionally, *C. butyricum* suppressed tumorigenesis *in vitro* and *in vivo* by decreasing cancer cell proliferation, triggering cell cycle arrest and increasing apoptosis.[22,23,110,111] Two studies showed that treatment of CRC cells *in vitro* with *C. butyricum*-conditioned media significantly inhibited cell proliferation and increased cell apoptosis.[22,110] This effect was driven by an increased expression of p21,[WAF1114] a cell cycle inhibitor, dependent on the activation of SCFA receptor GPR43.[22,99] *In vivo, C. butyricum* suppressed the Wnt pathway,[115,116] a signaling cascade essential for cell proliferation and tissue homeostasis.[22] Additionally, another study reported an increased proportion of apoptotic cells in the colonic tissues of *C. butyricum* treated CAC mice, supported by decreased Bcl-2 (an anti-apoptotic protein) and increased Bax (a pro-apoptotic protein) levels.[23]

Bladder Cancer

Another common form of cancer affects the bladder. Approximately 70% of newly diagnosed bladder cancers are non-muscle invasive (superficial), for which the standard of care treatment is Bacillus Calmette-Guérin (BCG) intravesical therapy.[117] This form of immunotherapy involves intravesical injection of a "vaccine" derived from *Mycobacterium bovis*. However, BGC can have serious side effects, including sepsis. This has

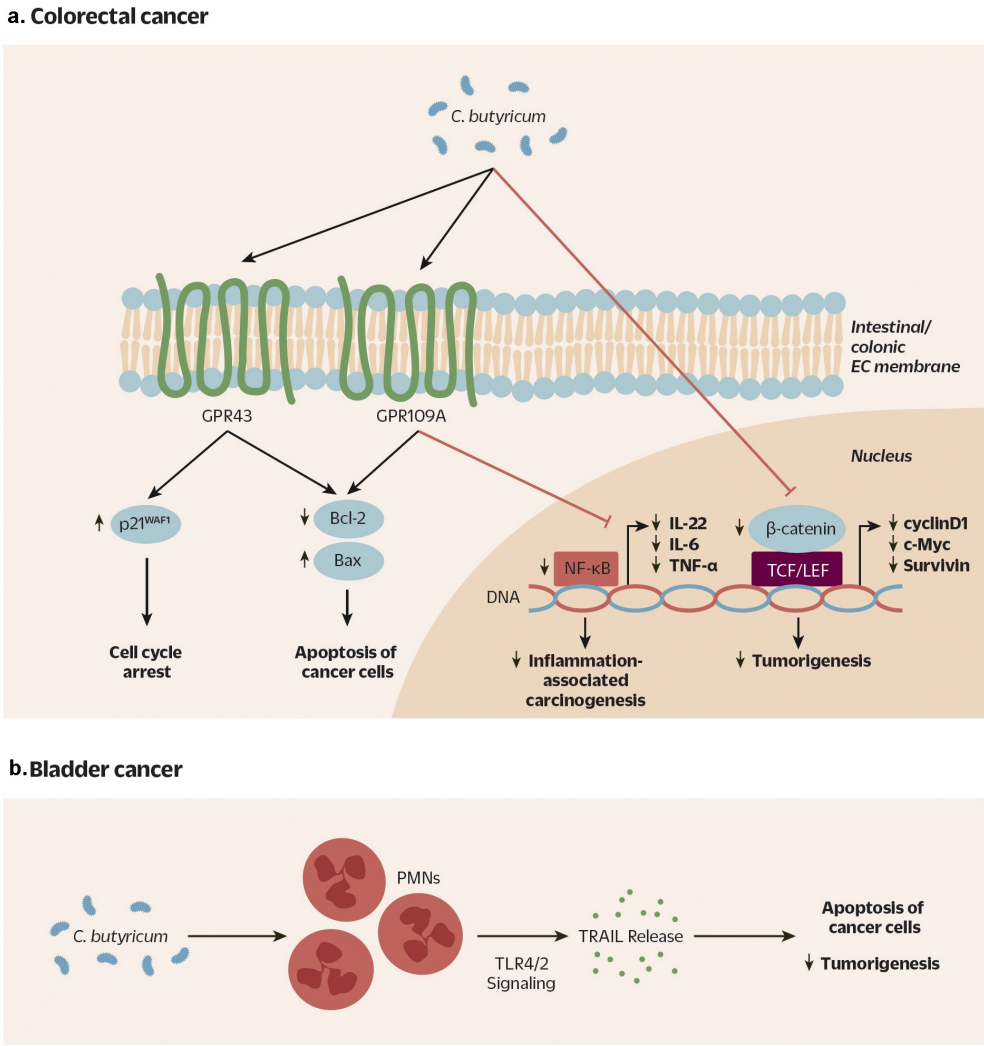

Figure 6. Proposed mechanism of action of beneficial *C. butyricum* strains across animal models of A) colorectal cancer (CRC) and B) bladder cancer. A) *C. butyricum* increases expression of SCFA receptors GPR43 and GPR109A in colonic and intestinal epithfiguelial cells. Activation of GPR43 eventually leads to an increased expression of $p21^{WAF1}$ and cell cycle arrest in cancer cells. Again likely via activation of GPR43 and GPR109A, *C. butyricum* triggers a decrease in anti-apoptotic proteins Bcl-2, and an increase in pro-apoptotic protein Bax, resulting in the apoptosis of cancer cells. Moreover, *C. butyricum* inhibits NF-κB signaling (TLR4-MyD88-NF-κB) and decreases certain proinflammatory factors (IL-22, IL-6, TNF-α), potentially leading to a decrease in inflammation-associated carcinogenesis. Finally, *C. butyricum* may also act to decrease CRC development by inhibiting the Wnt signaling pathway (β-catenin, cyclinD1, c-Myc, Survivin etc.). Cross-talk among these pathways is likely, but not yet fully elucidated. B) *C. butyricum* treatment of PMNs stimulates their release of TRAIL, a cytokine that specifically induces apoptosis in tumor cells. CRC: colorectal cancer; SCFA: short chain fatty acids; GPR43/109A: G-protein-coupled receptor 43/109A; EC: epithelial cell; Bcl2: B cell lymphoma 2; Bax: Bcl2 associated x protein; TLR4: Toll-like receptor 4; MyD88: Myeloid differentiation primary response 88; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; IL-22: interleukin-22; IL-6: interleukin-6; TNF-α: tumor necrosis factor alpha; PMN: polymorphonuclear leukocyte; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

motivated a search for other bacteria with anticancer effects, which could act as a safer replacement for *Mycobacterium bovis*. Given the anticancer properties of *C. butyricum* outlined above, Shinnoh and colleagues investigated its efficacy as a novel intravesical therapy against superficial bladder cancer.[118] Previous work in this area has demonstrated that post BCG treatment, polymorphonuclear neutrophils (PMNs) migrate to the bladder and release tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a cytokine that specifically induces apoptosis in tumor cells.[119–121] Shinnoh *et al.* find that, similar to the BCG mechanism outlined above, *in vitro* treatment of PMNs with CBM 588 enhanced their release of TRAIL (through TLR2/4 signaling

pathways) and that the released TRAIL stimulated apoptosis of human cancer cells (Figure 6b). The induction of TRAIL release from PMNs was unique to BCG and *C. butyricum*, and did not occur with other strains or anticancer compounds (*Lactobacillus*, Krestin, and Lentinan). Researchers further confirmed these effects *in vivo*: the anti-cancer effects of intra-tumor injection of *C. butyricum* and BCG were compared in C3H/HeN mice inoculated with murine bladder cancer cells. *C. butyricum* proved to be more effective than BCG, nearly completely inhibiting tumor growth rather than partially suppressing it.[118] Thus, *C. butyricum* was found to suppress the growth of bladder cancer cells both *in vivo* and *in vitro*, and may represent a promising new intravesical intervention against superficial bladder cancer.

C. butyricum *in immune checkpoint blockade therapy*

Immune checkpoint blockade (ICB) is a revolutionary cancer therapy that has been under extensive investigations in the past 20 years. ICB utilizes the immune checkpoint proteins of T cells that are designated to eliminate cancer cells. When ICB inhibits negative immune checkpoint proteins such as programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4), T cells can continuously kill the cancers without a "brake."

Microbiome differences have been proposed to directly affect the effectiveness of ICB.[122,123] Exposure to antibiotics prior to receiving ICB therapy is especially detrimental to the outcome.[124] Recently, first-in-human clinical trials showed that fecal microbiota transplantation (FMT) improved the efficacies of anti-PD-1 immunotherapy in patients with anti-PD-1-refractory metastatic melanoma, while also reprogramming the gut microbiome.[125,126] As *C. butyricum* supplementation has been repeatedly shown to improve symptoms related to microbial dysbiosis, Tomita *et al.* retrospectively analyzed the survival of non-small lung cancer patients who received ICB therapy, with or without CBM 588 within 6 months prior to and concurrently with ICB.[127] Taking CBM 588 significantly improved the progression-free survival (PFS) and overall survival (OS) in patients that have been treated with ICB compared to no CBM 588, even in the population that had been exposed to antibiotics prior to ICB. While this retrospective study did not investigate the effect of *C. butyricum* on the gut microbial composition or the patient's immune profile, several studies as presented in this review support that the underlying mechanism may be due to the promotion of certain beneficial taxa such as *Bifidobacterium*, strengthening of the intestinal barrier, and modulation of the immune system. An on-going Phase I clinical trial (NCT03829111) is aimed to test the effect of CBM 588 in kidney cancer patients being treated with a combination of CTLA-4 and PD-1 inhibitors. Stool microbial composition and systemic immunological shifts are to be assessed along with the efficacy of the ICB drugs. More human intervention trials confirming the *C. butyricum*-improved efficacies of ICB therapies and investigating the mechanism behind the benefits will open a novel avenue of overcoming non-responsiveness to cancer immunotherapies with probiotics administration.

C. butyricum in human clinical trials

As detailed above, numerous studies in murine models have shown protective or ameliorative effects of *C. butyricum* across a range of disease models. In humans, we could find seven additional papers examining *C. butyricum*'s potential as a probiotic in clinical trials of gastrointestinal, psychiatric, and metabolic disorders (Table 2).

Two of these clinical studies pertain to antibiotic-associated diarrhea (AAD), both in children[8] and in adult patients with gastro-duodenal ulcers undergoing *Helicobacter pylori* eradication therapy.[128] The primary endpoint, diarrhea incidence, decreased across both open-label trials: Seki *et al.* reported a decrease from 59% in the antibiotics-only arm, to 5% in children receiving *C. butyricum* for three days, and to 9% in those that received treatment for all six days.[8] Imase *et al.* similarly saw a decrease from 43% in the antibiotics-only group to 14% in low dose and 0% in high dose *C. butyricum* supplementation groups; the *H. pylori* eradication rate was unaffected by the supplementation.[128] Similarly, Sun *et al.* reported improved diarrhea symptoms (IBS symptom severity scale) and quality of life ((IBS-QOL scores) in a double-blind, placebo-controlled study of subjects with diarrhea-predominant IBS receiving

Table 2. The effect of *C. butyricum* supplementation on human health and gut microbiome composition across human clinical trials.

Study	Probiotic	Dose (daily)	Duration of treatment	Disease	Study size	Study design	Primary end-point(s)	Community analysis method	Community analysis primary result	Other analyses
Seki *et al.* 2003	CBM588	1–4 × 10^7 CFU	3 days; 6 days	Antibiotic – associated diarrhea (AAD) in children	n = 27 (antibiotics); n = 38 (3 day *C. butyricum*); n = 45 (6 day *C. butyricum*)	randomized, open label	decreased incidence of diarrhea in both *C. butyricum* treatment arms vs. antibiotic-only	culture-based	treatments prevented antibiotic-induced reduction in total anerobe counts and levels of *Bifidobacterium* spp., *Eubacterium* spp., *Lactobacillus* spp.	
Imase *et al.* 2008	CBM588	6x10^7 (low); 1.2x10^8 CFU (high)	1 week	Antibiotic – associated diarrhea (AAD)	n = 7 (antibiotics); n = 7 (low-dose) n = 5 (high-dose *C. butyricum*)	randomized, open label	decreased incidence of diarrhea in both *C. butyricum* treatment arms vs. antibiotic-only	culture-based	treatments prevented antibiotic-induced reduction in total anerobe counts and levels of *Bifidobacterium* spp., *Lactobacillus* spp.	Treatment decreased the detection rate of *C. difficile* toxin A
Sun *et al.* 2018	*C. butyricum*	5.67x10^7 CFU	4 weeks	IBS-D	n = 95 (placebo); n = 105 (*C. butyricum*)	multi-center, randomized, double-blind, placebo-controlled	reduction of IBS symptom severity scale (IBS-SSS) in treatment group vs. placebo	16 s rRNA PCR and Illumina sequencing; metagenomic (shotgun) Illumina	PCoA shows groups diverge after treatment; 45 OTUs changed from baseline to week 4 between arms (e.g. *Clostridium sensu stricto* reduced in treatment group); several KEGG pathways distinguish arms post treatment	improvement in change of overall IBS-QOL score and stool frequency in treatment vs. placebo arm; no difference in change of Bristol stool scale between arms;
Yasueda et al. 2016	CBM588	CFU not specified; 3 tablets 3x/day	24 months (post hospital discharge)	pouchitis	n = 8 (placebo); n = 9 (*C. butyricum*)	randomized, placebo – controlled	1/9 subjects in the treatment arm and 4/8 subjects in the placebo arm developed pouchitis (NS)	T-RFLP	obligate anaerobes increase in treatment arm group (confounded by unknowns); *Clostridium coccoides* group increased in the placebo group; *Escherichia* subgroup decreased in the treatment group	serum CRP levels decreased with treatment; safe and well-tolerated
Miyaoka *et al.* 2018	CBM588	CFU not specified; 60 mg/day	8 weeks	treatment-resistant major depressive disorder (TRD)	n = 20 (control); n = 20 (*C. butyricum*)	randomized; open label	treatment improved depression (reduced median HAMD-17, BDI, and BAI scores)			safe and well tolerated
Xia *et al.* 2018	*C. butyricum* CGMCC0313.1 and *B. infantis* CGMCC0313-2	>4.5 x10^7 CFU (*C. butyricum*) and >4.5x10^6 CFU (*B. infantis*)	3 months	minimal hepatic encephalopathy (MHE)	n = 37 (control); n = 30 (*C. butyricum* & *B. infantis*)	randomized; open label	treatment improved the results of the psychometric tests for MHE (NCT-A and DST)	taxa-specific qPCR	total bacterial number similar between arms; treatment increased *Clostridium* cluster I and *Bifidobacterium, and* decreased *Enterococcus* and *Enterobacteriaceae*	mean venous ammonia level, and serum LPS, D-lactate and DAO decreased with treatment

(*Continued*)

Table 2. (Continued).

Study	Probiotic	Dose (daily)	Duration of treatment	Disease	Study size	Study design	Primary end-point(s)	Community analysis method	Community analysis primary result	Other analyses
Perraudeau & McMurdie *et al.* 2020	WBF-11: *Akkermansia muciniphila*, *Anaerobutyricum hallii*, *Clostridium beijerinckii*, *Clostridium Butyricum* CBUT, *Bifidobacterium infantis*); WBF-10: excludes *A. muciniphila* and *A. hallii*	$1.2x10^9$ AFU (*A. muciniphila*); 9.0×10^8 AFU (*A. hallii*); 1.6×10^{10} AFU (*C. beijerinckii*); 3.3×10^9 AFU (*C. butyricum*); 2.0×10^9 AFU (*B. infantis*)	12 weeks	type 2 diabetes	n = 26 (placebo); n = 27 (WBF-10); n = 23 (WBF-11)	multi-center, randomized, double-blind, placebo – controlled	treatment improved total glucose AUC	16S rRNA (V4) PCR and Illumina sequencing	no change in alpha-diversity (Shannon) or beta-diversity (weighted UniFrac distance) among arms or timepoints; no separation of arms in PCoA analysis	both formulations safe and well tolerated; no change in CRP and other inflammatory markers for either formulation vs. placebo

C. butyricum daily for 4 weeks, compared to the placebo group.[129] Finally, *C. butyricum* has also been investigated in humans for the management of pouchitis. Total proctocolectomy with ileal pouch anal anastomosis (IPAA) is the standard surgical procedure for patients with UC, whereby surgeons create a pouch to replace the damaged colon and rectum. However, the pouch can become inflamed and damaged in a complication known as pouchitis.[130] Yasueda *et al.* found that commercially available CBM 588 tablets reduced the incidence of pouchitis in UC patients from 50% in the placebo group to 11% in the *C. butyricum*-treated group.[83] Due to a small sample size (9 treatment, 8 placebo patients), this decrease was non-significant.

C. butyricum has also been investigated in humans as a treatment for psychological disorders: treatment-resistant major depressive disorder (TRD) and minimal hepatic encephalopathy (MHE). Miyaoka *et al.* found that subjects with TRD receiving *C. butyricum* in addition to SSRI (selective serotonin reuptake inhibitors) antidepressants[131] reported significantly lower median scores across several indices compared to the control group.[131] Along these lines, Xia *et al.* reported that a probiotic multi-strain formulation featuring *C. butyricum* CGMCC0313.1 and *B. infantis* CGMCC0313-2 improved cognition (measured via the number connection test A and digit symbol test) in subjects with minimal hepatic encephalopathy (MHE),[132] a complication of liver cirrhosis that leads to mild cognitive and motor impairment.[133]

Finally, Perraudeau & McMurdie *et al.* investigated *C. butyricum* strain CBUT as part of a 5-strain and 3-strain consortium in a double-blind, placebo-controlled study of glycemic control in subjects with type 2 diabetes.[134] Researchers concluded that the 5-strain consortium containing CBUT alongside strains of *Akkermansia muciniphila, Anaerobutyricum hallii, Clostridium beijerinckii*, and *Bifidobacterium infantis* caused a significant improvement in total glucose $AUC_{0\text{-}180min}$ relative to placebo following 12 weeks administration, as well as nominally-significant improvements in incremental glucose $AUC_{0\text{-}180min}$ and hemoglobin A1c. Although CBUT was present in both formulation arms and therefore it could not be determined whether it was a necessary and direct contributor to the observed improvements, butyrate production is among the hypothesized mechanisms.

Across these studies, a similar dose of the chosen strains of *C. butyricum* (~ 10^7 CFU/g) administered orally was well tolerated and deemed safe, and had a positive impact across the primary endpoints of each study (Table 2). However, of the seven studies, only three were placebo-controlled and only two were double-blind; hence, interpretations of benefit must be made with caution. Additional double-blind and placebo-controlled clinical studies are needed across indications to resolve and expand upon these findings.

While these clinical studies have shown safety and efficacy of *C. butyricum* in various human diseases, the mechanism behind improvements in clinical endpoints remains unconfirmed. Mirroring results from animal studies, culture-based and taxa-specific qPCR analyses show *C. butyricum* administration in humans restoring total fecal anaerobe counts after antibiotic treatment, as well as increasing levels of certain beneficial species (*Bifidobacterium, Lactobacillus*) and decreasing *Enterococcus*/*Enterobacteriaceae*.[8,128,132] Sequencing of the 16S rRNA gene has not shown a change in overall gut microbiota diversity. Sun *et al.* via PCoA detected a compositional shift after 4 weeks of administration, whereas Perraudeau & McMurdie *et al.* did not detect a separation of study arms in their PCoA analysis.[129,134] Among the putative mechanisms, butyrate is often regarded as causal to the effects of *C. butyricum* supplementation. We previously described studies in murine models suggesting that *C. butyricum* can not only directly produce butyrate, it can also stimulate additional butyrate-producing taxa.[20,21,24] Yet among human clinical trials, only one study measured fecal butyrate levels after supplementation, finding only weak trends of increase that were not statistically significant.[134] Mechanistic insight can also be derived via a suite of secondary inflammatory endpoints. Three clinical studies measured changes in serum markers of inflammation and gut barrier function following *C. butyricum* administration: Yasueda *et al.* found a reduction in CRP,[83,132] and Xia *et al.* found decreased D-lactate, LPS, and diamine oxidase[83,132] Perraudeau & McMurdie *et al.* did not detect a significant change in inflammatory markers (CRP, IL-6, IL-10, TNF-α, or TGF-β). Future clinical research should move toward evaluating mechanistic hypotheses: using high-resolution methods to monitor changes in gut microbiota, probing for changes in various fecal and serum metabolites, and including markers of host immune response, barrier function, and metabolism.

Conclusions and Perspective

The gut microbiota and its effect on host health is increasingly relevant and supported by an ever growing number of investigations in model systems and proof-of-concept clinical trials. As summarized above, strains of *C. butyricum* appear to enhance the gut's ability to reduce microbial and immunological perturbations induced by antibiotics, pathogens, or other factors, making them interventions of potential importance in a number of health conditions. For humans, probiotic *C. butyricum* strains are promising in the management of gastrointestinal symptoms, psychological conditions, and metabolic disease. According to the available literature, C. butyric*um* strains have shown beneficial effects in a wide range of animal models of human disease, yet their beneficial effects in humans for these same diseases remains underexplored in clinical trials. Although authors across preclinical and clinical studies suggest butyrate as causal to the effects of *C. butyricum* supplementation, direct evidence of this still remains elusive.[134] Mechanisms and effects on host physiological pathways also appear to differ depending on the disease state or model. For example, *C. butyricum* supplementation has been shown to increase apoptosis in cancer models, leading to suppression of tumorigenesis; however, in models of neurodegenerative diseases, the effect on the same apoptosis-related proteins (Bcl-1, Bax) is reversed, leading to decreased apoptosis and consequently decreased neuronal death. Another largely unresolved effect of *C. butyricum* treatment is the extent to which it shifts the overall diversity or composition of the gut microbiota. Currently available studies have used vastly different metrics to assess gut microbial composition, and hence the effect of a specific *C. butyricum* strain is difficult to define. Most studies suggest shifts in specific taxa following supplementation, but the mechanism behind this modulation is unknown.

Dosage and consistency of effects across strains have yet to be resolved: human clinical studies across diseases tend to examine only a single dose of a single strain. Optimized dosing could increase

beneficial effects; similarly, with a more in-depth understanding of strain-level differences and a more diverse strain culture collection, disease-specific, or perhaps tailored strain selection may broaden the benefits of supplementation.

The preceding review of the literature indicates that the full potential of *C. butyricum* to improve human health has not yet been fully elucidated. While the human trials completed to date are consistent with the larger body of preclinical data for this species, fundamental differences in the human and murine microbiota, as well as in the anatomy and physiology of the gastrointestinal tract, pose inevitable limitations in fully recapitulating the biology of the human gut-microbiome interaction. Further human studies designed around mechanistic hypotheses are needed to ascertain the true clinical utility of specific *C. butyricum* strains. Disease areas of great interest include metabolic diseases, due to their prevalence across the globe and the need for new clinical interventions with lower risk profiles; and psychological disorders, given their complex nature and the extent to which these disorders affect individuals' quality of life.

As we look toward the future of health benefits conferred by *C. butyricum* and other live microbes, studies need to be conducted in a more strategic manner with more advanced tools than in previous decades of probiotic research. Creating new microbiota-focused products that convey health benefits requires, at minimum, (1) the use of genomic analysis for the selection of novel strains and initial safety assessments, and (2) rigorous, double-blind, placebo-controlled human clinical trials. Additionally, refining the technology essential to *in situ* measurements will enable a more complete understanding of complex microbiome-host interactions – for example, new ingestible sensor technologies that can profile *in situ* microbiomes[135] and perhaps spatially resolve SCFA profiles in the gut in real time.[136] Since microbial metabolites may be key players in the host health benefits conferred by microbes, increased serum and fecal metabolomic profiling of human clinical subjects may provide further mechanistic insight into the effects of these strains in host physiology and pathophysiology. By employing advanced technologies and co-ordinated approaches to discovering and testing novel strains, scientists in the field will successfully bridge the gap between rodent and human studies and make more rapid progress in clarifying the relationships between live microorganisms, host signaling pathways, and health outcomes.

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